The Inner Loop of Tetraspanins CD82 and CD81 Mediates Interactions with Human T Cell Lymphotrophic Virus Type 1 Gag Protein*

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Dmitriy Mazurov, Gisela Heidecker, and David Derse

From the HIV Drug Resistance Program, NCI-Frederick, Frederick, Maryland 21702-1201

The tetraspanin superfamily proteins play important roles in organizing membrane protein complexes, modulating integrin function, and controlling T cell adhesion. Tetraspanins such as CD82 contain two extracellular loops with its N terminus, C terminus, and inner loop exposed to the cytoplasm. The matrix (MA) domain of human T cell lymphotrophic virus, type 1 (HTLV-1), Gag interacts with the cytoplasmic face of the plasma membrane and is concentrated at tetraspanin-enriched microdomains. To understand the basis of this association, we generated site-directed mutations in the various domains of CD82 and used coimmunoprecipitation and colocalization approaches to examine interactions with HTLV-1 MA. The large extracellular loop of CD82, which is important for interactions with integrins, was not required for the association with HTLV-1 MA. The cytoplasmic N terminus and C terminus of CD82 were also dispensable for CD82-MA interactions. In contrast, mutations of conserved amino acids in the inner loop of CD82 or of palmitoylated cysteines that flank the inner loop diminished CD82 association with MA. HTLV-1 MA also interacted with the inner loop of CD81. Thus, association of HTLV-1 Gag with tetraspanin-enriched microdomains is mediated by the inner loops of CD81 and CD82.

The tetraspanins are a large family of four transmembrane domain-containing proteins that are widely expressed in many cell types and tissues. The proteins of this family interact laterally with each other and with other transmembrane proteins to form tetraspanin-enriched membrane microdomains (TEMs), also referred to as the tetraspanin web (1–4). Tetraspanins modulate the activities of molecules that are organized within the web and alter their function. They help to provide a scaffold of adhesion proteins associated with signaling complexes that link cell surface molecules to the cytoskeleton. The molecular partners for various tetraspanins include integrins, growth factor receptors, major histocompatibility complex molecules, CD4, CD8, Ig-like protein EWI-2, EWI-F, uroplakins, rhodopsin, and others (5-8). Cells of the human immune system express specific tetraspanins, such as CD82, CD81, CD53, CD63, and CD231, which play important roles in cell spreading, adhesion, motility, synapse formation, BCR and TCR signaling, and antigen presentation (2, 9, 10).

Tetraspanins contain four transmembrane domains, two extracellular loops, a small inner cytoplasmic loop, and intracellular N and C termini (Fig. 1) (7, 11, 12). The large extracellular loop (TEL) shows the greatest sequence diversity among tetraspanin domains and is responsible for lateral interactions with specific membrane partners such as integrins. The TEL has between two and four intramolecular disulfide bonds, depending on the tetraspanin, that are essential for the structure required for specific protein-protein interactions. Less is known about the small extracellular loop except that it is believed to be required for optimal function of the TEL (10). The hydrophobic transmembrane domains (TM1 to TM4) stabilize individual tetraspanins during biosynthesis and also contain polar residues that promote associations within and between tetraspanins and other transmembrane molecules. Tetraspanins are palmitoylated on highly conserved cysteine residues located on the cytoplasmic side of each transmembrane domain (Fig. 1). Palmitoylation of tetraspanins is important for their proper function and associations with integrins and other tetraspanins (13–17). Tetraspanins also associate with cytosolic proteins such as phosphoinositide 4-kinase and protein kinase Cα, and these associations appear to be mediated by the N or C termini of tetraspanins (18–20). Some tetraspanins have a tyrosine-based internalization motif in the C terminus that is involved in protein trafficking between plasma membrane and intracellular membrane compartments (Fig. 1) (21). Specific functions or interactions of the inner loop have not been demonstrated.

Human T cell leukemia virus, type 1 (HTLV-1), is a deltaretrovirus, etiologically associated with adult T cell leukemia and HTLV-1-associated myelopathy/tropical spastic paraparesis (22, 23). The virus infects both CD4+ and CD8+ T cells in vivo and appears to spread by cell-cell contact. The viral Gag protein is necessary and sufficient to direct virion assembly and release at the plasma membrane (24). Recently, we reported that HTLV-1 Gag assembly and release are targeted to TEMs (25). In particular, we showed that the MA domain of HTLV-1 Gag interacts with CD82-containing regions of the plasma mem-
brane, because antibody-mediated cross-linking of CD82 on the cell surface caused intracellular Gag to concentrate at the patches of CD82. Also, Gag trafficked with CD82 to the site of immune synapse formation, suggesting that this association may facilitate HTLV-1 transmission. Finally, CD82 and HTLV-1 MA were immunoprecipitated from cell extracts under Brij97 detergent lysis conditions, which preserve the tetraspanin web. It has been reported that HIV-1 assembly and release also occur at tetraspanin-enriched membrane microdomains (26, 27), but it is unclear whether these are the same TEMs that are targeted by HTLV-1 Gag. The mechanisms by which retroviral Gag proteins are targeted to and associate with TEMs are not known. In this study, we have analyzed the interaction of HTLV-1 Gag with TEMs by determining whether HTLV-1 Gag associates directly with CD82 or indirectly with a molecular partner of CD82. By mutating various domains of CD82, we show that the inner loop of CD82 is important for mediating the interaction with HTLV-1 Gag.

EXPERIMENTAL PROCEDURES

Cell Culture—Jurkat E6-1 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 units of penicillin/ml, and 0.1 mg of streptomycin/ml. Human 293T cells and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine, 100 units of penicillin/ml, 0.1 mg of streptomycin/ml, nonessential amino acids, and 1 mM sodium pyruvate (all tissue culture reagents were from Mediatech). TEMs that are targeted by HTLV-1 Gag. The mechanisms by which retroviral Gag proteins are targeted to and associate with TEMs are not known. In this study, we have analyzed the interaction of HTLV-1 Gag with TEMs by determining whether HTLV-1 Gag associates directly with CD82 or indirectly with a molecular partner of CD82. By mutating various domains of CD82, we show that the inner loop of CD82 is important for mediating the interaction with HTLV-1 Gag.

Antibodies and Reagents—Anti-human tetraspanin antibodies were anti-CD82 clone B-L2 (Serotec, UK) and clone MC8D12 (gift from Filatov A.V., Institute of Immunology, Moscow, Russia). Monoclonal anti-FLAG clone M2 was purchased from Sigma. Anti-HTLV-1 p19 (MA) monoclonal antibody 46/6.11.1.3 was from Zeptometrix (Buffalo, NY), and rabbit polyclonal anti-HTLV-1 p19(MA) SP-61 was a gift from S. Oroszlan (Frederick, MD). Secondary antibodies were donkey anti-FLAG Alexa Fluor 488, goat anti-mouse Alexa Fluor 350, 488, and 546 (all from Molecular Probes), horseradish peroxidase-conjugated anti-mouse IgG antibodies (Cell Signaling Technology), and mouse TrueBlot™ horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) for direct immunoblots or with TrueBlot™ for immunoprecipitation; the latter recognizes only native mouse IgG after immunoprecipitation. Blots were washed again and immunoreactive bands were detected with ChemiGlow® reagent on an Alphalager (Alpha Innotech). The Spot Denso analysis tool in FluorChem SP software was used to measure the chemiluminescent intensity of bands on immunoblots. Because mutations often affected expression or immunoprecipitation of CD82, we expressed the amount of p19 (MA dimer) that coprecipitated with CD82 as a relative colP value. The relative colP is defined as the ratio of the immunoprecipitated p19 (MA dimer) relative to the amount of wild type or mutated CD82 that was immunoprecipitated. This value was normalized to the level of p19 detected by immunoblotting of each cell extract. The relative colP values were normalized to the value determined with wild type CD82, which was set at 1. In experiments where CD82 was immunoprecipitated with anti-FLAG antibody, the integrated density of both mature and immature proteins was used.

[3H]Palmitate Labeling—25–30 h after transfection, 293T cells were washed with serum-free DMEM and incubated in 10 ml of DMEM for 3 h (starvation). Cells were then metabolically labeled in 4 ml of DMEM with 5% dialyzed fetal calf serum, containing 0.2 mCi/ml [3H]palmitic acid (PerkinElmer Life Sciences) for 3 h. Cells were washed twice with PBS, lysed in 1% Triton X-100, 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA with inhibitor mixture (Complete Mini, Roche Applied Science) and immunoprecipitated with antibodies as described above. Samples were resolved by 4–12% SDS-PAGE under
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reducing conditions. The gel was fixed in isopropyl alcohol: water:acetic acid (25:65:10) for 1 h, soaked in Amplify reagent (Amersham Biosciences) for 30 min, and dried under vacuum at 80 °C for 2 h. Kodak-MR film was directly exposed to the gel at ~70 °C for 2–4 weeks.

**Immunofluorescent Microscopy**—Sixteen hours after transfection, live Jurkat cells were isolated by gradient centrifugation using Lymphocyte Separation Medium (ICN) and washed with PBS. Cells were adhered to poly-l-lysine (Sigma)-coated cover-slips in PBS for 30 min, fixed with 4% paraformaldehyde (Sigma) in PBS for 30 min, washed, and permeabilized for 30 min with 0.1% saponin (Sigma) containing 1% normal goat serum in PBS. HTLV-1 matrix protein was stained with polyclonal rabbit anti-HTLV-1 p19 antibody, and CD82 was stained with mouse anti-FLAG antibody; antibodies were added directly to permeabilization solution for 30 min and washed once with permeabilization solution and twice with PBS. Secondary donkey anti-rabbit Alexa 488 antibody and goat antimouse Alexa 546 antibody were added in permeabilizing solution in the presence of 1% normal donkey serum for another 30 min. After washing, cover-slips were transferred to slides and maintained in the ProLong antifade kit (Molecular Probes). Slides were analyzed on a Delta Vision RT deconvolution microscope (Applied Precision, LLC). Image data were analyzed with the SoftWoRx Imaging Work station. Colocalization of HTLV-1 p19 with CD82 was estimated through all Z-stacks of individual cells as the Pearson Coefficient of Correlation (full colocalization is 1.0).

**RESULTS**

We previously showed that HTLV-1 Gag localizes to tetraspanin-enriched microdomains in the plasma membrane and that a dimer of HTLV-1 MA coimmunoprecipitates with CD82 from cell extracts prepared in extraction buffers containing Brij97 detergent (25). If HTLV-1 Gag associates directly with CD82, it is likely to interact with a region of the protein exposed from cell extracts prepared in extraction buffers containing Brij97 detergent (25). If HTLV-1 Gag associates directly with CD82, it is likely to interact with a region of the protein exposed from cell extracts prepared in extraction buffers containing Brij97 detergent (25). If HTLV-1 Gag associates directly with CD82, it is likely to interact with a region of the protein exposed from cell extracts prepared in extraction buffers containing Brij97 detergent (25).

**Mutation of the Large Extracellular Loop of CD82 Does Not Alter Gag-CD82 Association**—The LEL of CD82 contains four cysteines, which form two disulfide bonds that are invariant among tetraspanins and are essential for forming the tertiary structure of this domain. Mutation of either pair of cysteines results in dramatic structural changes of the LEL and abolishes interaction of the tetraspanin protein with its partners, as was shown for the lateral association of CD151 with α,β, γ-integrin (33). The predicted disulfide bonds for CD82 are Cys149–Cys216 and Cys150–Cys176 (11). The mutated CD82 protein designated as SSG was altered such that cysteines at positions 149 and 150 were replaced with serines (Fig. 1). Human 293T cells were cotransfected with an HTLV-1 Gag expression vector in combination with either wild type or SSG CD82 expression plasmids in which a FLAG epitope tag was appended to the N terminus of CD82. The MC8D12 antibody recognizes a structural epitope on CD82 LEL and does not recognize the reduced form of CD82 on immunoblots (25). As shown in Fig. 2A, the MC8D12 antibody did not recognize SSG CD82 on immunoblots, indicating that the SSG mutation altered the structure of the LEL. Immunoprecipitation of CD82 with anti-FLAG antibody followed by immunoblotting with anti-HTLV-1 p19(MA) antibody revealed that the MA dimer coimmunoprecipitated with both wild type and SSG CD82 (Fig. 2A). When expressed as the ratio of MA to CD82 in immunoprecipitates (in order to account for differences in expression and immunoprecipitation of wild type and mutated CD82 proteins), there was no significant difference in the amounts of MA coprecipitated with wild type and SSG CD82 proteins (Fig. 2A, bottom panel). These data suggest that the LEL, or cellular proteins that associate with the LEL, are not involved in the interaction between CD82 and HTLV-1 Gag. This result was somewhat surprising because SSG CD82 showed drastic changes in cellular localization compared with wild type CD82 when the YFP-tagged versions of the two proteins were expressed in HeLa or Jurkat cells (Fig. 2B). In contrast to wild type CD82-YFP, which localized to the plasma membrane, the SSG-YFP protein was localized to the endoplasmic reticulum (ER). Retention of the SSG protein in the ER and accumulation of immature (unglycosylated) protein in the cell (Fig. 2A) likely result from misfolding of the LEL (34).

**The Cytoplasmic Termini of CD82 Are Not Required for Association with HTLV-1 Gag**—To determine whether the N-terminal or C-terminal cytoplasmic domains of CD82 are involved in the association with HTLV-1 Gag, we generated three...
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mutated versions of CD82 shown at the top of Fig. 3. At the C terminus, a premature stop codon in C-tr CD82 deletes the last 13 amino acids of the protein. We also mutated the YXXØ internalization motif (where Ø is a large hydrophobic amino acid) in the C-terminal cytoplasmic tail of CD82; the YSKV sequence was mutated to LSKR (Fig. 3, top). In addition, we constructed a deletion mutant at the N terminus of CD82, designated as N-tr, which lacks the first 9 amino acids of CD82. The wild type and mutated CD82 expression plasmids were cotransfected into 293T cells with the HTLV-1 Gag expression vector. Proteins were examined by immunoblotting the cell extracts or the proteins recovered after immunoprecipitation with anti-CD82 antibody (Fig. 3). HTLV-1 MA was detected in cell extracts or the proteins recovered after immunoprecipitation with anti-CD82 antibody and immunoblotting with anti-HTLV-1 p19 (MA) antibody. Palm−CD82 was expressed at higher levels than wild type CD82, but roughly equal amounts of the two proteins were immunoprecipitated from cell extracts (Fig. 4). The amount of MA, relative to CD82, which coprecipitated with Palm−CD82 was only about 2% of the amount of MA that precipitated with wild type CD82 (Fig. 4). The mutation of the Cys in the N terminus of CD82 (P1) resulted in a decreased association with HTLV-1 MA to about 37% of the wild type. Mutations of the Cys residues flanking the cytoplasmic loop (P2) or in the C terminus (P3) of CD82 resulted in reductions in MA coprecipitation to values less than 20% compared with wild type CD82 (Fig. 4, histogram). Thus, palmitoylation has a cumulative effect on the ability of CD82 to interact with HTLV-1 MA. This is consistent with other reports showing the importance of palmitoylation on the functional activity and lateral interactions of tetraspanins with other membrane proteins (17).

We also examined the intracellular localization of Palm−CD82 in HeLa cells (Fig. 5B) and its colocalization with HTLV-1 Gag in Jurkat T cells (Fig. 5C). The Palm−CD82-YFP fusion protein was expressed at higher levels than the wild type and mutated versions of CD82 shown at the top of Fig. 3. At the C terminus, a premature stop codon in C-tr CD82 deletes the last 13 amino acids of the protein. We also mutated the YXXØ internalization motif (where Ø is a large hydrophobic amino acid) in the C-terminal cytoplasmic tail of CD82; the YSKV sequence was mutated to LSKR (Fig. 3, top). In addition, we constructed a deletion mutant at the N terminus of CD82, designated as N-tr, which lacks the first 9 amino acids of CD82. The wild type and mutated CD82 expression plasmids were cotransfected into 293T cells with the HTLV-1 Gag expression vector. Proteins were examined by immunoblotting and mutated versions of CD82 was examined by immunoprecipitation with anti-CD82 antibody and immunoblotting with anti-HTLV-1 p19 (MA) antibody. Palm−CD82 was expressed at higher levels than wild type CD82, but roughly equal amounts of the two proteins were immunoprecipitated from cell extracts (Fig. 4). The amount of MA, relative to CD82, which coprecipitated with Palm−CD82 was only about 2% of the amount of MA that precipitated with wild type CD82 (Fig. 4). The mutation of the Cys in the N terminus of CD82 (P1) resulted in a decreased association with HTLV-1 MA to about 37% of the wild type. Mutations of the Cys residues flanking the cytoplasmic loop (P2) or in the C terminus (P3) of CD82 resulted in reductions in MA coprecipitation to values less than 20% compared with wild type CD82 (Fig. 4, histogram). Thus, palmitoylation has a cumulative effect on the ability of CD82 to interact with HTLV-1 MA. This is consistent with other reports showing the importance of palmitoylation on the functional activity and lateral interactions of tetraspanins with other membrane proteins (17).

We also examined the intracellular localization of Palm−CD82 in HeLa cells (Fig. 5B) and its colocalization with HTLV-1 Gag in Jurkat T cells (Fig. 5C). The Palm−CD82-YFP fusion protein was expressed at higher levels than the wild type
Unlike the wild type CD82, Palm \(-/\) expression vector and vectors expressing FLAG-tagged versions of CD82 proteins, and both proteins localized to the microvilli of adherent cells. There was no discernable difference in the localization of the two fusion proteins detected by immunoblotting (Fig. 4). There was no statistically significant difference in the relative co-IP value between wild type CD82 and any of the mutated CD82 proteins. (p > 0.05).

CD82-YFP in HeLa cells (Fig. 5B), consistent with relative levels of the two proteins detected by immunoblotting (Fig. 4). There was no discernable difference in the localization of the two fusion proteins, and both proteins localized to the microvilli of adherent cells (Fig. 5B). Jurkat cells were cotransfected with HTLV-1 Gag expression vector and vectors expressing FLAG-tagged versions of wild type or Palm \(-/\) CD82. In Jurkat cells, both wild type and Palm \(-/\) CD82 were detected in the complex Golgi (round shape through the middle plane of the cell) and plasma membrane (Fig. 5C). Unlike the wild type CD82, Palm \(-/\) CD82 did not colocalize with HTLV-1 Gag in the plasma membrane (Fig. 5C). Together, these data indicate that palmitoylation of the juxtamembrane Cys residues in CD82 are essential for interactions with HTLV-1 Gag, and the Cys residues flanking the inner loop of CD82 appear to play a significant role in this association.

CD82 contains Cys to Ser substitutions at positions 5, 74, 83, 251, and 253. P1 contains only one Cys to Ser substitution at position 5 in the N terminus of CD82. P2 has two substitutions at positions 74 and 83 that flank the inner loop. P3 contains Cys to Ser substitutions at positions 251 and 253 in the C-terminal cytoplasmic domain. Human 293T cells were cotransfected with wild type (WT) or mutated versions of CD82 in combination with the HTLV-1 expression vector pCMV-HT1. Brij97 cell extracts were directly immunoprecipitated with anti-HTLV-1 p19 (MA) and anti-CD82 (MC8D12) antibodies or immunoprecipitated (IP) with anti-CD82 B-L2 antibody and then blotted with anti-HTLV-1 p19 (MA) or anti-CD82 MC8D12 antibody, as before. The histogram shows the mean relative co-IP values from three experiments, which were calculated as described in Figs. 2 and 3.

The Inner Loop of Tetraspanins Is Essential for Association with HTLV-1 Gag—In contrast to the N-terminal and C-terminal cytoplasmic domains of tetraspanins, which are implicated in interactions with the cytoskeleton and signal transducing kinases, much less is known about the inner loop. Even the number of amino acids that are exposed to the cytoplasm in this loop between transmembrane domains TM2 and TM3 is uncertain; some models predict a short, 4-amino acid loop consisting of Asn-Glu-Val-Arg, whereas others predict a longer cytoplasmic loop (11). For comparative purposes, we have depicted this domain as the 8 amino acids flanked by the Cys residues at positions 74 and 83 (Fig. 1 and Fig. 6). As shown in the amino acid sequence alignment in Fig. 1, the Cys residues at positions 74 and 83, the Gly at position 76, and the Glu at position 80 of CD82 are conserved in CD81, CD53, and CD63. Only CD231 (A15 or TALLA) differs from this consensus. To test the possibility that the inner loop is involved in the CD82-Gag interaction, we mutated the conserved amino acids in CD82 to Gly76 to Asp (G76D) and Glu80 to Ala (E80A) (Fig. 6). We also constructed ILC CD82, in which G76D and E80A mutations were combined with the Cys mutations in P2. Finally, the inner loop from CD81 was inserted into CD82 to give IL81.

Association of the mutated CD82 proteins with HTLV-1 Gag was determined by immunoprecipitation of transfected cell extracts with anti-CD82 antibody and immunoblotting with anti-HTLV-1 p19 (MA) antibody. Compared with wild type...
CD82, the relative coprecipitation of MA with CD82 was decreased to about 31% for E80A and to 16% for G76D (Fig. 6). The Cys mutations in P2 caused a decrease in relative coprecipitation of MA to 20% of the wild type level (Fig. 6 and Fig. 4). The combination of mutations in ILC nearly abolished its coprecipitation with MA (Fig. 6). Furthermore, the mutations in the inner loop of ILC also resulted in a significant decrease in the colocalization of HTLV-1 Gag and ILC in transfected Jurkat cells (Fig. 5).

The chimeric CD82/IL81 protein contains the inner loop of CD81, which essentially replaces the variable amino acids in the inner loop of CD82 with those of CD81. Although the CD82/IL81 protein was expressed at lower levels than wild type CD82, more HTLV-1 MA was immunoprecipitated with CD82/IL81 than wild type CD82 (Fig. 6, IL81). This result suggested that the variable amino acids in the inner loop influence its interaction with MA. To study this further, we examined MA coprecipitation with FLAG-tagged versions of CD81, CD82, and each protein with the heterologous inner loop. CD81/IL82 and CD82/IL81 (Fig. 7). The inner loop of CD81 yielded 2.5-fold higher relative MA coprecipitation compared with the CD82 inner loop, either in the context of CD81 or CD82 (Fig. 7). These data indicate that the inner loop with flanking, palmitoylated cysteines of CD82 and CD81 plays an important role in the targeting of HTLV-1 Gag to TEMs.
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FIGURE 7. HTLV-1 MA interacts differently with the inner loop of CD81 and CD82. The amino acid sequences of the inner loops (IL) of CD82 and CD81 are aligned at the top of the figure. The five amino acids in the inner loop of CD81 that differ from CD82 are highlighted; it is this group of amino acids that was switched in the chimeric proteins, CD82/IL81 and CD81/IL82. All constructs were cloned with a FLAG tag on the N terminus of the tetraspanin and expressed with pCMV-HT1 in 293T cells. Cells were lysed in Brij97 buffer and immunoblotted directly or immunoprecipitated (IP) with anti-FLAG M2 antibody. Proteins were separated by SDS-PAGE under nonreducing conditions and immunoblotted with anti-HTLV-1 p19 or anti-FLAG antibodies. The mean relative co-IP values were calculated from four independent experiments (as described under “Experimental Procedures”) and are presented in the histogram. The relative co-IP value for wild type CD82 was set at 1 for comparison. In calculating these values for CD82 constructs, we integrated the densities from both mature (glycosylated) and immature forms of the protein. Compared with the CD82 inner loop, proteins containing the inner loop from CD81 gave higher p19 co-IP values, and these were statistically significant for the CD81 and CD81/IL82 pair, p < 0.05, and for the CD82 and CD82/IL81 pair, p < 0.05.

DISCUSSION

We reported previously that HTLV-1 is assembled and released from CD82-containing TEMs at the plasma membrane (25). This was supported by evidence showing the following: (i) HTLV-1 Gag colocalized with cell surface tetraspanins (CD81, CD82, and CD53) and with tetraspanin partners (major histocompatibility complex and β3-integrin); (ii) Gag and CD82 trafficked to cell-cell contact sites after superantigen-induced synapse formation; and (iii) CD82 was concentrated in the membranes of HTLV-1 virus-like particles as they budded from cells. Targeting and assembly of virions at TEMs is not unique to HTLV-1 but has been reported for HIV-1 as well (26, 27). In addition to showing the colocalization of HTLV-1 Gag with CD82, we also reported that stable complexes containing CD82 and MA were recovered after extracting cells with buffer containing Brij97 detergent. In these experiments we show that the inner loop and palmitoylated cysteines of CD82 or CD81 are important for the association with HTLV-1 Gag. These results are directly related to general mechanisms of Gag-membrane association and Gag targeting to specific microdomains.

N-terminal myristoylation of retroviral Gag proteins and clusters of basic amino acids in MA are essential for membrane association. The underlying reasons for this were deduced from cellular and structural studies of HIV-1 Gag. HIV-1 Gag is targeted to membranes because its MA domain interacts with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), which is localized to specific sites on the inner leaflet of the plasma membrane (35). Structural studies revealed that a cleft in HIV-1 MA accommodates the 2′-fatty acid chain and phosphoinositol group of PI(4,5)P2, which helps anchor MA to the membrane (36). PI(4,5)P2 binding also induces a conformational switch that exposes the N-terminal myristate group on MA, thus providing an additional membrane tether. It is not yet known whether HTLV-1 MA binds PI(4,5)P2 or a different membrane lipid. Although the conserved structures of retroviral MA domains (37) suggest a common mechanism for membrane targeting, HTLV-1 and HIV-1 Gag target different membrane microdomains (25). HTLV-1 Gag could target specific TEMs if it were to bind a lipid, such as PI(4,5)P2, which is concentrated in TEMs. Alternatively, HTLV-1 Gag targeting to TEMs could result from MA binding to a lipid and also to the inner loop of certain tetraspanins.

Although HTLV-1 Gag colocalized with TEMs in cells, only the MA dimer coprecipitated with CD82 in the Brij97 extraction buffers that have been used extensively to study tetraspanin interactions with cellular proteins (15, 20, 25, 38). The structure of the MA domain, and its interaction with membranes, changes after Gag assembly and maturation. In HTLV-1, the MA dimer forms late in the budding process (39) and appears to interact with the Brij97-resistant membrane fraction more strongly than either Gag or the MA monomer. This may reflect a unique structure for the MA dimer, as well as the potential to form four membrane tethers, which results in a stable interaction with Brij97-resistant TEMs.

Other cytoplasmic proteins, such as protein kinase Cα or phosphoinositide 4-kinase, have been shown to form stable interactions with CD82 that persist in Brij97 detergent extraction conditions (18, 20, 40). The determinants in CD82 for these cytoplasmic interactions appear to reside in the N terminus and/or C terminus of the tetraspanin (20). These regions of CD82 were dispensable for interaction with HTLV-1 MA. The sequence of the LEL is highly variable among tetraspanins and is responsible for the specific interactions with integrins, receptors, and other membrane proteins (12). Mutation of the CD82 LEL did not affect its interaction with HTLV-1 Gag, consistent with this interaction being mediated by the inner loop. So far, HTLV-1 Gag is the only protein whose interaction with tetraspanins is affected by mutations of the inner loop. The conservation of the inner loop sequence among tetraspanins suggests that HTLV-1 MA is likely to interact with other family members. Indeed, the association of HTLV-1 MA with the CD81 inner loop sequence supports this notion.

The interaction of HTLV-1 Gag with CD82-containing TEMs is independent of the viral envelope glycoprotein (Env), yet Env has also been reported to associate with CD82 (41). In fact, monoclonal antibodies against CD81 and CD82 were shown previously to inhibit HTLV-1 Env-induced syncytia formation (42, 43). It will be interesting to determine how HTLV-1
Env is targeted to TEMs. The association of both Gag and Env proteins with the same membrane microdomain could ensure that the virion components assemble at the same membrane location. Furthermore, because CD82 couples cell surface protein complexes to the cytoskeleton (44, 45), virus assembly targeted to TEMs would coordinate and position virus release to sites of cellular adhesion. We are currently examining the effects of mutations in the MA domain of HTLV-1 Gag on TEM targeting.

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