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Secretory IgA Specific for a Conserved Epitope on gp41 Envelope Glycoprotein Inhibits Epithelial Transcytosis of HIV-1

Annette Alfsen, Pierre Iniguez, Edwige Bouguyon, and Morgane Bomsel

As one of the initial mucosal transmission pathways of HIV (HIV-1), epithelial cells translocate HIV-1 from apical to basolateral surface by nondegradative transcytosis. Transcytosis is initiated when HIV-1 envelope glycoproteins bind to the epithelial cell membrane. Here we show that the transmembrane gp41 subunit of the viral envelope binds to the epithelial glycosphingolipid galactosyl ceramide (Gal Cer), an alternative receptor for HIV-1, at a site involving the conserved ELDKWA epitope. Disrupting the raft organization of the Gal Cer-containing microdomains at the apical surface inhibited HIV-1 transcytosis. Immunological studies confirmed the critical role of the conserved ELDKWA hexapeptide in HIV-1 transcytosis. Mucosal IgA, but not IgG, from seropositive subjects targeted the conserved peptide, neutralized gp41 binding to Gal Cer, and blocked HIV-1 transcytosis. These results underscore the important role of secretory IgA in designing strategies for mucosal protection against HIV-1 infection. The Journal of Immunology, 2001, 166: 6257–6265.

Mucosal surfaces are the major site for HIV-1 entry (1). HIV-1 transmission occurs through exposure of mucosal surfaces to HIV-1-infected fluids, such as semen, colostrum, breast milk, and cervicovaginal fluid (2, 3). Mucosal Abs in seropositive subjects consistently contain S-IgA specific for HIV-1 envelope glycoproteins (4). Studies in both macaques and humans (5, 6) suggest that HIV-1 mucosal Abs participate in host resistance to infection. Indeed, HIV-1-specific S-IgA has been detected in mucosal fluids of uninfected individuals having unprotected sexual intercourse with HIV-1-seropositive partners, including sex workers and sero-different couples (7–9).

We previously showed that HIV-1 can cross the epithelium in vitro by transcytosis across epithelial cells, the most abundant cell type at mucosal surfaces (10, 11). In these studies, we reconstituted the first step of HIV-1 infection in an in vitro model using a monolayer of stratified epithelial cell line barrier. PBMCs infected with HIV-1 primary isolates (HIV-1) PBMCs) adhered to the apical surface of the epithelial cells (12), establishing a microenvironment similar to the “immunological synapse” at the dendritic cell-T lymphocyte interface during Ag presentation (13). Contact between HIV-1-infected PBMCs and epithelial cells induces massive polarized budding of HIV-1 in the microenvironment of the synapse. The newly formed virus is rapidly internalized by the epithelial cells, transcytosed to the basolateral pole of the cell, and released as infectious virions into the basolateral (serosal) environment where the virions could infect lamina propria mononuclear cells. The relevance of this in vitro model using human transformed cell lines as target cells to in vivo early mechanism of HIV-1 entry was confirmed using specimens of human mucosal tissue (11), as infection in human is absolutely nonethical.

Two molecules are required for the transcytosis of HIV-1 across CD4-negative epithelial cells (10): the HIV-1 surface envelope glycoprotein subunit gp120 and glycosphingolipid galactosyl ceramide (Gal Cer),3 which is thought to act as gp120 epithelial cell receptor (14, 15). Gal Cer is markedly enriched at the apical surface of epithelial cells (16) and participates in the establishment of microdomains referred to as rafts, which act as platforms for endocytosis (17, 18) and transcytosis (19, 20). Here we show that a conserved epitope on gp41, the transmembrane subunit of the HIV envelope glycoprotein, mediates gp41 binding to epithelial cell Gal Cer and that S-IgA neutralizes this binding, thereby preventing HIV-1 transcytosis across epithelial cell monolayers. In addition, we identify a conserved epitope on gp41 to which the neutralizing S-IgA is directed and that this conserved epitope is immunodominant for the humoral mucosal response in HIV-seropositive patients. Finally, we demonstrate that both the binding of HIV-1 envelope glycoproteins to Gal Cer and the organization of Gal Cer in raft microdomains is required for effective HIV-1 transcytosis.

Materials and Methods

Antibodies

Cervicovaginal secretions were collected from eight HIV-1 (clade E)-seropositive women at the Institut Pasteur de Pnom Phen, Cambodia. Secretions were collected after 2 days of sexual abstinence using 3 ml of sterile PBS. Samples were centrifuged and frozen and stored at −80°C. Sample contamination by sperm was measured using the seminal fluid detection assay (SEMA; Humangen Fertility Diagnostics, Charlottesville, VA) according to the manufacturer, but optimized by increasing the incubation times and using 0-phenylenediamine dihydrochloride as substrate. Contaminated samples were discarded. Colostrum samples were collected from five HIV-1 (clade A)-infected women aged 25–40 years from the Institut National de la Sante et de la Recherche Medicale, Unité 332, Institut Cochin, Paris, France.

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3 Abbreviations used in this paper: Gal Cer, glycosphingolipid galactosyl ceramide; HIV-1, PBMC, PBMC infected with HIV-1 primary isolates; mAbs, methyl-β-cyclodextrin; TEB, transepithelial resistance; NC, nitrocellulose; CT-B, the B subunit of cholera toxin; PC, phosphatidylcholine.

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Pastor de Bangui, Cambodia (21). All subjects were at non-AIDS stages of HIV infection. Control specimens consisted of two pools of 10 colostrums from healthy seronegative volunteers. S-IgA and IgG were purified, as described earlier (21). 2FS is a human mononuclear IgG (26). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63).

Antigens
Recombinant HIV-1 envelope glycoprotein gp160 HIV-1 MN/LAI (V.V.T.G.9150) was obtained from Pasteur-Mérieux Sérum et Vaccins (Lyon, France) or from Advanced BioScience Laboratories (Kensington, MD). Recombinant HIV-1-gp 41 and gp160 were obtained from Intrascel (Cambridge, MA).

ELISA
ELISAs were performed as described previously (21). Briefly, plates were coated overnight at 4°C with 100 µl/well of either recombinant HIV-1 envelope glycoprotein gp160 (2.5 µg/ml) or HIV-1 peptides (0.2 µg/ml) diluted in PBS. After blocking the plates with 3% skimmed milk and washing, purified S-IgA or IgG were added and incubated for 1 h at 37°C. After washing, rabbit HRP-labeled Abs to human Fab(′)2 (1/2000) were added for 1 h at 37°C. HRP activity was detected with o-phenylenediamine (Sigma, St. Louis, MO) and read at 492 nm with a Titertek Multiskan spectrophotometer (Flow Laboratories, Glasgow, U.K.). The results were compared with a standard curve of a pool of whey from human milk containing 460 µg/ml of S-IgA. A large pool of 250 normal human sera containing 14 mg/ml of IgG was used as serum standard. The specific activities of S-IgA and of IgG against gp160 were expressed as arbitrary units per mg of Igs.

The ratio of S-IgA/IgG anti-gp160 = S-IgA specific activity for gp160/ IgG specific activity for gp160 represents the relative amount of Abs in the two Ig subclasses. For the peptide specific activity analysis, the results for each colostrum sample and cervicovaginal secretion (θ) are given by the ratio of S-IgA/IgG anti-peptide = Qi/ratio of S-IgA/IgG anti-gp160, where Qi = S-IgA specific activity for peptide/ IgG specific activity for peptide i is the ratio of the specific activity of S-IgA to that of IgG for the various HIV-1 envelope glycoprotein peptides i. Therefore, the ratio of S-IgA/IgG anti-peptide is Qi normalized to the total amount of anti-HIV-1 envelope glycoprotein Abs contained in S-IgA subclass relative to IgG.

HIV-1† PBMC
HIV-1† PBMC were prepared as described previously (11). Primary isolates were obtained by culture of PBMCs from HIV-1-infected Cambodian women (subtype A, nonsyncytia-inducing phenotype) and from HIV-1-infected Italian mothers (subtype B, syncytia-inducing and nonsyncytia-inducing phenotypes) (64).

Epithelial cell transcytosis assay and neutralization of transcytosis by S-IgA and IgG or by methyl-β-cyclodextrin (mβCD)
HIV-1 transcytosis across epithelial cells and neutralization of transcytosis by Abs was performed as previously described (21). Briefly, the endometrial cell line HEC-1 or intestinal cell line HT-29 clone 19 (65) cells expressing apical Gal Cer (data not shown) were each grown as a tight, monolayer of S-IgA and of IgG against gp160 were expressed as arbitrary units per mg of Igs.

The ratio of S-IgA/IgG anti-gp160 = S-IgA specific activity for gp160/ IgG specific activity for gp160 represents the relative amount of Abs in the two Ig subclasses. For the peptide specific activity analysis, the results for each colostrum sample and cervicovaginal secretion (θ) are given by the ratio of S-IgA/IgG anti-peptide = Qi/ratio of S-IgA/IgG anti-gp160, where Qi = S-IgA specific activity for peptide/ IgG specific activity for peptide i is the ratio of the specific activity of S-IgA to that of IgG for the various HIV-1 envelope glycoprotein peptides i. Therefore, the ratio of S-IgA/IgG anti-peptide is Qi normalized to the total amount of anti-HIV-1 envelope glycoprotein Abs contained in S-IgA subclass relative to IgG.

Ligand blotting
Ligand blotting was performed as previously described (69). Briefly, lipids, Gal Cer (Avanti Polar Lipids, Alabaster, AL), monosialoganglioside GM1 (Sigma), or phosphatidycholine (PC; Serdary Research Laboratories, London, Ontario, Canada) were dissolved in chloroform/methanol (1:1) at 5 mg/ml and spotted onto nitrocellulose (NC) supports (Bio-Rad, Hercules, CA). The NC strips were incubated for 1 h at room temperature in PBS containing 8% BSA to saturate nonspecific binding sites. Recombinant gp140 (gp120, 4 nM) or gp120 (4 nM) was added to the apical chamber and incubated for 10 min at 37°C. To initiate virus transcytosis, 2 × 10⁶ HIV-1 PBMC were added to the apical chamber. Contact between HIV-1† PBMCs and the epithelial cell monolayer resulted in rapid budding of the HIV-1-virions, followed by their transcytosis from the apical to the basolateral pole of the epithelial cells. After 2 h, inhibition of transcytosis by Ab was determined by detection of p24 in the basolateral medium by ELISA (Pasteur Sanofi, France). After 2 h, the level of p24 in the presence of Ab or in the presence of control nonimmune clostral S-IgA or IgG was measured and found to be similar with a value of 150 pg/ml. This value was taken as 100% of transcytosis and used to express the results. When indicated, Ig was preincubated with the ELDKWA peptide to block the recognition site on the Ig molecule as described (11). As control, an irrelevant ELDKWA scrambled peptide was used. After overnight binding at 4°C, remaining unbound peptide was dialyzed before addition of the Ig fraction as described above. Assays were performed in duplicate for each of the variables: HIV subtype (PBMCs infected with HIV subtype A or B subtypes) and cell lines (HT29 or HEC-1), which is between four and ten different conditions. Interassay variation was <10%. Fig. 2 shows the mean values of these combined experiments.

To analyze the role on HIV-1 transcytosis of the cholesterol-depleting drug mβCD (Sigma), optimal drug concentration and incubation time were determined using previously described conditions (29, 66, 67). Tight HT29 cell monolayers were preincubated first for 30 min at 37°C in RPMI 1640 supplemented with 0.25% fatty acid-free BSA (Boehringer Mannheim, Germany). mβCD was then added at 1, 5, and 10 mM, and the cell monolayers were incubated up to 4 h at 37°C. Transepithelial resistance (TER) was measured every hour (10). Western blotting experiments obtained from immortalized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63). Monoclonal human IgGs C31 anti-gp120 and D1 anti-gp41 used in ligand blotting experiments were obtained from immobilized PBMCs of an HIV-1-seropositive patient (62, 63).
Results

Clostral and cervicovaginal S-IgA to the ELDKWA motif of gp41

We previously reported that clostral IgG and S-IgA from HIV-1-seropositive women bind to the HIV-1 envelope glycoprotein precursor gp160 (21). We now investigate the epitope(s) on gp160 that were recognized by the S-IgA and Ig, using Abs from both colostrum and cervicovaginal secretions (Fig. 1, b and d). The specific activity of S-IgA and IgG for four epitopes on the HIV-1 envelope glycoproteins implicated in HIV-1 infection was analyzed. Two epitopes were on gp120: a V3 loop (MN isolate) hypervariable region and the C5 constant region (22) shown to be associated with neutralization or with slow HIV-1 progression. The other two epitopes corresponded to conserved gp41 motifs: the principal immunodominant domain (PID) (23) and the ELDKWA motif. The ELDKWA motif (aa 662–667) is a conserved epitope recognized by human serum IgG 2F5, the only Ig thus far identified that is directed against the gp41 and capable of neutralizing infection by a large majority of primary HIV-1 isolates (24). ELDKWA is part of the larger peptide DP178 (also called T-20) that has been shown to inhibit HIV-1 infection at nanomolar concentrations in cell culture experiments and to have an antiviral effect in a phase I clinical trial in humans (25). We previously showed that this epitope was used by both dimeric IgA and IgM isolated from HIV-1-seropositive patients to neutralize intracellularly HIV-1 transcytosis by epithelial cells (11). The results in Fig. 1 clearly indicate that for each of the five colostrums and seven of eight cervicovaginal secretions tested, S-IgA anti-HIV-1 envelope bound mainly to the ELDKWA epitope of gp41. In contrast, IgG anti-HIV-1 envelope from the same specimen of secretion failed to bind this gp41 domain. For the one cervicovaginal sample (Fig. 1d, 118), the amount of S-IgA specific for ELDKWA was still significant and 13 times more abundant than the paired IgG. This difference in Ig subclass binding to ELDKWA peptide is due to a higher S-IgA and lower IgG reactivity for each sample. In contrast, the reactivity for gp160 was always higher for IgG than for IgA (Fig. 1, a and c). Indeed, the ratio of clostral S-IgA anti-gp160 to clostral IgG anti-gp160 was 0.015–0.1 (Fig. 1a), indicating a much lower quantity of S-IgA than IgG specific for HIV envelope glycoprotein in colostrum. A similar analysis of cervicovaginal S-IgA and IgG confirmed the ratio (ratio of S-IgA/IgG anti-gp160 < 1) (Fig. 1c), indicative of the higher IgG anti-gp160 content as compared with S-IgA anti-gp160 in mucosal secretions.

Transcytosis of HIV-1 across a tight epithelial barrier is impaired by S-IgA against the ELDKWA motif of gp41

We next investigated whether polyclonal S-IgA and IgG purified from the colostrum of HIV-1-seropositive women could neutralize transcytosis of HIV-1. For these experiments, we used our previously described transcytosis assay and PBMCs acutely infected with primary isolates of HIV-1 of subtype A or B (HIV-1+ PBMCs). The ability of mucosal Ig to inhibit transcytosis of HIV-1 was higher for S-IgA (80% mean reduction) than for IgG (49% mean reduction) (Fig. 2). The amount of virus produced at the apical pole of the epithelial barrier during the assay was similar in the absence or presence of the Abs (data not shown), indicating that the Abs did not alter PBMC virus production. In addition, no difference in neutralization of transcytosis was detected using PBMCs acutely infected with HIV-1 isolates of subtype A or B. Clostral S-IgA and IgG were purified from seropositive women infected with HIV-1 subtype A but could equally neutralize transcytosis of primary isolates of subtypes A, B, and D. This strongly suggested that the epitopes recognized by the neutralizing Abs are conserved regions of HIV-1 envelope glycoprotein. No difference in transcytosis could be detected using HEC-1 endometrial or HT29 (clone 19) intestinal cell lines. Such inhibitory efficiency for S-IgA even though not complete, is biologically significant, considering that the Ig samples were polyclonal; consequently, titration of specific Ab-mediated inhibition could not be performed.

Because S-IgA but not IgG from the different colostrums (Fig. 1b) and cervicovaginal secretions (Fig. 1d) strongly recognized the ELDKWA peptide, we investigate whether this epitope was recognized by the colostrum Igs that neutralized HIV-1 transcytosis.

**FIGURE 1.** The ELDKWA peptide from gp41 is a major epitope recognized by clostral and cervicovaginal S-IgA of HIV-1-seropositive women. For each clostral (a) and cervicovaginal (c) sample (#), S-IgA and IgG binding activity to HIV-1 envelope glycoprotein gp160 was compared. The relative amount of anti-HIV-1 envelope glycoprotein Abs contained in each Ig subclass sample is given by the ratio of S-IgA/IgG anti-gp160 calculated as in Materials and Methods. For each clostral (b) and cervicovaginal (d) sample (#), specific activities of S-IgA and IgG binding to various HIV-1 envelope glycoprotein peptides (i) were compared and are given by the ratio of S-IgA/IgG anti-peptide calculated as described in Materials and Methods.
As reference, serum-derived IgG 2F5 Ab (.1 mg) were added to the standard in which control clostral S-IgA and IgG were added to the apical medium. After a 2-h incubation at 37°C, the extent of viral transcytosis was evaluated from the serosal (basolateral) medium by detection of HIV-1 p24 Ag. The results are expressed as the percentage of transcytosis compared with the HIV-1 subtype used to infect PBMCs nor by the origin of the epithelial cell line used in the transcytosis assay. These results reinforce the concept that ELDKWA is a crucial epitope in HIV-1 infection. Indeed, it is the minimal hexapeptide recognized by the transcytosis-neutralizing colostral S-IgA (11), and neutralizing 2F5 IgG (26).

Both HIV-1 envelope gp41 and gp120 subunits interact with epithelial cell Gal Cer

The ability of anti-ELDKWA Abs to inhibit HIV-1 transcytosis suggested that the hexapeptide epitope of gp41 participates in the interaction between HIV-1 and its epithelial cell target. The glycosphingolipid Gal Cer has been shown to act as the gp120 receptor on epithelial cells (14, 15). We have previously shown that this interaction likely plays a role in transcytosis because Abs to both anti-gp120 and anti-Gal Cer completely blocked HIV-1 transcytosis (10). Based on structural and thermodynamic considerations (A. A. and M. B., manuscript in preparation), we investigated whether gp41 also interacts with Gal Cer and whether this interaction involves hexapeptide ELDKWA.

First, as shown morphologically in Fig. 3, recombinant gp120 as well as gp41 bind to the apical surface of a tight epithelial cell

In experiments designed to block neutralization, equal amounts of purified IgG and S-IgA were preincubated with ELDKWA peptide or with a ELDKWA scrambled irrelevant peptide for control (Fig. 2). After dialysis to eliminate excess free peptide, the Ig samples were assayed for their capacity to neutralize HIV-1 transcytosis. Neutralization was significantly decreased when S-IgA, but not IgG, had been preincubated with the ELDKWA peptide implicating ELDKWA as a key peptide involved in S-IgA inhibition of HIV-1 transcytosis. Transcytosis neutralization was not inhibited by an excess of peptide inadequately removed by dialysis because under the conditions referred to as “standard + ELDKWA” (Fig. 2), HIV-1 transcytosis was not affected. In this condition, before addition to the cell system, the ELDKWA peptide was incubated alone (without Abs) at 4°C overnight followed by dialysis with a cutoff at 10 kDa to remove the free peptide. Incubation with the ELDKWA scrambled peptide did not alter the neutralization efficiency, indicating the specificity of the ELDKWA peptide sequence in Ab-mediated inhibition of transcytosis. The ability of ELDKWA to more strongly diminish transcytosis inhibition by S-IgA than by IgG (55 vs 5%, respectively) is consistent with the ability of S-IgA to bind ELDKWA more strongly than IgG (Fig. 1, b and d). The amount of colostrum 48 and cervicovaginal fluids were insufficient for analysis in the transcytosis assay.

Expressing HIV-1 transcytosis inhibition as a function of the specific activity of the Ig for ELDKWA (Fig. 1) revealed that the capacity to neutralize transcytosis and the involvement of ELDKWA in this process is much more efficient for S-IgA than IgG. Prevention of neutralization by ELDKWA was affected neither by the HIV-1 subtype used to infect PBMCs nor by the origin of the epithelial cell line used in the transcytosis assay. The ability of ELDKWA to more strongly diminish transcytosis inhibition by S-IgA than IgG (55 vs 5%, respectively) is consistent with the ability of S-IgA to bind ELDKWA more strongly than IgG (Fig. 1, b and d). The amount of colostrum 48 and cervicovaginal fluids were insufficient for analysis in the transcytosis assay.
barrier. Both interactions were antagonized by monoclonal anti-Gal Cer Abs. In contrast, only the gp41 interaction with the apical epithelial surface was inhibited by a 100 molar excess of the EL-DKWA peptide, whereas the binding of gp120 was not altered by this peptide. That gp41 bound specifically to Gal Cer via EL-DKWA is also supported by the inability of an irrelevant or an EL-DKWA scrambled peptide to impair this interaction and the lack of binding of control human IgA to the apical surface of the cell (data not shown).

Second, by using a ligand blotting approach, we showed that recombinant gp41, similar to gp120, binds Gal Cer immobilized on a NC support (Fig. 4a). No binding to other glycolipids, such as the ganglioside GM1, or a phospholipid, such as PC, was detected (Fig. 4e). As a control for specificity, we also showed that CT-B bound only its known receptor GM1 (27) and not Gal Cer or PC (Fig. 4e).

Third, a 100 molar excess of the EL-DKWA peptide completely inhibited the interaction of gp41 with Gal Cer (Fig. 4a, arrow) but not the interaction between gp120 and Gal Cer. EL-DKWA scrambled peptide did not inhibit binding of gp41 to Gal Cer, and the binding of CT-B to GM1 was not sensitive to the presence of the EL-DKWA peptide, further indicating that gp41 binding with Gal Cer involved EL-DKWA.

We next compared the capacity of S-IgA and its paired IgG to neutralize HIV-1 transcytosis (Fig. 2) with their capacity to inhibit the binding of gp41 to Gal Cer. In this experiment, ligand blotting was performed in the presence of sample 63 or 64 S-IgA or IgG. Igs from these patients were chosen because a relatively large quantity of material was available, S-IgA bound EL-DKWA substantially better than its paired IgG (ratio of ~20, see Fig. 1b), and the S-IgA was highly effective in neutralizing HIV-1 transcytosis (>84%, see Fig. 2). Results obtained with samples from patients 63 and 64 were identical, and only those obtained with sample from patient 63 are shown on Fig. 4b. As shown in Fig. 4b, S-IgA alone efficiently blocked gp41 binding to Gal Cer (Fig. 4b, arrow) but not gp120 binding to Gal Cer. Additionally, Gal Cer binding of both gp41 and gp120 was unaffected by paired clostral IgG. Finally, we addressed whether the capacity of S-IgA to inhibit gp41 binding to Gal Cer could be ascribed to the efficiency of S-IgA binding to EL-DKWA (Fig. 1). S-IgA (63 or 64) or paired IgG were preincubated with EL-DKWA, and unbound peptide was removed by dialysis, as in Fig. 2. The Igs were assayed for inhibiting gp41 or gp120 binding to Gal Cer. S-IgA, not matched IgG, preincubated with EL-DKWA had lost its capacity to inhibit gp41 binding to Gal Cer (Fig. 4b). As additional control for specificity, an EL-DKWA scrambled irrelevant peptide was used and had no effect (data not shown). These results indicate that EL-DKWA is a gp41 epitope functionally involved in HIV-1 binding to and transcytosis across epithelial cells and that EL-DKWA is the epitope used by the S-IgA for neutralizing HIV-1 transcytosis.

Disruption of the organization of raft microdomains containing Gal Cer inhibits HIV-1 transcytosis

Glycolipids (mainly Gal Cer) and cholesterol form dynamic microdomains functioning at the apical epithelial membrane (16, 28). These microdomains create floating rafts in otherwise phospholipid-rich membrane environments and are involved in membrane trafficking to and from the apical membrane, including apical endocytosis (17, 20) and transcytosis (19, 20). These transient microdomains have been shown to be stabilized by cholesterol (29), and depletion of cholesterol from cell membrane destabilized these raft microdomains (29), therefore impairing their functional activity. To investigate the functional role of Gal Cer containing raft microdomains in HIV-1 transcytosis, the raft organization was disrupted by cholesterol depletion using mβCD.

We first established the optimal conditions for mβCD disruption of raft microdomains without affecting the integrity and tightness of the epithelial barrier (see Materials and Methods). Treatment for 1 h at 37°C with 1–10 mM mβCD, concentrations usually used to disrupt the domain (29), did not alter the TER of the epithelial barrier (Fig. 5). The drug was then removed and HIV-1 transcytosis was assessed as described above. As shown in Fig. 5, mβCD inhibited HIV-1 transcytosis in a concentration-dependent manner with almost total inhibition at 10 mM. mβCD pretreatment of epithelial monolayers did not modify the apical production by HIV-1+–PBMCs of HIV-1 presented for transcytosis (data not shown), and therefore could not account for the inhibition of transcytosis. The effect of mβCD on transcytosis was reversible when the drug was removed and epithelial cells were allowed to replenish their cholesterol content for 12 h prior the onset of the transcytosis assay (data not shown). These data suggest that HIV-1 transcytosis required the interaction of HIV-1 envelope glycoprotein subunits with the apical epithelial Gal Cer as well as the functional organization of Gal Cer in raft microdomains stabilized by cholesterol.
transcytosis in the absence of drug. TER was measured at the end of the experiment as the percentage of the transcytosis inhibition compared with control. The transcytosis results (closed bar) are expressed as the percentage of transcytosis inhibition compared with control. TER was measured at the end of the experiment.

Sphingolipids (35, 36). Gal Cer, a monohexosylceramide, is a glycolipid characteristic of the apical membrane epithelial cell. Transient lateral assemblies of glycosphingolipids stabilized by cholesterol, referred to as raft microdomains (18, 37, 38), function in epithelial cells as platforms for trafficking toward the apical membrane as well as in apical endocytosis (17, 20). Raft microdomains are also involved in transcytosis across endothelial cells (39). Apical lipid rafts invaginate forming caveolar structures that contain the cholesterol-binding protein caveolin (40, 41). Such dynamic structures are endocytosed allowing endocytosis and transcytosis (20). Cholesterol depletion completely abolishes the formation of apical caveolae and blocks internalization and transcytosis of clustered raft proteins (20), indicating the stabilizing role of cholesterol in raft structure and the highly dynamic nature of raft microdomains.

Here we have shown that the interaction between HIV-1, and more specifically ELDKW, on gp41 with epithelial cells is dependent on the raft organization on the apical epithelial membrane. Our studies reinforce the concept that HIV-1 is endocytosed at the apical surface and then transcytosed to the basal surface. First, HIV-1 envelope glycoprotein subunits bind specifically to Gal Cer, an essential component of raft structure and dynamics. Second, disruption of the organization of raft microdomains completely blocks transepithelial transcytosis of HIV-1. HIV-1 may enter epithelial cells via caveolar structures. The distance between the two Gal Cer binding sites on gp120 and gp41, estimated to be 5 nm (32, 34), is in good agreement with the proposed size of a stabilized raft microdomain having an estimated size between 3 and 50 nm in diameter (29, 37, 42). Therefore, the interaction of one spike of gp120-gp41 multimers at the surface of the virus with one domain of the target epithelial cell membrane could be a cooperative one. The primary contact of the gp120 part of the spike with a Gal Cer microdomain would stabilize this raft domain, allowing a favorable orientation and interaction of the gp41 exposed peptide within the same space. However, the involvement of clathrin-coated structures cannot be entirely excluded yet, as cholesterol depletion has also been shown to affect clathrin-dependent endocytosis in epithelial cells, although to a much lesser extent (20).

We and others have previously shown that transcytosis of the virus budding from HIV-1-infected mononuclear cells is dependent on the contact between the HIV-1-infected cell and the apical epithelial cell surface (10–12). Transcytosis, but not the viral budding event, is inhibited by Arg-Gly-Asp added to the apical surface. Arg-Gly-Asp peptides broadly inhibit cell-cell contact mediated by integrin-disintegrin interactions (M.B., unpublished data). Because epithelial cells are known to express adhesion molecules like ICAM-1 apically, these results suggest that the HIV-1 infection of mononuclear cells, which up-regulates surface expression of integrins like LFA-1 (43), interact with the target epithelial cell via integrin-disintegrin interactions. Such interactions would circumscribe a microenvironment between the two cells, which resembles the recently described immunological synapse (13, 44, 45). Actin is required for polarized HIV-1 budding from HIV-1-infected cells at the epithelial cell surface (46). Similarly, the dynamics of actin filaments polymerization, induced upon cell-cell contact and mediated by integrins, play an important role in the formation of the immunological synapse. Actin filaments organize the synapse into distinct supramolecular activation clusters that favor the initiation of signal transduction cascades. The actin scaffold would recruit or stabilize specialized membrane domains enriched in glycolipids (13, 47). The interaction of HIV-1-infected mononuclear cells with epithelial cell inducing HIV production and transcytosis may be similar to the formation of immunological synapse for T cell activation. This adhesion mechanism would
of HIV of the main viral subtypes (A-H), some rare isolates harbor the ELDKWA peptide has been found identically conserved in their respective functions and probably also account for their rare occurrence in patient sera (49). The existence of such specific epitope (57). Several non-mutually exclusive parameters can be invoked.

First, the structure of neutralizing Abs: the multimeric structure of S-IgA, as compared with monomeric IgG, most likely plays a role in the affinity/avidity of the Ab for the target (50) and, therefore, for the neutralizing function. HIV-neutralizing IgGs do not have a remarkably rapid mutational rate nor do they reveal striking pressure for replacement mutations in V region framework regions and complementary-determining regions. However, both the neutralizing 2F5 and 2G12 IgGs that recognize, respectively, the ELDKWA peptide and a region on SU-gp120 carry an unusual heavy chain complementary-determining region 3 that might mediate their respective functions and probably also account for their rare occurrence in patient sera (49). The existence of such specific structures in neutralizing S-IgA remains to be established.

Second, the compartmentalized induction of mucosal vs systemic humoral immunity (51, 52), and within the mucosal compartment itself, as has been recently demonstrated (53–55). However, mechanisms responsible for compartmentalization remain unclear.

Third, the tolerance induced by self-peptide (56): self-peptide could originate from a soluble protein like the human complement factor H present at high concentration in the plasma, which has been shown to bind to TM-gp41 at a site overlapping the 2F5 epitope (57).

Fourth, the structure of the epitope that elicits neutralizing Abs: ELDKWA is the minimal hexapeptide recognized by neutralizing 2F5 IgG (26) by clostral S-IgA (present studies) and by dimeric IgA as we have shown earlier (11). However, injection of this hexapeptide in animal models never leads to the induction of neutralizing Abs, whatever the site of induction and the class of Ab (IgG or IgA) induced. This indicates that either the immunogenically functional epitope is larger than the hexapeptide and/or that the conformational structure of this peptide within TM-gp41 is of great importance for its immunogenicity. Furthermore, even if the ELDKWA peptide has been found identically conserved in >85% of HIV of the main viral subtypes (A-H), some rare isolates harbor in this region a partial conservation with the leucine (L) and the tryptophan (W), as is also the case for HIV-2 (EH0) and SIV (mac239) (58). This suggests that not only the sequence but also the structure of the region encompassing ELDKWA is of importance and could define a mimotope, essential for HIV transcytosis via Gal Cer binding and induction of humoral immunity. It is striking that the overlapping peptide DP178/T20 (aa 638–673) is able to neutralize infection of cells in vitro by primary HIV isolates, and has been recently shown significantly to reduce the level of circulating HIV when administered to patients for 14 days (25). Indeed, DP178/T20 has been shown to adopt a helical structure and to assemble in tri- or tetrameric oligomers. Furthermore, the amino acid sequence 650–684 of TM-gp41 contains a stack of aromatic hydrophobic residues (W and F) that extend the DP178 sequence at its C-terminal end up to the transmembrane region (aa 684) (59). These tryptophans have been very recently found to be essential for HIV interaction with its target, at a post binding but pre-fusion step (58) as is the case here, because HIV do not fuse with the epithelial cell membrane during transcytosis (10). This suggests that this stack contributes to the structure of the sequence starting at the ELDKWA peptide and extending N terminally to the charged polar amino acid up to the reported Ca2+ binding sites (60) by organizing the covering water surface layer. It would create a water layer between the peptide and the cell membrane that in turn would facilitate charge transfer processes (A. A. and M. B., manuscript in preparation) being at the origin of the signal for endocytosis (61).

The data presented here suggest that the ELDKWA hexapeptide of gp41 is a potential candidate Ag for a mucosal HIV-1 vaccine. First, the peptide is a conserved epitope. Second, ELDKWA is involved in the binding of gp41 to its epithelial receptor Gal Cer and is functionally involved in HIV-1 translocation across a tight epithelial barrier. Third, ELDKWA is an immunodominant Ag in the mucosal humoral response in seropositive patients. Fourth, S-IgA developed against the hexapeptide functionally blocks transcytosis, likely by interfering with the binding of gp41 via ELDKWA to epithelial Gal Cer. Fifth, as part of the DP178/T20 peptide that inhibits HIV-1 infection in cell culture experiments, ELDKWA is reported to have an antiviral effect in a phase I clinical trial in humans (25). Finally, the peptide is recognized by the 2F5 IgG that recently was shown to participate in the protection of macaque against SIV or chimeric HIV-1/SIV mucosal infection (30, 31) when used in a passive immunization protocol. Although the precise structure of the ELDKWA containing peptide that elicits its in vivo such neutralizing S-IgA has to be determined, the information presented here indicates the critical role the ELDKWA plays in the interaction of HIV-1 envelope glycoproteins and epithelial cells, the first step in epithelial cell translocation of HIV-1.

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