HIV-1-infected Blood Mononuclear Cells Form an Integrin- and Agrin-dependent Viral Synapse to Induce Efficient HIV-1 Transcytosis across Epithelial Cell Monolayer

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The heparan sulfate proteoglycan agrin and adhesion molecules are key players in the formation of neuronal and immune synapses that evolved for efficient communication at the sites of cell-cell contact. Transcytosis of infectious virus across epithelial cells upon contact between HIV-1-infected cells and the mucosal pole of the epithelial cells is one mechanism for HIV-1 entry at mucosal sites. In contrast, transcytosis of cell-free HIV-1 is not efficient. A synapse between HIV-1-infected cells and the mucosal epithelial surface that resembles neuronal and immune synapses is visualized by electron microscopy. We have termed this the “viral synapse.” Similarities of the viral synapse also extend to the functional level. HIV-1-infected cell-induced transcytosis depends on RGD-dependent integrins and efficient cell-free virus transcytosis is inducible upon RGD-dependent integrin cross-linking. Agrin appears differentially expressed at the apical epithelial surface and acts as an HIV-1 attachment receptor. Envelope glycoprotein subunit gp41 binds specifically to agrin, reinforcing the interaction of gp41 to its epithelial receptor galactosyl ceramide.

INTRODUCTION

For efficient transmission of information between adjacent cells, neuronal cells have evolved the synapse, the elaborate structure at the site of cell-cell contact in which pre- and postsynaptic membranes are tightly apposed (Bezakova and Ruegg, 2003). Immunological synapses, which have been described more recently (Dustin and Dustin, 2001; Khan *et al.*, 2001), share some of the molecules and processes involved in the formation and functions of the neurological synapse.

Adhesion molecules play a critical role in the initial stages of synapse formation: they contribute to the recognition events between pre- and postsynaptic cells, resulting in turn, in tight apposition or locking of the pre- and postsynaptic surfaces. The adhesive clamp provides stability and aligns the presynaptic “active zones” and postsynaptic elements in relation to one another (Dustin and Dustin, 2001).

Additionally, aggregation of pre- and postsynaptic surface molecules together with reorganization of membrane components at the synaptic zone is essential for synapse formation and for ensuring the rapid exchange of information between neurons. One such aggregating factor is agrin, a heparan sulfate proteoglycan of ~300–400 kDa with two major conserved sites of glycanation (reviewed in Bezakova and Ruegg, 2003) that is active, glycosylated at the neuronal synapse, but only deglycosylated at the immunological synapse (Gesemann *et al.*, 1995; Khan *et al.*, 2001; Yang *et al.*,

2001). Different isoforms of agrin arise from alternative mRNA splicing. These include a transmembrane form and an isoform bound to the basement membrane (Hilgenberg *et al.*, 1999; Neumann *et al.*, 2001; Hoover *et al.*, 2003). Agrin contains one heparin- and one integrin-binding site. Integrins, including the beta-1 integrin, have been shown to modulate agrin activities (Martin and Sanes, 1997; Burkin *et al.*, 1998; Martin, 2002). In immune cells, agrin aggregating activity, in synergy with integrins, has been shown to stabilize glycosphingolipid-enriched microdomains, known as lipid rafts, at the immunological synapse (Dustin and Dustin, 2001).

Host cell interaction with virus has long been studied with cell-free viral particles. However, recently the role of infected cells, especially infected immune cells, in disseminating infection has been revisited. A close contact between virus-infected cell and target cell has been visualized and shown to stimulate virus transmission and infection of the target cell (Phillips *et al.*, 1994; Bomsel, 1997; Igakura *et al.*, 2003; Jolly *et al.*, 2004). By analogy to immunological and neuronal synapses, the concept of a virally induced synapse has emerged to describe this exquisitely targeted mode of virus transfer at infected cell contacts with target cells (Bomsel, 2000, 2002; McDonald *et al.*, 2003; Arrighi *et al.*, 2004; Jolly *et al.*, 2004). In more recent studies (reviewed in Jolly and Sattentau, 2004; Piguet and Sattentau, 2004) several different types of synapses involved in virus transfer have been described, all designated as “virological synapse.” A first type occurs between the infected and the target cell leading to the fusion of the synaptic cell partners and infection of the target cell then allowing direct cell-cell transfer of the viral genetic material and in turn efficient viral dissemination (Igakura *et al.*, 2003; Jolly *et al.*, 2004). In this case, the requirement of actual cell-free viral particle formation re-

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mains uncertain. In a second type of synapse, similar to neuronal and immunological synapses, synaptic cell partners establish close contact but do not fuse together. Such synapses have been described between HIV-infected cells and their uninfected target cells and facilitates recruitment of cell-free viral particles at the synaptic cleft and their transmission across it. A DC-SIGN-mediated infectious synapse is operational for the transfer to CD4⁺ target cells of HIV internalized within dendritic cells, resulting in HIV fusion with and infection of the CD4⁺ T-cells (McDonald *et al.*, 2003; Arrighi *et al.*, 2004). Alternatively, HIV-infected cell-mediated synapses form between HIV⁺ mononuclear cells and epithelial cells leading to local budding of viral particles, endocytosis and transcytosis of the virus without fusion with the epithelial cell before release of virus at the serosal pole of the epithelial cells, and subsequent viral spread (Bomsel, 2000, 2002). To avoid any confusion between these different types of "virological synapses," we used the term of "virally mediated synapse" for describing this last type of synapse.

Despite the differences between the various types of synapses operative in virus transfer, and in addition to their specific morphological characteristics, several features are common to all of them. Recently, the "presynaptic" effector structures in the infected cell have been studied in more detail. Recruitment of adhesion molecules and lipid raft microdomains to the synaptic contact has been detected together with a polarization of the actin cytoskeleton with the microtubule organizing center (MTOC; reviewed in Jolly and Sattentau, 2004; Piguet and Sattentau, 2004). The polarization of the HIV-infected cell governing viral budding at the cell-cell contact involves a reorganization of the actin cytoskeleton (Pearce-Pratt *et al.*, 1994; Perotti *et al.*, 1996).

Sexual transmission of HIV-1 most commonly occurs through exposure of a mucosal epithelial surface to HIV-1-infected cells that are present in secretions (e.g., semen, cervico-vaginal fluid, colostrum, and milk). Early studies and our own (Phillips *et al.*, 1994; Bomsel, 1997; Bomsel *et al.*, 1998) have shown that HIV-1 entry into mucosal epithelial cells is much more efficient when HIV-1 particles bud locally after contact between HIV-1-infected cells and uninfected epithelial cells than by direct entry of cell-free virus into the epithelial cells, as is also true for CD4⁺ T-cells. This observation led us to hypothesize the occurrence of an HIV-1 "virological synapse" (Bomsel, 2000, 2002). HIV-1 entry into epithelial cells relies on the interaction of both the HIV-1 envelope glycoprotein gp120 (Harouse *et al.*, 1991) and gp41 (Bomsel, 1997; Alfsen *et al.*, 2001; Alfsen and Bomsel, 2002) with the epithelial glycosphingolipid receptor galactosyl ceramide organized in lipid raft microdomains (Alfsen *et al.*, 2001). A similar phenomenon has also been observed in the effector cell in other virological synapses (Jolly and Sattentau, 2004; Piguet and Sattentau, 2004).

The paradigm of the use of a unique viral receptor for virus entry into epithelial cells has been challenged by the description of additional attachment receptors (Bomsel and Alfsen, 2003). Heparan sulfate proteoglycans (HSPG) represent one broad class of apical attachment receptors. Interestingly, glycosaminoglycans can act as coreceptor for HIV-1 in immune cells as well as in endothelial cells (Ohshiro *et al.*, 1996; Bobardt *et al.*, 2003), but their role in facilitating viral entry into the epithelial cell remains elusive. The previously unsuspected detection of adhesion molecules, including RGD-dependent integrins at the apical surface of epithelial cells (Sonnenberg, 1993; Schwartz *et al.*, 1995; Hynes, 2002),

has allowed us to envision these molecules as apical partners for virus entry into epithelial cells.

Such data have raised the question of the nature of the signals transmitted from the infected cell to the epithelial cell and have suggested the formation of a virally mediated synapse to increase the transmission efficiency of viral particles between the two types of cells without direct transfer of viral material between the two cytosols of adjacent cells, as recently described between HIV-1 (Lai X4 cell adapted virus) chronically infected cell lines and target CD4⁺ T-cells (Jolly *et al.*, 2004) or for HTLV-I (Igakura *et al.*, 2003). We therefore attempted to define the exact nature of the molecules involved in the attachment and transcytosis of the virus and their possible role in the formation and function of the virally mediated synapse using not only chronically infected cells but also human peripheral blood mononuclear cells (PBMCs) infected by HIV-1 R5 or X4 molecular clones.

MATERIALS AND METHODS

Antibodies and Reagents

In HIV-1 transcytosis and binding assays, reagents and antibodies were used as follows. Blocking mouse anti-human $\beta 1$ integrin monoclonal antibody (mAb; DE9, UBI, Lake Placid, NY; Bergelson *et al.*, 1992) nonblocking mouse anti-human $\beta 1$ integrin mAb (LM534, Chemicon International, Temecula, CA; Giacotti and Ruoslahti, 1990) were used at 2 and 20 μ g/ml. Anti-heparan sulfate antibodies (mouse IgM) were from Seigagaku (Tokyo, Japan) and were used at 2 μ g/ml. Mouse anti-GalCer mAbs (IgG3; Ranscht *et al.*, 1982) were used at 5 ng/ml. The following rabbit polyclonal antibodies were kindly provided by M. Ruegg, Basel, Switzerland: 204 (antibody against mouse agrin C-terminal), 3240 (against chick agrin C-terminal) and used at 1:500 dilution. Heparin Choay (Sanofi-synthelabo, Paris, France) 5000 IU/ml or 33 mg/ml, an HSPG polydisperse polymer, was used at concentrations from 1 to 10 mg/ml. Purified heparin was obtained as a 9 kDa mw and sulfated disaccharide units with a degree of polymerization (dp) from dp2 to dp18 (mw from 600 to 5400 kDa), all kindly provided by H. Lortat Jacob, Grenoble, France (Sadir *et al.*, 2001) were used in a concentration range from 10 to 20 nM and from 2 to 100 nM, respectively. Recombinant chick agrin proteoglycans: the full-length B/z+ (inserts 7.4.8) or B/z- (0.0.0) glycosylated forms, or the 95-kDa nonglycosylated N25C95 B/z+ or B/z- forms (Bezakova and Ruegg, 2003) kindly provided by M. Ruegg, were used at concentrations from 2.5 to 20 nM for spectroscopy (Denzer *et al.*, 1995), and at 1 μ g per spot for dot blot. As a control for agrin specificity, secretory component or syndecan-1 were used in the concentration range of 5–10 nM.

RGD and RGL peptides were from Sigma (St. Louis, MO). Biotinylated RGD peptide (CGRGDTPC-biotin) and its control RGL, cyclized by addition of two cysteines to mimic its conformation at the tip of a loop present on disintegrins (Bomsel *et al.*, 1998) was synthesized by Eurogentec (Seraing, Belgium), with 98% purity as shown by HPLC analysis, and used at concentration up to 100 μ M as indicated. When indicated, RGD tetramers were formed by incubation of biotinylated RGD peptide with streptavidin at 1:4 M ratio for 30 min at 37°C. As a control streptavidin alone was used. Alternatively, octamers of RGD peptides (RGDx8) were chemically synthesized with 98% purity by Eurogentec and used at concentration up to 25 μ M as indicated.

Heparinase I and III (Sigma) at 10 IU/ml were added apically, and epithelial cell monolayers were then incubated 30 min at 37°C in Hank's balanced salt solution with these enzymes as indicated (Saphire *et al.*, 2001).

P1 (651–685), P2 (660–679) from gp41 and a scrambled peptide with all amino acids from P1 in random order were synthesized by Eurogentec, with 98% purity as shown by HPLC analysis. In the concentration range used (50–125 μ M), no aggregation of the peptide could be observed in the absence or in the presence of GAG or HSPG, neither by fluorescence nor by measurement of circular dichroism.

Fluorescent Glycosphingolipids

Bodipy-galactosylceramide (BpGalCer*), and Bodipy-glucosylceramide (BpGluCer*) were purchased from Molecular Probes (Eugene, OR) and stored as ethanolic solutions. To study the interaction of Bp-glycolipids* and peptides, each ethanolic solution of Bp-glycolipid* was directly injected into the peptide-containing solution of phosphate-buffered saline (PBS) at a final concentration of 1–6 μ M of the Bp-glycolipid*. At these concentrations, no collisional quenching was observed.

In most of the experiments, peptides were first incubated at room temperature with the proteoglycan, the GAG, or the disaccharides for 1 h and then the BpGalCer* was added before measurement in Cary microcuvettes (60 μ l). When indicated, BpGalCer* was first added to the peptide before incubation

with HSPG, GAG, or dp. Alternatively, when indicated, BpGalCer* was first incubated with HSPG before addition of P1.

Epithelial Cell Transcytosis Assay

HIV-1 transcytosis across epithelial cells induced upon contact with HIV-1-infected PBMC was performed as previously described (Bomsel, 1997; Alfsen *et al.*, 2001). Briefly, the endometrial cell line HEC-1 or the intestinal cell line HT-29 clone 19 (Denning, 1996), expressing apical GalCer, was each grown as a tight, polarized monolayer for 7 d on a permeable filter support (0.45- μ m pore size), forming the interface between two independent chambers, the upper one bathing the apical surface of the epithelial monolayer and the lower one bathing the basolateral surface. When used, antibodies, heparan sulfate or heparin were added to the apical chamber and preincubated for 10 min at 37°C, remaining present during the transcytosis assay. For anti-beta 1 antibodies, when indicated, unbound antibodies were washed out before addition of HIV-1-infected PBMC. Alternatively, HIV-1-infected cells were preincubated for 1 h at 4°C with antibodies, and unbound antibodies washed out before addition of HIV-1-infected PBMC to the apical pole of epithelial cells. To initiate virus transcytosis, either HIV-1-infected PBMC (2 million cells/filter) prepared as described elsewhere using HIV-1 primary isolates R5 or X4 (Lagaye *et al.*, 2001), or HIV-1-infected CD4⁺ T-cell line (800,000 cells/filter of CEM chronically infected by the X4 NDK clone or the R5 YU2 primary clone), as indicated, were added to the apical chamber. Contact between HIV-1-infected PBMC and the epithelial cell monolayer resulted in rapid budding from the PBMC of HIV-1 virions, followed by their transcytosis from the apical to the basolateral pole of the epithelial cells. After 2 h, the extent of transcytosis was determined by detection of p24 in the basolateral medium by ELISA (Beckman Coulter, Villepinte, France) and the transepithelial resistance (TER) was measured as described (Bomsel, 1997). The presence of the various agents studied did not change TER significantly from the onset to the end of transcytosis, in the absence of antibody or in the presence of control nonimmune IgG or IgM (Sigma). The level of p24 was measured after 2 h. Transcytosis was evaluated, and p24 was found to be similar for many experiments with a value around 150 pg/ml. This value was taken to represent 100% of transcytosis and was used to calculate the results in experimental settings. Interassay variation was <10% and the mean values of these combined experiments (at least 3 independent) are shown.

HIV-1-binding Assay

Cell-free HIV-1 (YU2 or JR-CSF R5 tropic molecular clones or LAI X4 tropic molecular clone, obtained from the NIH repertoire) were produced by calcium phosphate transfection of HEK 293T cells with proviral DNA (30 μ g) according to standard techniques. Viral stocks (3 μ g/ml HIV-1p24) were aliquoted for single use, snap frozen, and stored at -80°C.

HIV-1 binding to epithelial cells was measured as follows. Tight epithelial cell monolayers were incubated at the apical pole, with (150 μ l) cell-free HIV-1 at indicated concentrations in RPMI 1640 without bicarbonate, supplemented with 0.65 g/l Na₂CO₃, glutamine 1 mM, and 2% bovine serum albumin (BSA; RPMI-BSA), and at the basolateral pole with 500 μ l plain RPMI-BSA. Incubation was for 1 h at 4°C with gentle rocking. Virus was then removed, and the epithelial apical surface was extensively washed (6 $\times$) with RPMI-BSA to removed unbound virus. The cell monolayer was then cut out of the filter holder and lysed with 0.5% NP40 in PBS, at 4°C for 45 min under vigorous agitation. Cell lysate was clarified by centrifugation at 10,000 $\times$ g at 4°C for 10 min, and p24 content was quantified by ELISA (Beckman Coulter) according to the manufacturer's instructions. When indicated, antibodies, heparin, or heparan sulfate were preincubated at the apical side of epithelial cell monolayers for 30 min at 4°C, before virus addition.

Cell-free HIV-1 Transcytosis Assays Induced by RGD Peptides

Filter-grown epithelial cell monolayers were preincubated with RGD or RGL as control, biotinylated-RGD, or its control biotinylated-RGL, complexed to streptavidin, or RGD (X8) at the indicated concentration for 15 min at room temperature before addition at the apical pole of 150 μ l cell-free HIV-1 (YU2 or JR-CSF R5 tropic molecular clones or LAI X4 tropic molecular clone), prepared as described above for the HIV-1 binding assay, at 400 pg/ml HIV-1 p24, unless indicated. Transcytosis was allowed to occur at 37°C for 90 min and was evaluated by quantification of HIV-1 p24 in the basolateral medium of the chamber using a commercial ELISA (Beckman Coulter) and according to the manufacturer's instructions. When indicated, antibodies, heparin, or heparan sulfates (HS) were preincubated at the apical pole of the epithelial cells for 30 min at 4°C, before virus addition.

Western Blot

HT29 and HEC or HEC-1 epithelial cells were grown as a tight monolayer on permeable support filters as described above. For each cell line, one filter was washed twice in PBS at 4°C, the cell-bearing filter was cut out of the filter holder, and cells were solubilized in 150 μ l Tris 50 mM, pH 7.4, 0.5% TX-100, supplemented just before use with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, benzamidine (360 μ g/ml), leupeptin (4 μ g/ml), pepstatin (4

μ g/ml), and aprotinin (4 μ g/ml), for 45 min at 4°C with vigorous shaking. Cell lysate was centrifuged at 14,000 $\times$ g, for 10 min at 4°C. Proteins from 200,000 cells were separated by 5% SDS-PAGE and transferred to PVDF membranes (Perkin Elmer-Cetus Life Science, Boston, MA) as described (Bomsel *et al.*, 1998) and saturated in PBS, 3% BSA. For agrin immunodetection, membranes were incubated overnight at 4°C with polyclonal rabbit anti-agrin antibody (204; 1:500 dilution), followed by HRP-labeled anti-rabbit antibody (Amersham, Piscataway, NJ; 1:5000 dilution) and ECL detection according to the manufacturer's instructions (RPN 2106, Amersham). Films (BioMax, Eastman Kodak, Rochester, NY) were exposed for 10 s before development.

Dot Blot

One microgram of proteins, secretory component, full-length glycosylated, or nonglycosylated N25C95 B/z- recombinant agrin, was spotted onto nitrocellulose membrane and immediately labeled with the transient stain Ponceau red. The membrane was saturated for 1 h at room temperature with PBS-5% dry skimmed milk. Membrane was incubated with biotinylated-P1 at 15 or 50 μ M in PBS-5% dry skimmed milk for 2 h at room temperature. After three washes in PBS-5% dry skimmed milk, biotinylated-P1 was revealed by incubation with HRP-labeled streptavidin (Rockland, Gilbertsville, PA; 1:50 000 dilution) for 1 h at room temperature and by ECL and autoradiography as above.

Immunofluorescence Detected by Confocal Microscopy

Filter-grown epithelial cell monolayers were processed for immunofluorescence as described (Bomsel, 1997) using rabbit anti-chicken agrin (204; 1/500) as primary antibody followed by FITC-labeled anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, 1:300). Cells were double-labeled for actin with Texas Red-labeled phalloidin (Molecular Probes, 1:100). Colon biopsies from healthy donors were frozen and sectioned (10 μ m thickness; provided by Dr. B. Terris. Cochin Hospital, Department of Anatomical Pathology, Paris, France) before fixation in 4% paraformaldehyde. Sections were labeled for galactosyl ceramide (an apical marker) detection with mouse monoclonal anti-GalCer antibody (10 μ g/ml; Ranscht *et al.*, 1982) followed by anti-mouse IgG3 FITC (Southern Biotechnology Associates, Birmingham, AL, 1:100) and for agrin detection followed by rabbit anti-chick agrin antibody Texas Red (Jackson ImmunoResearch, 1:300), as described above. Labeling was observed using confocal microscopy on a Bio-Rad 1024 confocal microscope (Richmond, CA) as described (Bomsel, 1997). Sections were taken 0.5 μ m apart. For epithelial cell lines, apical labeling corresponded to the vertical projection of the five most apical section recorded and basolateral to the one recorded between the tight junction and the filter.

When indicated, filter-grown epithelial cell monolayers were first incubated with HIV-1-infected PBMC for 45 min at 37°C as described for transcytosis assays. Epithelial cells were fixed with 4% paraformaldehyde for 45 min at room temperature. Cells were then double-labeled with anti-agrin IgG and anti-gp41 2F5 IgG (5 μ g/ml), followed by Texas Red-labeled anti-human IgG as described above prior to analysis by two-color confocal microscopy. Overlap and colocalization of the two markers for each section was obtained using the dedicated Bio-Rad 1024 software.

To quantify the number of HIV+ cells adherent to epithelial cell surface, HIV-1 JRCSF-infected or -uninfected PBMCs were first labeled with Cell Tracker Orange CMTMR 6 μ M (Molecular Probes) for 30 min at 37°C as previously described (Lagaye *et al.*, 2001). Fluorescently labeled HIV+ or uninfected PBMCs were inoculated apically and contact was allowed for 2 h before fixation with 4% paraformaldehyde as described above. Cell coculture were labeled for agrin and observed by two-color confocal microscopy. Ten random low-magnification images were recorded and the ratio of PBMCs (orange cell tracker positive) to epithelial cells (agrin positive) was quantified. Epithelial cells are easily enumerated because of the agrin pattern. Two independent experiments were performed and the mean is given. SE was 18%.

Electron Microscopy

When indicated, filter-grown epithelial cell monolayers were first incubated with cell-free virus for 1 h at 4°C or HIV-1-infected PBMC for 45 min at 37°C as described for binding or transcytosis assays, respectively. Epithelial cells were fixed with 0.5% glutaraldehyde for 45 min at room temperature. After three washes in PBS, cells were labeled using the preembedding technique as described below. When indicated, primary antibodies were applied for 1 h at 37°C in PBS, 0.66% fish skin gelatin (Sigma), 0.025% saponin (Sigma). Primary antibodies were rabbit anti-chicken agrin (1/100), human 2F5 anti-gp41 antibody (10 μ g/ml), nonimmune human (10 μ g/ml), or nonimmune rabbit immunoglobulin. Primary antibodies were detected with 5 or 10 nm gold-labeled anti-rabbit or anti-human antibodies (1/50; British BioCell International, Cardiff, United Kingdom) applied for 1 h at 37°C, as indicated. After three washes, cells were postfixated for 1 h at room temperature, washed, and embedded in Epon. Ultrathin sections were examined with an electron microscope Philips CM 10 (Philips, Eindhoven, The Netherlands) after staining with uranyl acetate and lead citrate.

Spectroscopy

Circular Dichroism Measurements. The circular dichroic spectra of peptides in the far UV (200–260 nm) was obtained on a Mark V dichrograph (Jobin Yvon, Edison, NJ), equipped with software for the acquisition of the data. The concentration of peptides varied from 10 to 500 μ M, using a different path length from 0.01–2 cm. Blanks for buffer were subtracted, and spectra were analyzed as previously described (Alfsen and Bomsel, 2002).

Absorption Spectra. They were recorded on a UV-Visible Varian DMS 70 spectrophotometer (Varian SA, Les Ulis, France).

Fluorescence Measurements. Steady state fluorescence emission and excitation spectra were obtained on a Cary eclipse spectrofluorimeter (Varian SA). All emission spectra were obtained at 22°C using the scan mode with an excitation wavelength of 280 nm and emission wavelength between 300 and 600 nm. Excitation and emission slit width were 5 and 10 nm, respectively.

Fluorescence quantum yield (Q_A) and efficiency of the transfer were calculated as indicated in Alfsen and Bomsel (2002).

RESULTS

Efficient Virus Transcytosis Depends on the Contact of HIV-1-infected Cells and the Apical Epithelial Surface

Viral transcytosis generated upon contact of HIV-1-infected cells with epithelial cells appears much more efficient than does transcytosis of cell-free virus as previously described (Bomsel, 1997) by a quantitative measurement of the virus (estimated by HIV-1 p24 content) in the basolateral medium after 2 h of apical contact between epithelial cells and HIV-1-infected PBMC as compared with the same assay with epithelial cells in contact with cell-free virus originating from the same HIV-1-infected cells. Accordingly, cell-free virus entry into epithelial cells was subsequently shown (Hocini *et al.*, 2001) to require inocula 100–1000-fold higher in HIV-1 p24 U (~ 20 pg/ml HIV-1 p24 after 2 h, for a total of 20 ng inoculum added apically in 200 μ l), than the inocula required for infection using cell incorporated HIV-1 when the infected cells were placed in contact with the same epithelial cells. Standard assay is defined as the amount of virus detected in basolateral medium after 2 h of apical contact between the epithelial cell line and HIV-1-infected cells at a 1:1 ratio. The fraction of HIV-1-transcytosed in this assay is up to 5% of the inoculum, whereas the virus in the basolateral chamber is estimated by p24 quantification (Bomsel, 1997).

HIV-1 is an enveloped virus. Its membrane is composed of lipids derived from the host cell plasma membrane to which not only viral envelope glycoproteins gp41 and gp120, but also host cell proteins have been recruited. Consequently, the nature of the host cell is one critical factor in the composition of the viral membrane. Additionally, the recruitment of defined host cell protein species into the viral membrane has been shown to be altered, for a defined HIV-1 genome and a defined host cell, upon contact between the host cell and an uninfected target cell (Shattock *et al.*, 1996a, 1996b). To investigate whether direct contact between HIV-1-infected cells and the apical epithelial surface was indeed required to initiate virus transcytosis as opposed to just the presence in the viral membrane of cellular components recruited there upon contact with the epithelial surface, cell-free HIV-1 were produced as follows. First, HIV-1 (R5 JR-CSF molecular clone)-infected peripheral blood mononuclear cells (PBMC; 2×10^6) cultivated for 2 h at 37°C were allowed to bud viruses (referred to as cell-free virus). Second, HIV-1-infected PBMC (2×10^6) were allowed to interact with epithelial cells for the same amount of time (2 h at 37°C) and to initiate virus budding. The apical medium was collected and centrifuged to discard infected cells. This centrifuged medium contains free viruses (referred to as

virus on epithelia). As shown on Figure 1A, none of these cell-free virus particles (originating from the same number of HIV-1-infected cells and corresponding to 500 pg/ml) exhibited efficient transcytosis. Even uninfected PBMC added apically together with cell-free viruses could not rescue efficient transcytosis of either cell-free virus fraction. Similar results were obtained using the X4 NDK isolate subtype for 45 min at 37°C or using HIV-1 chronically infected CD4⁺ T-cell lines to produce cell-free virus fractions, in the presence or absence of uninfected CD4⁺ T-cell lines (unpublished data).

We therefore hypothesized that the actual contact between HIV-1-infected cell and the apical epithelial surface played a critical role in HIV-1 binding and endocytosis in epithelial cells, similar to the role of the immunological synapse in the activation of CD4⁺ T-cell during MHC class II-restricted antigen presentation by dendritic cells. To challenge this hypothesis, HIV-1 (R5 JR-CSF molecular clone)-infected PBMCs (2×10^6) were allowed to interact with the apical pole of polarized epithelial cells for 45 min at 37°C to allow initiation of HIV-1 transcytosis before fixation of the coculture. Ultrastructural studies were performed by electron microscopy after thin serial sectioning to visualize focal contacts between the HIV-1-infected cell and the epithelial surface (Figure 1B, a and a'); virions (Figure 1Ba', black arrows), probably newly formed, are visible in the intercellular space. Because these circular shapes are only visualized on one of numerous consecutive cell sections, they cannot represent sectioned microvilli. Higher magnification of virions present at synaptic contacts (Figure 1Ba', inset) indicates that part of the virions display a central dark core characteristic of mature virions, whereas others do not display the central dark core but rather a circular dark ring, signature of immature virions. Two consecutive sections, 0.5 μ m in thickness, demonstrate intimate contacts between the two cell membranes: i) HIV-1-infected PBMC and ii) epithelial cell (Figures 1B, b, b', c, and c'). Apposed membranes appeared to be separated by 1.5–2.0 nm. For approximately 30% of the cells exhibiting apical contacts, virus could be detected at the site of contact. However, it should be mentioned that thickness of the observed section is ~ 0.5 μ m, 20 times smaller than the diameter of the HIV+ cell. In contrast, we showed that uninfected CD4⁺ T-cells added in the same number as HIV-1-infected cells to the epithelial cells were unable to adhere in significant numbers to the epithelial surface (unpublished data), as previously reported (Phillips *et al.*, 1994). The contact between the HIV-1-infected cell and the epithelial cell will therefore be referred to below as the virally mediated synapse, according to the definition given for the prototypic synapse (Dustin and Colman, 2002): the presence of points of contiguity between the two individual cells but not continuity and the modifications induced in both cell membranes allowing signal transduction from the presynaptic cell to the postsynaptic one.

Taken together these results suggest that the contact between HIV-1-infected PBMCs and uninfected epithelial cells, leading to formation of the mucosal epithelial synapse, induces modifications in the cell membranes of both cell types that permit signal transduction from the infected cell to the epithelial cell to promote efficient HIV-1 endocytosis and transcytosis into the latter.

The beta-1 Integrin and Its RGD Disintegrin Counterpart Control Virological Synapse Formation

By analogy to the immunological synapse, because the beta-1 integrin subunit is expressed at the epithelial apical surface (Sonnenberg, 1993; Schwartz *et al.*, 1995) and its

Figure 1. (A) Cell-free HIV-1 is not efficient for transcytosis and uninfected T-lymphocytes cannot substitute for the synapse formation. HIV-1-infected cells (1 million; Standard), cell-free HIV-1 (500 pg/ml p24), cell-free virus produced upon contact with the epithelial cell apical pole (500 pg/ml p24; virus on epithelial cells), in the absence or presence (1 million) of uninfected PBMC (uninfected cells) were inoculated at the apical pole of epithelial cells. Transcytosis was permitted for 2 h at 37°C. Transcytosis, estimated by the p24 content in the basolateral medium, was expressed as % of transcytosis observed in standard conditions. (B) Virological synapse between HIV-1 (R5 JR-CSF molecular clone)-infected PBMCs and the apical pole of epithelial cells. HIV-1-infected cells were inoculated at the apical pole of epithelial cells and allowed to initiate HIV-1 transcytosis for 45 min at 37°C. Cells were then fixed and processed for electron microscopy examination after ultrathin sectioning. (a, b, c) Low magnification micrograph (×6200) showing an HIV-1-infected cell (HIV-1+ cell) interacting with the epithelial surface (EC). (a'-c' and a''-c'') the black boxed areas in (a-c) have been enlarged by ×5 and ×25, respectively. In a': black arrows indicate viral particles that are shown enlarged in the inset. In a''-c'', the microvilli are evidenced around the cell, outside the synaptic cleft. Bar, a' 100 nm; a' inset 50 nm

signaling is RGD dependent, we next asked whether RGD-dependent integrins participated in virally mediated synapse formation and signaling. RGD peptide (5 μ g/ml) added at the apical pole of epithelial cells was able to inhibit induced synapse formation, whereas control peptide RGL was inefficient as an inhibitor (Figure 2A). Quantification of the virus that budded in the apical chamber during the incubation of HIV-1+ cell and epithelial cell was equivalent whether contact took place in the presence of RGD or RGL peptides (unpublished data). Transcytosis of HIV-1 was also significantly inhibited by function-blocking anti-beta-1 integrin antibody in a concentration-dependent manner (Figure 2B). The effect of monomeric RGD peptide on HIV-1 transcytosis is specific, because neither nonblocking anti-beta-1 integrin antibody (Figure 2B) nor other anti-beta-integrin subunits (beta-2 or beta-4; Dustin and Dustin, 2001; Lanza-vecchia and Sallusto, 2001), have any effect on the virus transcytosis (unpublished data). Integrin-disintegrin interaction between HIV-1-infected cells and epithelial cells appears therefore one of the critical processes for virally mediated epithelial synapse signaling function.

Oligomeric RGD Peptide Restores Cell-free HIV-1 Transcytosis (by Simulating Synaptic Signaling)

The analysis of the mechanism of HIV-1 transcytosis generated upon contact of a HIV-1-infected cell with an epithelial cell has been hampered because of several biological factors,

including the lag time required for the synapse formation and the difficulty to synchronize the system. Indeed the establishment of the contact between infected and epithelial cells, i.e., synapse formation and function, requires a 20-min lag time before the initiation of viral budding and detection of transcytosis (Phillips *et al.*, 1994; Bomsel, 1997). Furthermore the contact between individual HIV-1-infected PBMC and epithelial cells is transient, and HIV+ cells have been shown to crawl over several adjacent epithelial cells before completion of polarized virus budding and subsequent detachment. Furthermore, the use of reversible drugs affecting epithelial cell trafficking, is precluded because of their possible additional effects on viral budding from HIV-1-infected cells. Instead, in an attempt to overcome these obstacles, using cell-free virus, we took advantage of the RGD dependency of viral transcytosis as described below. Integrins become active upon interaction with an oligomeric disintegrin ligand (Ruoslahti, 1996, 2003; Hynes, 2002) inducing integrin oligomerization, transconformation, and, in turn, signal transduction. To mimic this oligomeric disintegrin signal, a tetrameric RGD complex was formed between four biotinylated RGD-peptides and one streptavidin molecule [(RGD-b) $\times$ 4-SA] able to bind four biotins. When inoculated apically before adding cell-free HIV-1, [(RGD-b) $\times$ 4-SA] was able to promote transcytosis of cell-free HIV-1 in a concentration-dependent manner (Figure 2C). In contrast, monomeric RGL-b was unable to promote cell-free virus transcy-

Figure 2. (A) Monomeric RGD peptides inhibit transcytosis induced upon HIV-1+ cell contact with epithelial cells. Monomeric RGD peptide, at the indicated concentration, was preincubated at the apical pole of epithelial cell monolayers before inoculation of HIV-1(X4 NDK clones)-infected cells. RGL was used as control of specificity. Transcytosis was measured as described above. (B) Transcytosis induced upon HIV-1+ cell contact with epithelial cells depends on beta-1 integrin. Blocking (DE9) or nonblocking (LM534) anti-human beta-1 integrin antibodies were preincubated at the apical pole of epithelial cell monolayers, for 20 min at 37°C, before inoculation of (X4 NDK clones) HIV-1-infected PBMC (Anti-beta-1, blocking or nonblocking). When indicated the apical epithelial surface was washed three times before virus inoculation to eliminate unbound blocking beta-1 antibodies (Anti-beta-1, blocking on Epithelial Cells). Alternatively, HIV-1-infected PBMC were preincubated with blocking anti-human beta-1 integrin antibodies followed by three washes to eliminate unbound antibodies, before inoculation onto the epithelial apical surface of the endothelial monolayers (Anti-beta-1, blocking on HIV-1+ PBMC). Transcytosis was allowed

to occur for 2 h at 37°C and was measured as described above and compared with standard conditions. Error bars represent the mean of at least three independent experiments performed using X4 (NDK clone) or R5 (YU2 clone), HIV-1-infected CD4⁺ cells or the cell-free X4 HIV-1 (LAI clone) or R5 HIV-1 (YU2 clone) with similar results. (C) Efficient cell-free virus transcytosis is induced by oligomeric RGD peptides. Cell-free HIV-1 (the X4 LAI or the R5 YU2 clone; 500 pg/ml) were inoculated at the apical pole of epithelial cells alone (virus) or after apical treatment for 20 min at 37°C with monomeric biotinylated-RGD (100 µM; RGD-b), streptavidin (25 µM; SA), RGD-b tetramers complexed with streptavidin (RGD-b+SA), or synthesized RGD octamers (RGDx8). Transcytosis was estimated after 1 h 30 min at 37°C by quantifying HIV-1 p24 in the basolateral medium. Error bars represent the means of at least three independent experiments performed using the cell-free X4 HIV-1 (LAI clone) or R5 HIV-1 (YU2 clone) with similar results.

HSPG Acts as an Attachment Receptor for Binding of HIV-1 to the Host Cell Membrane

We have recently shown that a conserved motif of gp41 (amino acid 650–685; Alfsen *et al.*, 2001), referred to as P1, interacted specifically with galactosylceramide (GalCer) on the host cell membrane (Alfsen and Bomsel, 2002). In contrast to binding of gp120 (Harouse *et al.*, 1991), binding of P1 to GalCer exhibits the characteristics of a lectin-sugar interaction, P1 acting as a lectin (Alfsen and Bomsel, 2002). Because of the nature of the lectin-sugar interaction, it is likely that environmental factors characteristic of mucosal surfaces such as HSPG, play an important role in viral entry.

To test whether HSPG could act as attachment receptor for HIV-1, we developed a binding assay in which cell-free HIV-1 was allowed to bind to the apical surface of epithelial cells at 4°C (see *Materials and Methods*). Using this assay, with an inoculum of 500 pg of p24, 80 pg (16%) attached specifically to the epithelial surface (Figure 3A). Such binding saturated above 10 ng of p24 inoculum (unpublished data). Anti-HSPG antibody added apically before the virus blocked HIV-1 binding, suggesting that an HSPG served as attachment receptor. Furthermore, a washing step at acidic pH performed after the HIV-1 binding step entirely abolished virus adhesion to the epithelial cell surface. Interestingly, a monoclonal anti-GalCer antibody, added apically before the virus, had no effect in a similar experiment (Figure 3A).

The specificity of the sugar chains in the glycosaminoglycan (GAG), the ratio of sulfated to N-acetylated saccharides, as well as the length of the polysaccharide chain were then analyzed for their role in HIV-1 binding to the apical surface of epithelial cell by using HS of increasing size or heparin (9 kDa). Unexpectedly, instead of competing with HIV-1 binding, addition of exogenous heparin or HS of chain length from 12 to 18 disaccharide polyunits (dp) increased by 2.5-fold the extent of HIV-1 bound to the epithelial surface. HS of smaller chain lengths had no effect (Figure 3B)

Taken together these results suggest strongly that surface HSPG present at the apical epithelial surface acts as an HIV-1 attachment receptor on epithelial cells, independently of integrin activation by disintegrin.

Figure 2 (cont). to occur for 2 h at 37°C and was measured as described above and compared with standard conditions. Error bars represent the mean of at least three independent experiments performed using X4 (NDK clone) or R5 (YU2 clone), HIV-1-infected CD4⁺ cells or the cell-free X4 HIV-1 (LAI clone) or R5 HIV-1 (YU2 clone) with similar results. (C) Efficient cell-free virus transcytosis is induced by oligomeric RGD peptides. Cell-free HIV-1 (the X4 LAI or the R5 YU2 clone; 500 pg/ml) were inoculated at the apical pole of epithelial cells alone (virus) or after apical treatment for 20 min at 37°C with monomeric biotinylated-RGD (100 µM; RGD-b), streptavidin (25 µM; SA), RGD-b tetramers complexed with streptavidin (RGD-b+SA), or synthesized RGD octamers (RGDx8). Transcytosis was estimated after 1 h 30 min at 37°C by quantifying HIV-1 p24 in the basolateral medium. Error bars represent the means of at least three independent experiments performed using the cell-free X4 HIV-1 (LAI clone) or R5 HIV-1 (YU2 clone) with similar results.

Figure 3. (A) Cell-free HIV-1 bind to the apical epithelial surface in an HSPG-dependent but GalCer-independent mechanism. Cell-free HIV-1 (the R5 JR-CSF or the R5 YU2 clone; 500 pg/ml) were directly inoculated at the apical pole of epithelial cells (Standard). Alternatively, virus inoculation was preceded by apical treatment for 20 min at 37°C with anti-GalCer mAb or anti-HSPG IgM. HIV-1 was allowed to bind for 1 h at 4°C with gentle rocking. In one case a washing step with acidic buffer (glycine 100 mM, pH 2.2) was performed after the HIV-1 binding step. Binding was estimated by quantification of HIV-1 p24 associated with the cell monolayer after extensive washing of unbound virus and also expressed as % of virus bound in absence of treatment (Standard). Error bars represent the mean of at least four independent experiments performed using R5 HIV-1 (JR-CSF or YU2 clone). (B) Heparan sulfate and heparin enhance cell-free HIV-1 binding to the apical epithelial surface. In a similar binding assay as described in panel a, epithelial cells were preincubated with HS of different size from disaccharide subunit (dp) 8–18 or with heparin 9 kDa. Error bars represent the mean of at least three independent experiments performed using the cell-free R5 HIV-1 (YU2 clone).

GAG Interacts with P1 to Stabilize Its Structure and Optimize P1 Lectin Site Orientation for Its Binding to GalCer

From these results, two epithelial receptors for HIV-1 have emerged: the HSPG attachment receptor and the glycolipid GalCer. To analyze the role of HS/heparin on HIV-1 binding to GalCer, we used the biophysical assay that we have previously established to measure directly the molecular interaction of gp41 peptide P1 with GalCer, by fluorescence resonance energy transfer (FRET) from P1 tryptophan to biophysically-labeled GalCer (BpGalCer*).

Incubation of P1 in a dimeric configuration (125 μ M; Alfsen and Bomsel, 2002) with HS of increasing chain lengths from 14 to 18 dp (10–100 μ M) or heparin 9 kDa (10–20 nM) or heparin Choay (1–10 μ g/ml), before addition of the BpGalCer*, increases from 200 to 600% the efficiency of energy transfer from the tryptophans of the peptide to BpGalCer* (Figure 4A), indicating that GAG are indeed

acting on the P1 lectin site. In contrast, no effect was observed on FRET either when BpGalCer* was added to P1 before incubation with HSPG, GAG, or HS dp, or when one of the GAG was first added to BpGalCer* before interaction with P1. As controls, gp41 P2 peptide (660–679) or scrambled P1, which both contain the same number of Trp as P1, were incubated with the GAG but did not exhibit FRET. The incubation with heparin of P1 at P1 concentration below 50 μ M, where the peptide is no longer a dimer (Alfsen and Bomsel, 2002), does not modify the absence of FRET.

The most effective GAG at promoting FRET from dimeric P1 is high MW heparin (heparin Choay) compared with heparin 9 kDa or HS dp 14–18 at the same molar concentration. Because heparin contains more sulfated than acetylated sugars compared with HS, the role of disulfate ions appears predominant in the interaction with P1. These acidic groups could bind P1 on its QxxKN motif (656–660). Energy transfer between two molecules relates to the Forster equation in which the only variable parameters are the orientation factor and then the dipole-dipole interaction between the aqueous medium and the fluorescent species (Chen *et al.*, 1969). Hence, a multimolecular interaction can be proposed: first between GAGs and P1 favoring in turn an interaction with GalCer. Indeed, binding of GAG from HS or heparin to P1 by an ionic interaction with positively charged groups of the QxxKN motif could modify the orientation and/or the environment of P1 for its subsequent interaction with BpGalCer*, either by stabilizing its oligomeric structure leading to a more favorable presentation and orientation toward the GalCer and/or by decreasing the radiationless loss of energy of the complex. According to this model, we observed that the dimeric conformation of P1 as measured by circular dichroism (Alfsen and Bomsel, 2002), was not modified in presence of heparin or HS (unpublished data).

Viral Synapse Signaling and HIV-1 Transcytosis Are Dependent on HSPG

To investigate the potential involvement of HSPG in synapse signaling and HIV-1 transcytosis, anti-HSPG antibody was applied to the apical surface of epithelial cells together with RGDx8 before addition of cell-free virus (Figure 4B) or before addition of HIV-1-infected cells. After a 2-h incubation at 37°C, transcytosis was significantly inhibited, by >90%. Similarly, treatment of epithelial cell with heparinase before addition of HIV-1-infected cells significantly inhibited transcytosis. All together, these results suggest that synapse signaling as well as transcytosis of HIV-1 were both HSPG-dependent.

Furthermore, as shown for HIV-1 binding, heparin stimulated by more than 2.5-fold HIV-1 transcytosis induced either by RGDx8 or by contact of HIV-1-infected cells with epithelial cells (Figure 4B).

HSPG Acts in Cooperation with Integrins to Transduce Signal for HIV-1 Transcytosis

To study the respective role of GalCer- and RGD-induced signals in HIV-1 transcytosis, transcytosis of cell-free virus induced by RGDx8 was assayed in the presence of Mab anti-GalCer in the apical compartment of epithelial cells. It resulted in a reduction in transcytosis of 60% compared with standard conditions (unpublished data), which was less than the almost complete inhibition caused by Mab anti-GalCer on transcytosis by HIV-1-infected cells (Bomsel, 1997). Furthermore when both heparin and Mab anti-GalCer were added in the apical compartment, transcytosis induced by RGDx8 or HIV-1-infected cells was still present but was reduced by 50% compared with standard conditions. These

Figure 4. (A) Heparan sulfate and heparin increase the interaction between P1 and GalCer. P1 (125 μ M) was preincubated with heparan sulfate (HS) from 14 to 18 disaccharide units (dp; 20 nM) or Heparin, either 9 kDa or Choay (20 nM), before addition of BpGalCer*. Fluorescence Resonance Energy Transfer (FRET), from the tryptophan present in P1 to the Bodipy of BpGalCer* was measured. P2 and a scrambled peptide containing the same number of tryptophan as P1 were used as control. Results are expressed as % of FRET from P1 to BpGalCer* (Standard). (B) HIV-1 transcytosis is stimulated by heparin and dependent on an HSPG. Transcytosis of HIV-1 from HIV-1-infected PBMC or cell-free virus transcytosis initiated by RGDx8 was measured as described above after preincubation of the apical surface of the epithelial cell monolayer with heparin (33 μ M), heparinase, or anti-HSPG antibodies for 20 min at 37°C. Transcytosis was measured as described above and compared with standard conditions. Error bars represent the mean of at least three independent experiments performed using X4 HIV-1 (NDK clone) or R5 (YU2 clone)-infected CD4⁺ cells or the cell-free R5 HIV-1 (YU2 or JR-CSF clone).

results suggest that integrin oligomerization acts in cooperation with heparin to promote HIV-1 binding to the epithelial surface receptor.

Agrin Is Expressed at the Epithelial Apical Surface

The need for HSPG in the formation of virological synapses for efficient transcytosis of HIV-1 resembled the mechanisms described for neurological and immunological synapse formation and function. Indeed, in these latter cases, two cell types interact and HSPG participates in signal transduction from one cell to the other. One HSPG constantly present in several types of synapses: the neuronal synapse, the neuromuscular junction, and, more recently, the immunological synapse, is agrin (Burgess *et al.*, 2000; Erickson and Couchman, 2001). Furthermore, agrin is also expressed at the renal epithelial plasma membrane (Groffen *et al.*, 1998). We there-

fore hypothesized that agrin is the HSPG present at the virological synapse for HIV-1 transcytosis. Agrin was detected by western blot on both epithelial cell lines tested; i.e., HEC-1 (endometrial origin) and HT29 (colonic origin) as a band of ~300–350 kDa (Figure 5A) as described for CD4⁺ T-cell agrin (Erickson and Couchman, 2001). Difference of agrin apparent molecular weight between HT29 and HEC-1 may be due to glycosylation extent. Similar differences have been reported between different cells (Khan *et al.*, 2001). After indirect immunolabeling and confocal microscopy analysis, agrin appears apically distributed on colonic HT29 or endometrial HEC-1 polarized monolayers (Figure 5B, left), and not on the basolateral pole, where actin is present (Figure 5B, right), as well as on primary human colon epithelial cells (in situ in colon tissue section) where it colocalizes with the apical marker galactosyl ceramide (Figure 5C).

Agrin Acts as an Attachment Receptor for HIV-1, a Step Required for Transcytosis

We next addressed the role of agrin in HIV-1 binding to and transcytosis across epithelial cells. As shown in Figure 6A, anti-agrin antibodies directed against the C terminal protein core, block HIV-1 binding to the epithelial surface and HIV-1 transcytosis induced either by RGDx8 or contact of HIV-1-infected cells with epithelial cells.

Double immunogold-labeling experiments for agrin and gp41, carried out by electron microscopy, revealed occasional colocalization of agrin and HIV-1 particles on apical microvilli (Figure 6Bd), strongly suggesting that agrin plays a determinant role in HIV-1 attachment. Unfortunately, because of the fixation protocol required for immunolabeling, internal viral structures are not clearly visible.

To confirm the colocalization of epithelial agrin and HIV-1 at the apical surface, the interaction of HIV-1-infected and epithelial cells was analyzed by double immunofluorescence to detect agrin and gp41 visualized by confocal microscopy. Serial sections throughout the contact zone between HIV-1-infected and epithelial cells revealed focal double-labeled points at the cell-cell interface (yellow dots in Figure 6C) and unexpectedly a reorganization of agrin pattern in small patches (compare Figures 5B and 6C). To explain these variations one can hypothesized that the way the HIV-infected cell (visualized in red) is positioned relative to the apical surface influences the agrin distribution on epithelial apical surface. Indeed, in the first of the series (II), the HIV-infected cell is more visible, in the area of cell-cell contact, hence closer to the apical surface, and agrin adopts a more patched pattern. In contrast, in the second of the series (III), only a small area of the HIV-infected cell seems to interact with the epithelial surface, allowing agrin to maintain its more diffuse pattern, as in the absence of HIV-infected cells (see Figure 5B). Because infected PBMCs do not express agrin and epithelial cells do not express the HIV envelope, the only site of overlap is the interface. Quantification of the adherent HIV+ cells onto the epithelial monolayer indicated that the ratio of HIV+ cell (positive for HIV-1 envelope glycoprotein) per epithelial cell, quantified 30 min after HIV+ cell inoculation in the apical chamber, was of 1/15. In contrast, this ratio was only of 1/100 when uninfected PBMCs were inoculated apically in a similar experiment. This value did not vary significantly with time, i.e., at 45 and 90 min. These results are in agreement with the reported ability of HIV+ cell to sequentially interact with several cells of the epithelial monolayer (Pearce-Pratt and Phillips, 1993), as well as with the limited time (~2 h) during which HIV+ cells in contact with epithelial cells are able to bud viruses (Phillips, 1994) and induce transcytosis (Bomsel, 1997).

Figure 5. (A) Epithelial cells express agrin. Filter grown HT29 (left lane) and HEC-1 (right lane) cells were solubilized, proteins separated by SDS-PAGE 5% and after electrotransfer, agrin was immunodetected by Western blot using a rabbit polyclonal anti-chick agrin antibody (antibody 3240). The migration pattern of molecular-weight standards run simultaneously is shown on the right. Note that no trace of agrin is visible at the loading position. (B) Agrin is expressed at the apical pole of epithelial cells. Filter grown HT29 (top row) and HEC-1 (bottom row) cells were fixed and labeled for agrin by indirect immunofluorescence with a rabbit polyclonal anti-chick agrin antibody revealed by FITC-labeled secondary IgG. To visualize the basolateral region of the monolayer, epithelial cells were double-labeled with Texas Red-labeled phalloidin. Agrin and actin labeling were detected by dual-color confocal microscopy. Agrin "Apical" corresponds to the labeling detected in the upper 2 μm (5 consecutive sections, 0.5 μm apart) of the cell above the tight junction and "Basolateral" to the labeling detected below the tight junction down to the filter (15 consecutive sections, 0.5 μm apart). To visualize the basolateral region the actin labeling is shown (Actin Basolateral). Bars, 10 μm . (C) Agrin is expressed at the apical pole of human colon epithelial cells. Human colon biopsies were frozen, sectioned, fixed, and labeled for galactosyl ceramide or agrin by indirect immunofluorescence with mouse monoclonal anti-GalCer antibody and rabbit polyclonal anti-chick agrin antibody, respectively. Labeling was detected by confocal microscopy. GalCer (green labeling) and agrin (red labeling) colocalized (merged) at the apical pole of epithelial cells (EC), facing the mucosal lumen (mucosal) in a single optical section (0.5 μm). Magnification, $\times 100$.

Agrin is the Proteoglycan, which Interacts with P1, Specific Both for Its Sugar Chains and for Its Protein Core

Finally, we asked whether the increase in P1 interaction with GalCer in the presence of agrin was specific to agrin as a proteoglycan and whether the protein core of the glycosaminoglycan also played also a specific role. To test this hypothesis, we first analyzed by dot-blot the specific binding of agrin to P1. Recombinant full-length glycosylated and 95-kDa nonglycosylated chick agrin and IgA secretory component, a highly glycosylated protein expressed by epithelial cells were spotted onto nitrocellulose paper. Biotinylated-P1 was allowed to bind to immobilized proteins. As shown in Figure 7A, biotinylated P1 was only able to bind to glycosylated agrin. Furthermore, biotinylated P1 interaction with agrin occurred only at concentration of P1 at which it forms oligomers (Alfsen and Bomsel, 2002), confirming binding specificity. Second, FRET was measured in the presence of various forms of recombinant full-length chick agrin: glycosylated agrin (B/z+ and B/z- isoforms); nonglycosylated -B/z+ agrin; syndecan I, another HSPG, with HS similar to agrin, which is a fusion coreceptor to HIV-1 envelope subunit gp120 (Saphire *et al.*, 2001); and another nonrelated protein: IgA secretory component. Proteins were added to P1 before interaction with BpGalCer* (Figure 7B). Glycosylated agrin isoforms (B/z+ or B/z-) were the only proteoglycans active in promoting binding of P1 to GalCer. This activity was concentration dependent, saturating at 16 nM. It occurred through the sugar chains bound to the core protein, because neither nonglycosylated agrin (B/z+) nor similar glycans bound to the syndecan protein core, nor the glycan chains bound to the IgA protein core of the secretory component, were effective.

DISCUSSION

Previous works have shown that direct contact between HIV-1-infected PBMCs and the apical epithelial surface played a critical role in HIV-1 binding and endocytosis in epithelial cells (Phillips *et al.*, 1994; Bomsel, 1997). The present work presents evidence for morphological, structural, and functional similarities between such HIV-1-infected cell-epithelial cell adhesion and the neuronal and immune synapses that have evolved for efficient communication between pre- and postsynaptic cell surfaces. Indeed, the site of contact between HIV-infected cells and the mucosal epithelial surface, when visualized at the ultrastructural level, closely resembles neuronal and immune synapses and requires the synapse-specific scaffolding molecule agrin. Furthermore, the process of information transfer from the HIV-1-infected cells to the epithelial cells shares most of the important steps involved in the formation and function of both neuronal and immunological synapses. We propose, therefore, a new efficient mechanism for mucosal entry and efficient transcytosis of HIV-1 through its epithelial target cell via the formation of a "virally mediated synapse" between HIV-infected and epithelial cells.

In the virally mediated synapse described here, it is the infectious process that confers to the HIV-1-infected cell the capacity to act as epithelial cell partner. Several factors could confer to the HIV+ cell the ability to establish a synapse: first, the virally encoded envelope glycoproteins gp120/gp41; second, an increased expression of cell surface adhesion molecules on HIV+ cell as compared with uninfected cells (Shattock *et al.*, 1996b); and third, an actin cytoskeleton-mediated process through adhesion-induced polarized secretion of HIV-1 from activated mononuclear cells onto epithelia.

Figure 6. (A) HIV-1 binding to and transcytosis across epithelial cells involves agrin. HIV-1-infected cell derived transcytosis, cell-free virus transcytosis initiated by RGDx8, or cell-free HIV-1 binding was measured as described above, after preincubation of a rabbit polyclonal anti-chick agrin antibody (antibody 3240), at the epithelial cell apical pole, for 20 min at 37°C. Transcytosis was measured as described above and compared with standard conditions. Error bars represent the mean of at least three independent

The present data, therefore, not only have described the synapse formed between an HIV-1-infected cell and an epithelial cell, one of the principal primary target cells of HIV upon sexual and vertical transmission, but also have allowed us to unravel the machinery on which the virally mediated synapse is based.

Three epithelial molecules, namely, agrin as HIV-1 attachment receptor, beta-1 integrin/RGD containing factors and GalCer, the previously described endocytic receptor for HIV-1 in epithelial cells (Bomsel, 1997; Alfsen *et al.*, 2001), are required for virally mediated synapse formation, stabilization, and initiation of efficient HIV-1 endocytosis/transcytosis.

The presence of the scaffolding HSPG agrin in the synaptic cleft is a hallmark of immunological and neuronal synapses (Bezakova and Ruegg, 2003). We now show that agrin is expressed with a polar distribution on the apical epithelial surface and around the microvilli on a polarized monolayer of HT29 or HEC-1 cells. Agrin is also present on primary epithelial cells purified from human colon (Figure 5C). Agrin participates in the virological synapse and its signaling, because anti-agrin antibodies inhibit HIV-1 transcytosis (Figure 7A). As in the immunological synapse (Batista *et al.*, 2001; Bromley *et al.*, 2001), agrin would act in an autocrine manner in the virally mediated synapse. However, in contrast to the immunological synapse, but similar to the neuronal synapse, only glycosylated agrin is active at the virological synapse between PBMCs and epithelial cells.

In addition to a scaffolding role within the synapses (Dustin and Colman, 2002), agrin appears to function as an HIV-1 attachment receptor. Indeed, colocalization of gp41 and agrin is detected in a punctuated pattern at the interface of the HIV+ cell and the apical epithelial cell surface (Figure 6B), most likely representing the synaptic cleft. In addition, newly budded HIV-1 particles are detected to colocalize

Figure 6 (cont). experiments performed using X4 (NDK clone) or R5 (YU2 clone) HIV-1-infected CD4⁺ cells or the cell-free R5 HIV-1 (YU2 or JR-CSF clone). (B) (a) and (b) Agrin is expressed apically on epithelial cells. HT29 (1) and HEC-1 (2) cells grown on filters were fixed, labeled for agrin, and processed with a rabbit polyclonal anti-mouse agrin antibody (antibody 204) whose binding was detected by 5-nm gold labeled anti-rabbit antibody and then preembedded and processed for ultrastructural analysis. Gold labeling is detected apically (a), but not basolaterally (b). Magnification: (a) $\times 70,000$; (b) $\times 40,000$. (c) Controls: control human and rabbit IgG were used together as described above as primary antibodies, followed by 5-nm gold labeled anti-rabbit and 10-nm gold labeled anti-human antibodies. No labeling is detected on HT29 (1) and Hec-1 (2) cells. Magnification, $\times 40,000$. (d) HIV-1 colocalized with agrin on epithelial cells. HIV-1 (NDK clone)-infected CD4⁺ cells were inoculated apically on filter-grown HT29 (1) or Hec-1 (2) cells for 50 min at 37°C. Cells were fixed and processed for agrin (5-nm gold) and HIV-1 gp41 (10-nm gold) labeling as described above. Apical detection of both colocalized markers, is shown. Magnification, $\times 60,000$. (C) Gp41 on HIV+ PBMCs colocalized with epithelial agrin at the cell-cell contact zone. HIV-1 (JR-CSF)-infected PBMCs were inoculated apically on filter-grown HT29 cells for 45 min at 37°C. Cells were fixed and processed for labeling of epithelial agrin (revealed by FITC-labeled anti-rabbit IgG) and HIV-1 gp41 at the surface of HIV+ cells or on viral particles (revealed by Texas Red anti-human IgG), as described above. Cells were observed by two-color confocal microscopy. Overlapped images of only the cell-cell contact zone are shown (section 1–10: 0.3 μ m steps) onto which colocalization, analyzed with the Bio-Rad 1024 software, appears as yellow dots. Position of the sections shown is boxed in the schematized coculture system (I). Two examples are shown (II and III). For the first of the series, the vertical projection is shown (P). Bars, 10 μ m.

Figure 7. (A) P1 binds to glycosylated agrin. Secretory component, glycosylated and nonglycosylated agrin were spotted onto nitrocellulose membrane. Spotted proteins were by Ponceau red transient staining (Ponceau) before membrane saturation and incubated with monomeric biotinylated-P1 (15 μ M) or oligomeric biotinylated-P1 (50 μ M). Biotinylated-P1 was revealed with HRP-streptavidin. (B) Glycosylated agrin specifically potentiates P1 interaction with BpGalCer*. P1 (125 μ M) was preincubated with 10 nM of glycosylated agrin, nonglycosylated agrin, or syndecan-1, or the unrelated heavily glycosylated secretory component, before addition of BpGalCer*. FRET from the W present in P1 to the Bodipy of BpGalCer*, was measured. Results are expressed as % of FRET (Standard) from P1 to BpGalCer*. Only glycosylated agrin (B/z+ or B/z-) increases FRET.

with agrin on microvillousities at the ultrastructural level (Figure 6A) but cell-free HIV-1 also bind epithelial cells at 4°C in an agrin-dependent manner (Figure 7A).

The gp41 region interacting with agrin appears restricted to the conserved region P1, as demonstrated by biochemical and biophysical studies. P1 contains epitopes critical for HIV neutralization by one of the rare IgG antibodies (2F5) able to neutralize HIV primary isolates in vivo (Baba *et al.*, 2000) and by the other gp41 specific neutralizing IgG, 4E10 (Zwick *et al.*, 2001) as well as by mucosal IgA from seropositive individuals (Bomsel *et al.*, 1998; Alfsen *et al.*, 2001) and from highly exposed but persistently IgG seronegative subjects resistant to AIDS (Devito *et al.*, 2000). The present data demonstrate a direct interaction of P1 with HSPG and specifically with agrin, before P1 binding to its epithelial endocytic receptor GalCer (Figures 4A and 7, B and C). Such an interaction between agrin and P1 provides a novel explanation for the exceptional antiviral properties of 2F5-like antibodies. Actually, the negative charge of agrin HS would modify the orientation of the P1 lectin site orientation, in turn strengthening P1 interaction with GalCer. Accordingly, only glycosylated agrin bound to P1 and strengthened P1 binding to GalCer. As in CNS neurons (Hilgenberg *et al.*,

1999), there was no distinction between B/z+ and B/z- agrin isoforms. Moreover, the agrin core protein also appeared critical in this activity as neither syndecan-1 or similar HS bound to another protein core, nor other IgA glycans were effective (Figure 7, B and C).

The long chains of polysaccharides in HSPG provide much versatility in biological information storage and transfer which justifies the new concept of "sugar code" (Gabius *et al.*, 2002). How the organization of these long polysaccharide chains on a specific protein core adds to the specificity of the molecule, remains to be investigated.

Binding of HIV-1 to its agrin attachment receptor is not sufficient to induce endocytosis/transcytosis. Hence, the attachment efficiency of cell-free HIV-1 to the epithelial membrane is several times higher than the efficiency of transcytosis (compare Figures 2C and 3A). An additional signal provided by the virological synapse framework, possibly initiated by RGD-containing proteins, is required.

Cell-cell adhesion leading to recognition between and locking of the two cell surfaces is due, at least in part, to integrin-disintegrin interactions between epithelial cells and HIV-1-infected PBMCs. Indeed, function-blocking beta-1 integrin antibodies or a monomeric RGD disintegrinlike peptide, applied at the epithelial cell apical surface, inhibit HIV-1 transcytosis induced upon contact of HIV-1-infected PBMCs with epithelial cells (Figure 2, A and B). Reciprocally, because beta-1 integrin ligand is RGD dependent, a multibranched RGD (octamer) peptide is able to promote efficient cell-free HIV-1 transcytosis (Figure 2C). Functional integrins are alpha/beta heterodimers and the beta-1 subunit can only associate with a limited set of alpha subunits, namely alpha V, 8 and 5 (Hartner *et al.*, 1999; Skrzypczak *et al.*, 2001; Proulx *et al.*, 2003) and bind to RGD-containing ligands. These three alpha subunits are expressed on epithelial cells including endometrial cells and therefore could participate in the virally mediated synapse. Integrins are not constitutively active. The interaction of the disintegrin with the alpha/beta interface induces a conformational change in the integrin dimer and propagation of signaling. Many function-blocking and -activating antibodies bind the same part of the interface, also suggesting a propagated conformational change in this region related to integrin activity (Hynes, 2002). Here, disintegrin on HIV-1-infected cells PBMCs, by interactions with epithelial cell integrin, could activate the integrin conformational change.

Within the neuromuscular junction synapse, integrins not only promote cell-cell contact but also interact with agrin to trigger acetylcholine (ACh) receptor clustering on the postsynaptic muscle cell in an RGD-independent manner; agrin here appears as an aggregating factor (Martin and Sanes, 1997). In the current model, because cell-free HIV-1 does not efficiently transcytose despite the presence of agrin on the epithelial surface, an RGD-independent interaction of agrin with integrin does not appear sufficient to initiate HIV-1 transcytosis.

However agrin and integrin are both required for the virally mediated synapse formation and signaling, probably not in a direct synergistic way. Rather, the interaction of integrin with the RGD-containing molecules on the infected cell membrane or delivered in the synaptic cleft as soluble factors by the HIV+ cell (Rusnati and Presta, 2002) could initiate the signaling pathway.

Regarding the results reported for cell-free virus transcytosis (Hocini *et al.*, 2001) the inoculum needed is 100-1000 fold higher in p24 U than that required for transcytosis of virus generated when HIV-1-infected PBMC are in contact with epithelial cells. These data suggest that transcytosis in

such cell-free conditions occurs nonspecifically by fluid phase transcytosis (Bomsel *et al.*, 1989), and therefore no mechanistic comparison can be made with the present data.

Taken together, the present data suggest that direct contact of HIV-1-infected PBMC with epithelial cells, leading to the virally mediated synapse formation, induces a segregation and concentration of molecules such as adhesion molecules (Shattock *et al.*, 1996b), with the recruitment of raft microdomains in both cell membranes (Nguyen and Hildreth, 2000; Alfsen *et al.*, 2001), in the synaptic cleft as defined for the prototypic synapse (Dustin and Colman, 2002). Additionally, HIV-1, through gp41, attaches to an epithelial HSPG, as do several other viruses with epithelial tropism (reviewed in Rabenstein, 2002; Bomsel and Alfsen, 2003), here further identified as agrin. Such interaction modifies the gp41-lectin site (P1) orientation toward GalCer, the HIV-1-endocytic receptor in epithelial cells, and strengthens the P1/GalCer interaction. In turn this would favor and stabilize the recruitment of GalCer to apical raft microdomains (Alfsen and Bomsel, 2002) together with the additional interaction of GalCer with HIV-1 envelope gp120 (Harouse *et al.*, 1991). Therefore agrin appears not only as a HIV-1 attachment receptor but also as an aggregating factor that mediates GalCer recruitment into raft microdomains via an interaction with gp41. The modifications induced in both cell membranes, together with HIV-1 binding, allow signal transduction from the infected PBMC to the epithelial cell to promote efficient HIV-1 transcytosis.

Hence, our present results indicate that viruses can induce and opportunistically utilize the specific molecular synapse scaffold and molecular machinery for entry into and transcytosis through their initial target cells at mucosal site, the epithelial cells.

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