Expression of the Human Immunodeficiency Virus Envelope Glycoprotein Is Restricted to Basolateral Surfaces of Polarized Epithelial Cells

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Polarized epithelial cells exhibit apical (luminal) and basolateral (serosal) membrane domains that are separated by circumferential tight junctions. In such cells, enveloped viruses that mature by budding at cell surfaces are released at particular membrane domains. We have used a vaccinia virus recombinant to investigate the site of surface expression of the human immunodeficiency virus type 1 envelope glycoprotein in Madin-Darby canine kidney cells. Cells were infected with the vaccinia virus recombinant, and surface expression of the glycoprotein was analyzed by indirect immunofluorescence, ^{125}I -protein A binding, and immunoelectron microscopy. The glycoprotein appeared exclusively at the basolateral surface as early as 2 h postinfection and reached a maximum level at 8 h postinfection. The gp120 glycoprotein was found to be secreted efficiently into culture medium, and this secretion occurred exclusively at the basolateral surface.

Epithelial cells exhibit polarization of their plasma membranes into two functionally different domains, the apical and basolateral domains, which are separated by well-defined tight junctions. Enveloped viruses which form by budding at the cell surface are assembled exclusively at apical or basolateral membrane domains of such polarized epithelial cells (8, 24, 25, 27). Paramyxoviruses and orthomyxoviruses mature at apical plasma membranes, whereas C-type retroviruses or rhabdoviruses such as vesicular stomatitis virus have been shown to bud preferentially from basolateral surfaces. The available evidence indicates that the maturation of enveloped RNA viruses at restricted membrane domains is dependent on the site to which the viral glycoproteins are transported and that the viral glycoproteins themselves contain all the information necessary for directional transport to either apical or basolateral surfaces (10, 18, 19, 26, 28, 29). Previous reports from our laboratory (11, 29) have shown that directional transport of viral glycoproteins can be studied in Madin-Darby canine kidney (MDCK) cells, a polarized line of canine kidney cells, by expression using recombinant vaccinia viruses. These expression systems enable studies of directional transport in the absence of other viral proteins.

The human immunodeficiency virus (HIV) envelope glycoprotein has many similarities to other retroviral glycoproteins. It is synthesized as a large precursor (gp160) that is, gp120 and gp41 proteolytically cleaved into two functionally active subunits (3, 4, 20, 23, 30); gp120 is a highly glycosylated extracellular subunit that interacts with a receptor molecule, enabling the virus to bind to susceptible host cells (5, 12, 17), and gp41 anchors the glycoprotein complex on the virion surface and appears to be involved in fusing the viral envelope to a cellular membrane, leading to release of the viral nucleocapsid into the cytosol (13, 30). However, the HIV envelope glycoprotein differs from other retroviral glycoproteins in that it has an unusually long C-terminal domain (approximately 160 amino acids), the function and transmembrane topology of which are not yet fully understood. In addition, other structural features of the lentivirus

HIV differ significantly from those of the C-type retroviruses. Two recent reports show that HIV can directly infect bowel epithelial cells and suggest that this may be responsible for some of the gastrointestinal disorders of acquired immune deficiency syndrome (AIDS) patients (16, 21). The

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of HIV envelope gene products in MDCK cells infected with wild-type vaccinia virus (lane a) and recombinant vaccinia virus (VVenv1) (lane b). Cells were labeled with [^3H]leucine for 1 h at 7 h p.i. and immunoprecipitated with HIV-1-positive human antiserum. MDCK cells were infected with wild-type vaccinia virus or VVenv1 at a multiplicity of infection of 10. At 6 h p.i., cells were labeled with [^3H]leucine (50 $\mu\text{Ci}/\text{ml}$) in leucine-free Eagle minimal essential medium supplemented with 2% fetal bovine serum. The cells were incubated for an additional 1 h at 37°C, washed three times with cold phosphate-buffered saline, lysed in 1 ml of lysis buffer (50 mM Tris hydrochloride [pH 7.5], 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 20 mM EDTA), and vigorously vortexed, and lysates were centrifuged at 13,000 $\times g$. Radiolabeled polypeptides were immunoprecipitated with Sepharose-protein A that had been preincubated with antiserum from AIDS patients for 16 h at 4°C. The precipitates were washed three times with cold lysis buffer, suspended in sample reducing buffer, boiled for 5 min, and analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel (14). The protein bands were visualized by fluorography.

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FIG. 2. Indirect surface immunofluorescence of MDCK cells infected with VVenv1. (A) Absence of fluorescence in intact monolayers of MDCK cells infected with VVenv1 at 7 h p.i.; (C) basolateral fluorescence of MDCK cells infected with VVenv1 and treated with 30 mM EGTA for 30 min at 4°C at 7 h p.i.; (E) focal area of fluorescence around a lesion in a monolayer of cells infected with VVenv1 at 12 h p.i.; (B, D, and F) phase contrasts of fields shown in panels A, C, and E, respectively. Results with wild-type vaccinia virus are not shown. For indirect surface immunofluorescent staining, MDCK cells were grown on glass cover slips (18-mm circle) in 12-well tissue culture cluster plates and infected with wild-type or recombinant vaccinia virus at a multiplicity of infection of 10 in a volume of 0.2 ml for 1 h at 37°C. The inoculum was removed and replaced with 0.5 ml of Dulbecco modified Eagle medium supplemented with 2% fetal bovine serum, and the cells were incubated at 37°C until used for immunofluorescence studies. Immunofluorescence studies were performed by using anti-HIV human sera (diluted 1/5 in phosphate-buffered saline-bovine serum albumin) as primary antibody, incubated for 30 min at 4°C, and then washed extensively with phosphate-buffered saline-bovine serum albumin. A fluorescein-conjugated goat anti-human immunoglobulin was used as secondary antibody (diluted 1/20 in phosphate-buffered saline-bovine serum albumin), incubated for 30 min at 4°C, and washed extensively with phosphate-buffered saline-bovine serum albumin to remove any unbound label. Cells were then fixed in 95% ethanol-5% acetic acid at -20°C, mounted, and then observed for fluorescence and photographed with a Nikon Optiphot microscope.

presence of HIV has also been noted in capillary endothelial cells of the brain (31). Because infection of nonlymphoid cells by HIV may be important in the pathogenesis of AIDS, we have investigated the transport of the HIV envelope glycoprotein in polarized epithelial cells.

The HIV type 1 envelope gene was excised from plasmid pBH10 (kindly provided by B. Hahn, University of Alabama at Birmingham) by using *SaI*I and *Xho*I and ligated to *SaI*I-cut pGEM-3. After cleavage with *Sac*I and *SaI*I, unidirectional digestion with exonuclease III and S1 nuclease was carried out as previously described (7) to delete most of the 5' noncoding sequences. The envelope gene was then inserted into the vaccinia virus recombination plasmid pSC11 and used to generate a recombinant vaccinia virus designated VVenv1. MDCK cells were infected with the recombinant virus and analyzed for expression of *env* gene products by radioimmunoprecipitation and polyacrylamide gel

electrophoresis (Fig. 1). Three bands that correspond to gp160, gp120, and gp41 which are not present in wild-type vaccinia virus-infected cells are seen in the recombinant.

To determine whether the envelope glycoprotein was transported to a specific membrane domain, monolayers of MDCK cells were infected with wild-type vaccinia virus or VVenv1 and analyzed for surface expression of the envelope glycoprotein at 7 h postinfection (p.i.) by indirect surface immunofluorescence (Fig. 2). No fluorescence was observed at this time in intact monolayers infected with VVenv1, indicating an absence of the glycoprotein on the apical surface. To determine whether the glycoprotein was expressed basolaterally, monolayers infected with the recombinant or wild-type virus were treated with EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], a chelating agent that disrupts tight junctions and exposes basolateral surfaces to antibodies. After EGTA treatment,

FIG. 3. ^{125}I -protein A-binding assay of MDCK cells infected with VVenv1. MDCK cell monolayers were grown on cellulose nitrate-acetate filters, infected with VVenv1, and tested for surface expression of the envelope protein at 2-h intervals by treatment with HIV-positive human antisera and then with ^{125}I -protein A. At appropriate times p.i. either the top or bottom surface of the filters was incubated in primary antibody (human antisera diluted 1/5 in phosphate-buffered saline–1% bovine serum albumin) for 30 min at room temperature. Filters were washed three times with phosphate-buffered saline–bovine serum albumin and then incubated for 30 min at room temperature with 100,000 cpm of ^{125}I -protein A prepared in phosphate-buffered saline–bovine serum albumin. The label was removed, filters were washed three times with phosphate-buffered saline–bovine serum albumin, and the amount of bound radioactivity was determined. Levels of nonspecific binding of ^{125}I -protein A were determined by infection with wild-type vaccinia virus followed by reactions with primary antibody and then with ^{125}I -protein A as described above, and these values were subtracted from experimental values obtained with recombinant vaccinia virus-infected cells.

cells infected with the VVenv1 recombinant virus exhibited a characteristic ring of fluorescence typical of a protein localized on basolateral membranes (Fig. 2C). In addition, surface fluorescence was detected in the absence of EGTA treatment when infection with VVenv1 was allowed to proceed for 12 h, when focal areas of virus cytopathology developed and hence tight junctions were disrupted (Fig. 2E). No fluorescence was observed in control cells infected with wild-type vaccinia virus (data not shown). Thus, the immunofluorescence results indicate that the HIV envelope glycoprotein is transported exclusively to the basolateral surface of MDCK cells. Similar results were obtained in immunoelectron microscopy studies performed on monolayers grown on nitrocellulose filters, to enable immunological reagents to gain access to basolateral membranes. Monolayers infected with VVenv1 showed immunoferritin labeling predominantly on basolateral plasma membranes, whereas little or no label was present on apical surfaces (data not shown). These results indicate that the envelope glycoprotein is transported to basolateral surfaces in intact MDCK cell monolayers. Therefore, because enveloped viruses mature at the site to which their glycoproteins are transported, infection of epithelial cells by HIV would be expected to result in virus maturation and budding at the basolateral membrane domain. Efforts are currently under way to identify the site of HIV maturation in epithelial cell lines and tissues.

To quantitate the amount of envelope glycoprotein present on apical or basolateral cell membranes, ^{125}I -protein A-binding assays were performed on nitrocellulose filter-grown monolayers of MDCK cells infected with VVenv1 (Fig. 3).

FIG. 4. Polarized secretion of gp120. Cell lysates (lanes c), basolateral media (lanes b), and apical media (lanes a) were analyzed for envelope glycoprotein products by immunoprecipitation. Monolayers of MDCK cells were grown on Millipore filters and infected with 10 PFU of VVenv1 per cell as described above. At 6 h p.i. the media were replaced on both surfaces with 0.5 ml of methionine-free Eagle medium and incubated for 1 h at 37°C. The cells were then pulsed with 400 μCi of [^{35}S]methionine for 5 min at 37°C and chased with 0.5 ml of Eagle medium per surface for 1 and 3 h at 37°C. The media were collected separately from the apical and basolateral chambers and centrifuged to remove any cell debris, and the supernatant was adjusted to 0.1% sodium dodecyl sulfate. The cells were washed with phosphate-buffered saline and removed from the filter by incubation for 5 min on ice with 0.5 ml of lysis buffer. Rabbit antiserum to gp160 (1 μl) was added to 0.5 ml of each sample (basolateral or apical medium or cell lysate) and incubated for 4 h at 4°C, and then 30 μl of a 50% suspension of protein A-Sepharose was added and incubated for 4 h at 4°C. The immunoprecipitates were washed three times with cold lysis buffer, suspended in sample reducing buffer, boiled for 5 min, and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The total protein present in the sample was applied to each lane.

The glycoprotein was detectable on the basolateral surface as early as 2 h p.i. and increased to a maximum at 8 h p.i., at which time the level of expression remained relatively constant until the appearance of cytopathology at 12 h p.i. Comparison of the amount of antibody bound on the apical surface with that bound to both apical and basolateral surfaces revealed that 94% of the total antibody was bound to the basolateral surfaces at 8 h p.i.

It has been reported that gp120 is secreted into the media of HIV type 1-infected cells (22). To determine whether gp120 was being secreted in a polarized manner from the apical or basolateral surface, MDCK cells were grown on filters, infected with VVenv1, and analyzed for the presence of the envelope glycoproteins in the cell-associated fraction or in apical or basolateral medium (Fig. 4). After a 5-min pulse, a prominent band of gp160 was observed in the cell-associated fraction and no proteins were found in immune precipitates from culture media. After a 1-h chase period, the majority of molecules were cleaved into gp120

and gp41. About 50% of the gp120 was recovered in the immune precipitates from basolateral medium, whereas none of the protein was detected in the apical medium. By 3 h, very little uncleaved gp160 was detected, and the majority (75%) of gp120 was secreted into the basolateral medium, whereas gp41 remained entirely cell associated. No apical secretion of gp120 was detected. Thus, the secretion of gp120 occurs at the same membrane domain as the surface expression of the membrane-anchored glycoprotein molecules. A likely mechanism for this directional secretion would involve transport of gp120 to basolateral surfaces as a membrane-anchored protein, in association with the gp41 subunit, followed by dissociation of the subunits after arrival on the plasma membrane. Alternatively, it is possible that the subunits dissociate intracellularly and that the gp120 molecule alone possesses signals for directional secretion at the basolateral membrane. Further experiments with truncated forms of the envelope protein, produced by recombinant DNA procedures, should provide further information on the possible existence of signals for directional secretion.

There have been several recent reports concerning infection of nonlymphoid cells by HIV both in vitro and in vivo and the pathogenesis associated with such infections. Nelson et al. (21) reported that HIV was recovered from bowel biopsy specimens from AIDS patients with chronic diarrhea and that the virus was detected predominantly in epithelial cells. A prominent feature in AIDS patients is a generalized encephalopathy, and in several cases, HIV was found to directly infect capillary endothelial cells in the brain (31). In addition, several epithelial cell lines have been reported to be susceptible to HIV infections in vitro (1, 2, 15). These include both naturally occurring colorectal carcinoma cell lines and cells which have been engineered to express the CD4 receptor on their surfaces. Several laboratories have reported the presence of HIV in the semen and saliva of patients with AIDS (6, 9, 32). Nelson et al. (21) have suggested that infection of the rectal mucosa could take place via direct contact of the epithelial cells with infected seminal fluid. This is consistent with a recent report that simian immunodeficiency virus infection can be transmitted to either male or female rhesus macaques with cell-free virus by simple application to the genital mucosa without damage to the epithelium (C. J. Miller, N. Alexander, M. Jennings, A. G. Hendrickx, and P. A. Marx, Abstr. IVth Int. Conf. AIDS 1988, p. 2581). The results presented above suggest that HIV probably matures at the basolateral membrane domains of epithelial cells. Taken together, these observations indicate a mechanism by which HIV can traverse an epithelial cell layer and gain entry into the circulatory system by a pathway that does not involve a physical breakdown of the blood barrier.

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