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Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells

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ABSTRACT **In polarized epithelial cells, the release of enveloped viruses by budding at the cell surface is restricted to a specific cell membrane domain, either the apical or basolateral domain. To investigate the role of the envelope glycoprotein and the capsid proteins of human immunodeficiency virus type 1 (HIV-1) in determining the site of virus assembly, we analyzed virus maturation in a polarized monkey kidney cell line. A line of cells harboring the HIV-1 provirus (VERO-pFN) was found to differentiate into polarized epithelial cell monolayers upon reaching confluency. By electron microscopy, virus maturation was observed predominantly at the basolateral membranes of VERO-pFN cells. Analysis of HIV-1 proteins revealed that virtually all of glycoprotein gp120 and capsid protein p24 were found in the basolateral medium, while no HIV-1 proteins were detected apically. A recombinant vaccinia virus (VV) expressing the HIV-1 gag polyprotein (VV_{gag}) was used to determine the site of release of HIV-1 core particles in polarized epithelial cells in the presence or absence of envelope glycoproteins. When cells were infected with VV_{gag} in the absence of envelope proteins, similar amounts of the p24 capsid protein were released into virus particles at the apical or basolateral surface. In contrast, when cells were doubly infected with VV_{gag} and a recombinant VV expressing the HIV-1 envelope glycoprotein (VV_{env}), 94% of p24 and all of gp120 were found to be associated with particles released into the basolateral medium. These results indicate that the HIV-1 envelope glycoprotein directly influences the site of release of virus particles containing the gag protein, probably via a specific interaction between the envelope protein and the gag protein.**

Many animal viruses obtain a lipid envelope by a process of budding at a cellular membrane. The viral envelope contains one or more virus-encoded glycoproteins, which are essential for virus attachment and penetration into a host cell. The precise interactions that lead to virus budding have not been clearly defined, and virus budding may occur at the plasma membrane or at an intracellular membrane, depending on the virus family (1). The assembly process is highly specific in that host-cell membrane proteins are effectively excluded from the completed virus particles. Available evidence suggests that direct interactions between the viral envelope proteins and internal proteins may be involved in virus maturation. In the case of alphaviruses, evidence for interactions between envelope and core proteins has been obtained by crosslinking studies (2), and recently it was shown that the nucleocapsid of Semliki Forest virus could interact *in vitro* with the cytoplasmic domain of the envelope protein p62/E2 (3). The transmembrane protein (p15E) and the matrix protein (p15) of Moloney murine leukemia virus appeared to be colocalized at the plasma membrane of infected cells as determined by immunofluorescence (4, 5), and the transmembrane glycoprotein (gp35) and matrix protein (p19) of Rous sarcoma virus were shown to be closely associated by chemical crosslinking analysis (6). Altered processing of the cytoplasmic domain of the Mason–Pfizer monkey virus glycoprotein was found recently in matrix protein mutants of this virus (35). Although such interactions between the envelope and core proteins are likely to be important in virus assembly, the assembly and release of retrovirus particles by budding at the cell surface also occur in the absence of viral glycoproteins (7–12).

Epithelial cells exhibit two functionally distinct plasma membrane domains, the apical and the basolateral domains, which are separated by well-defined tight junctions. Enveloped viruses that bud from the plasma membrane assemble preferentially at either the apical or the basolateral membrane domains in polarized epithelial cells, depending on the virus type (13–15). C-type retroviruses and rhabdoviruses such as vesicular stomatitis virus (VSV) bud from the basolateral surface, whereas paramyxo- and orthomyxoviruses bud from the apical membrane. The envelope glycoproteins of several viruses have been shown to be transported to either apical or basolateral membranes in polarized cells in the absence of other viral proteins, indicating that the glycoproteins themselves contain the necessary signals for their polarized transport (1, 16–21). The membrane domain to which these glycoproteins are transported is correlated with the site of virus assembly. Much less information has been obtained about the distribution of viral core proteins in polarized epithelial cells. It was reported that the M protein of VSV is associated with basolateral membranes of infected epithelial cells (22). A similar distribution of M protein was also observed in a temperature-sensitive mutant defective in glycoprotein transport, suggesting that the polarized expression of the VSV M protein can occur independently of glycoprotein expression. These observations raise the possibility that, at least in the case of VSV, viral core proteins may be targeted to a specific membrane domain by a mechanism independent of the directional transport of viral glycoproteins.

In this study, we used expression systems to investigate the role of envelope and core proteins of human immunodeficiency virus type 1 (HIV-1) in determining the site of virus assembly and release in polarized epithelial cells. The finding that HIV core proteins expressed from vaccinia virus (VV) recombinants can be assembled into virus particles in the presence or absence of envelope proteins (8–10) provides an important advantage for these studies. We have reported previously that the HIV-1 envelope glycoprotein was transported to the basolateral membrane of polarized epithelial cells in the absence of other viral components (18). Here we report further investigation of the release of HIV-1 particles in polarized epithelial cells by producing an epithelial cell line that has an integrated HIV-1 provirus. In addition, we used

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Abbreviations: HIV-1, human immunodeficiency virus type 1; VV, vaccinia virus; VV_{gag} and VV_{env}, recombinant VV expressing the gag and env proteins; VSV, vesicular stomatitis virus; RIP, radioimmunoprecipitation.

VV recombinants to investigate the expression of HIV-1 gag proteins and their incorporation into released virus particles in polarized epithelial cells and to determine the effects of envelope glycoprotein expression on the transport and release of gag proteins.

MATERIALS AND METHODS

Cells and Viruses. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum. VERO C1008 cells were obtained from the American Type Culture Collection, and the recombinant VV-K1 (9) expressing the HIV-1 gag polyprotein (referred to as VV_{gag} hereafter) was obtained through the AIDS Research and Reagent Program (Bethesda, MD). Procedures for the growth of VVs and construction of the recombinant designated VV_{env1} expressing the envelope protein of HIV-1 (called VV_{env} hereafter) and a control designated VV_{sc11} that expresses β-galactosidase have been described (18).

Radioimmunoprecipitation (RIP) and SDS/PAGE. Cells were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine (New England Nuclear) for various time intervals and lysed with Nonidet P-40 lysis buffer (1% Nonidet P-40/150 mM NaCl/50 mM Tris·HCl, pH 7.4). Cell lysates were pelleted in a microcentrifuge for 2 min to remove any cell debris. Supernatants were treated with pooled sera from AIDS patients (obtained from patients at the Birmingham AIDS Outpatient Center, Birmingham, AL) and protein-A-conjugated Sepharose (Pharmacia) for 16 hr at 4°C. Immune complexes were washed three times with lysis buffer and resuspended in sample-loading buffer for SDS/PAGE with 10% gels as described (23).

VERO-pFN Cells. The plasmid pFN was constructed by subcloning the 5′ region of the HXB2Dgpt genome (*Hpa* I–*Sal* I) and removing the *Kpn* I fragment (nucleotides 3829–4157) by cutting with *Asp*718. To maintain the downstream coding region in frame, the *Asp*718 ends were filled in by using dGTP and the Klenow fragment of DNA polymerase I, and the remaining three nucleotides of the overhang were removed by digestion with S1 nuclease to produce blunt ends and religation with T4 ligase. This resulted in the deletion of exactly 100 codons from the reverse transcriptase gene. The reconstructed genome was then inserted into pSP72 (Promega) into which the neomycin-resistance gene from pSV2-neo had been cloned. VERO C1008 cells were transfected with pFN by the lipofection method (24) (Bethesda Research Laboratories) and were maintained in DMEM supplemented with 10% fetal calf serum and 300 μg of G418 per ml for 30 days. Individual colonies were expanded and analyzed for HIV-1 protein expression by RIP and SDS/PAGE. A cell line designated VERO-pFN was selected for subsequent studies.

Electron Microscopy. Cells were grown to confluency on mixed-ester cellulose nitrate/acetate filters (type Millicel-HA, 0.45 μm; Millipore), fixed with glutaraldehyde, postfixed for 1 hr at 4°C in osmium tetroxide in phosphate-buffered saline (PBS), and prepared for electron microscopy as described (25). Specimens were examined with a Philips EM301 microscope.

p24 Antigen Assay. Cell culture media was analyzed for p24 antigen levels by a commercially available ELISA kit (DuPont).

RESULTS

HIV-1 Proviral Gene Expression in an Epithelial Cell Line. A polarized monkey kidney cell line (VERO C1008) was transfected with a plasmid containing a *pol* deletion mutant of the HXB2D clone of HIV-1 (pFN), so that assembly of virus particles expressed from an HIV provirus could be analyzed. A cell line (VERO-pFN) was clonally derived that was found to express the *gag* and *env* gene products by RIP and SDS/PAGE analysis. When lysates of [^{35}S]methionine-labeled VERO-pFN cells were analyzed by immunoprecipitation with AIDS patients' pooled antisera (Fig. 1), three polypeptides with estimated molecular masses of approximately 160, 120, and 41 kDa were observed that correspond to the envelope glycoprotein precursor and its cleavage products. In addition, two other bands were observed corresponding to cleavage products of the p55 gag precursor, p39 and p24. These results indicate that a stable epithelial cell line was produced that expressed and processed HIV-1-specific protein precursors.

Virus Maturation in Polarized Epithelial Cells. VERO-pFN and VERO C1008 cells were grown to confluency on Millicel-HA filter chambers, and their polarity was analyzed by measuring electrical resistance and [^{3}H]inulin exclusion. Both cell types were found to be capable of excluding >99% of the apically added [^{3}H]inulin from the basolateral medium chamber (and vice versa) for up to 5 days. Additionally, these cell monolayers maintained a transmembrane resistance greater than 100 ohms/cm^2 during this time, indicating that VERO-pFN cells form a polarized cell monolayer. To determine the site of virus release, filter-grown VERO-pFN cell monolayers were embedded and sectioned for electron microscopic analysis (Fig. 2). Virus budding of the type characteristically found in lentivirus-infected cells was observed predominantly at the basolateral membrane domains of VERO-pFN cells. Virus particles were observed in all stages of assembly, ranging from early membrane budding to cell-free virions with mature capsids. Apical assembly was detected in less than 5% of the cells that showed basolateral virus assembly, and no virus assembly was observed intracellularly. We also prepared concentrated virus particles from the filter-grown VERO-pFN cells by pelleting media from the apical and basolateral chambers through 10% sucrose, and the pellets were resuspended and examined by negative-staining electron microscopy. VERO-pFN-derived virus particles with an average diameter of 100 nm and a characteristic layer of surface spikes were observed in basolateral samples (not shown). Only spikeless particles of similar dimensions were occasionally observed in the apical sample. No particles of similar morphology were detected in uninfected VERO C1008 cells. These results indicate that expression of HIV-1 proteins from a provirus leads to assembly and release of virus particles predominantly at basolateral membranes in polarized epithelial cells.

To quantitate assembly and release of viral proteins, VERO-pFN cells were either grown to confluent polarized monolayers on Millicel-HA filter chambers over a 5-day period or were seeded at a high density and analyzed 8 hr

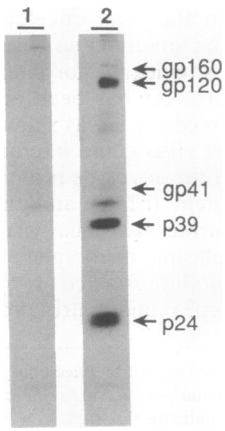

FIG. 1. Analysis of HIV-1 proteins expressed from an integrated provirus in polarized epithelial cells. VERO C1008 cells (lane 1) and VERO-pFN cells (lane 2) were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine for 1 hr and chased in unlabeled medium for 6 hr. Cells were lysed, and radiolabeled polypeptides were immunoprecipitated and analyzed by SDS/PAGE as described.

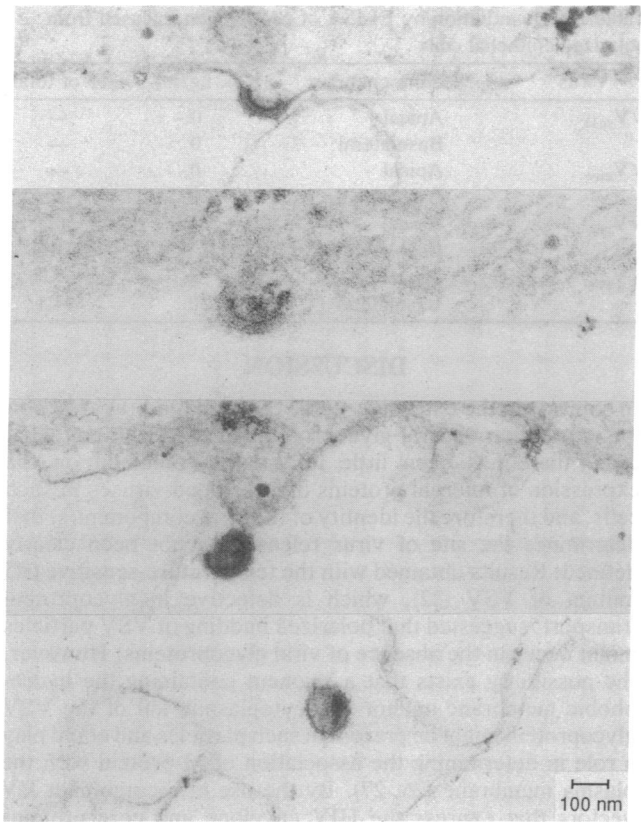

FIG. 2. HIV-1 maturation at basolateral surfaces of polarized epithelial cells. VERO-pFN cells were grown to confluency on Millicel-HA filters and prepared for electron microscopy. All stages of virus assembly were observed from the first signs of budding (*Top*) to the final release of progeny virions (*Bottom*).

later. The freshly seeded cells did not exclude apically added [^{3}H]inulin from the basolateral medium chamber and exhibited an electrical resistance no greater than 20 ohms/cm^2, indicating that they had not yet differentiated into a polarized epithelium. The cells were labeled with [^{35}S]methionine for 5 hr, and the media were collected separately from the apical and basolateral chambers, followed by immunoprecipitation and SDS/PAGE analysis (Fig. 3). A nonpolarized distribution of gp120 and p24 was observed in the medium of such freshly seeded VERO-pFN cells. The levels of each HIV protein released into the culture media were quantitated by laser densitometry. Only 42% of p24 and 54% of gp120 were found in the basolateral chamber of freshly seeded VERO-pFN cells, indicating that virus release was nonpolarized. In contrast, gp120 and p24 were only detected in the basolateral medium chamber of fully differentiated VERO-pFN cells. These results further support the conclusion that HIV-1 particle release occurs predominantly at basolateral plasma membranes in a polarized kidney epithelial cell line.

***gag* and *env* Gene Expression from Recombinant VVs.** To obtain higher levels of protein expression and to analyze gag and env protein expression individually, recombinant VVs were used. Previous reports indicated that expression of gag proteins from VV recombinants results in the release of enveloped core particles by budding at the cell surface (8–10). VERO C1008 cells were grown to confluency on Millicel-HA filters and maintained for 5 days to establish polarity. Following infection by recombinant VVs, membrane resistance remained above 100 ohms/cm^2 up to 12 hr postinfection. In cells infected with VV$_{gag}$, p55 and its cleavage products p24 and p39 were observed, whereas the envelope precursor gp160 and its cleavage products gp120 and gp41 were observed in VV$_{env}$-infected cells (Fig. 4). When cells were doubly infected with VV$_{gag}$ and VV$_{env}$, all of the above proteins were synthesized at levels similar to those found in cells infected with individual VV recombinants.

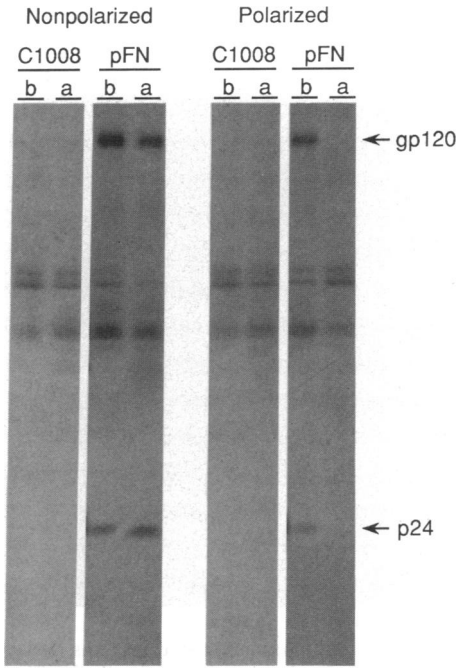

FIG. 3. Analysis of HIV proteins released into the medium of VERO-pFN cells. VERO-pFN and VERO C1008 cells were grown to confluency over a 5-day period (lanes designated "Polarized") or were seeded at a density equal to the number of cells present in a fully differentiated monolayer and analyzed 8 hr later (designated "Nonpolarized"). The cells were labeled with [^{35}S]methionine/[^{35}S]-cysteine for 5 hr, and media were collected separately from the apical (lanes a) and basolateral (lanes b) chambers for analysis by RIP and SDS/PAGE as described.

Release of Virus Particles from Recombinant VV-Infected Cells. To determine whether virus-like particles were released and to analyze polarity of release of gag proteins in the presence or absence of envelope glycoproteins, the apical and basolateral media from recombinant VV-infected VERO C1008 cells were analyzed. After metabolic labeling of filter-grown cells, virus particles in media from the apical and basolateral chambers were individually pelleted through 10% sucrose, followed by SDS/PAGE of the pellets for analysis of virion proteins (Fig. 5). No envelope proteins were observed in the samples from VV$_{env}$-infected cells, although

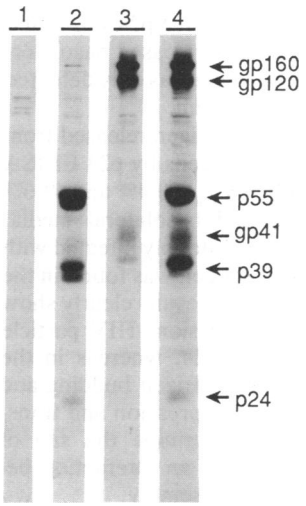

FIG. 4. Analysis of recombinant VV-expressed HIV-1 proteins. VERO C1008 cells were infected with recombinant VVs and 6 hr later were pulse-labeled with [^{35}S]methionine/[^{35}S]cysteine for 15 min. The cells were then chased with unlabeled medium for 4 hr, followed by lysis, immunoprecipitation, and SDS/PAGE analysis as described. Lanes: 1, VV$_{sc11}$; 2, VV$_{gag}$; 3, VV$_{env}$; 4, VV$_{gag}$/VV$_{env}$.

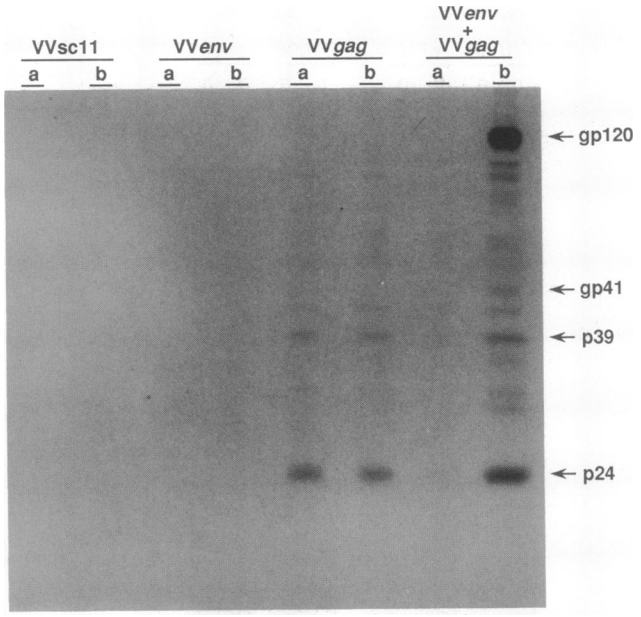

FIG. 5. Analysis of virion-associated proteins derived from recombinant VV-infected polarized epithelial cells. VERO C1008 cells were grown to confluency on Millicel-HA filters and were infected with recombinant VVs. Six hours later, cells were pulse-labeled with [^{35}S]methionine/[^{35}S]cysteine for 15 min and chased in unlabeled medium for 4 hr. Media were collected separately from the apical (lanes a) and basolateral (lanes b) chambers and centrifuged through 10% sucrose for 2 hr at 20,000 rpm in an SW-41 Beckman rotor. The pellets were resuspended in sample-loading buffer and analyzed by SDS/PAGE.

such cells secrete gp120 into the culture media (18), indicating that the conditions used for analysis were sufficient to exclude soluble, non-virion-associated proteins. When VV_{gag}-infected samples were analyzed, a major protein band corresponding to p24 was observed in approximately equal amounts in both the basolateral and apical media samples, indicating that the transport of the gag protein and its incorporation into released virus particles are nonpolarized under these conditions. In contrast, when the virion pellets from cells doubly infected with the VV_{gag} and VV_{env} recombinants were analyzed, both the envelope glycoprotein and the gag protein were found to be incorporated into particles predominantly at basolateral membranes. The levels of virion-associated p24 were measured in each sample by densitometry. In the absence of envelope glycoprotein, apical and basolateral levels of p24 were found to be 48% and 52% of total, respectively. However, in the presence of envelope glycoprotein, only 3% of the virion-associated p24 was found in the apical medium, whereas 97% was released basolaterally. In addition, the envelope glycoprotein was only detected in virus particles in the basolateral medium.

We also quantitated the level of p24 antigen released from recombinant VV-infected VERO C1008 cells by p24 ELISA (Table 1). In cells infected with only VV_{gag}, 50.4% and 49.6% of p24 were found in the apical and basolateral media, respectively. However, when cells were doubly infected with VV_{gag} and VV_{env}, 94.1% of the core antigen was found in the basolateral media. Taken together, these results clearly show that in the presence of *env* gene expression, HIV particle maturation is polarized in epithelial cells, whereas in the absence of *env* gene expression, nonpolarized budding and release of core particles occur. Thus, expression and transport of the envelope glycoprotein determine the site of release of gag proteins, presumably via an interaction between these components during virus assembly.

Table 1. Quantitation by ELISA of p24 antigen released from polarized epithelial cells

Virus	Medium chamber	p24, ng/ml	% of total
VV_{sc11}	Apical	0	—
	Basolateral	0	—
VV_{env}	Apical	0	—
	Basolateral	0	—
VV_{gag}	Apical	227	50.4
	Basolateral	223	49.6
VV_{gag}/VV_{env}	Apical	22	5.9
	Basolateral	350	94.1

DISCUSSION

In contrast to the extensive studies carried out previously on the expression of viral glycoproteins in polarized epithelial cells, there has been little information obtained on the expression of internal proteins of enveloped viruses in such cells, and therefore the identity of the viral component(s) that determines the site of virus release has not been clearly defined. Results obtained with the temperature-sensitive 045 mutant of VSV (22), which is defective in glycoprotein transport, suggested that polarized budding of VSV particles might occur in the absence of viral glycoproteins. However, the possibility exists that a segment containing the hydrophobic membrane anchor and cytoplasmic tail of the VSV glycoprotein might be present in such particles and could play a role in determining the association of M protein with the plasma membrane (26, 27). By the use of recombinant VV vectors that express the HIV envelope and core proteins individually or in combination, we have now been able to investigate the role of each component in determining the site of virus release. We also investigated the assembly and release of HIV-1 virus particles in polarized epithelial cells, using an integrated provirus. In both cases, assembly and release of virus particles occurred at the basolateral membrane domain when the envelope glycoprotein was present. In a previous report we showed that envelope glycoprotein transport was directed to the basolateral membrane in the absence of other viral proteins, indicating that this protein contains the necessary signals for polarized transport (18). In contrast, when release of virus particles was analyzed in the absence of the envelope glycoprotein by expressing the *gag* gene from a recombinant VV, we found that release of such particles occurred at approximately equivalent levels at apical and basolateral membranes. These results indicate that the envelope glycoprotein is directly responsible for the polarized maturation of HIV at basolateral membranes of epithelial cells.

Several groups have previously reported that synthesis of HIV-1 gag proteins by a variety of expression systems led to the maturation and release of HIV-like particles in the absence of envelope protein (7–11). The release of envelope-deficient particles has also been known to occur in both the avian and mammalian retrovirus systems (12, 28), although the normal maturation process for retroviruses is thought to involve a specific association between the envelope protein and the core proteins (5, 6, 29). Recently, it was reported that virus particles that contained both the gag and envelope proteins were assembled when both the HIV-1 *gag* and *env* genes were coexpressed in the same cell from separate recombinant VVs (8). The present finding that the release of HIV particles is polarized in the presence of the envelope protein demonstrates that the envelope protein affects the site of assembly and release of capsid proteins. It is possible that the cytoplasmic domain of the envelope protein is providing a high-affinity recognition site for the association of gag proteins. The presence of such a site, which would result in preferential association of gag proteins with basolateral

membranes, could explain the dramatic change in the site of release of viral core particles which results from coexpression of the envelope protein. Alternatively, the gag protein could be associating in the cytoplasm with envelope protein-containing membrane vesicles that are specifically targeted to the basolateral plasma membrane. It has been reported that chimeric molecules between the gag protein of murine leukemia virus and β-galactosidase associate with the endoplasmic reticulum, Golgi complex, and vesicle membranes; however, it was not determined whether the envelope glycoprotein was involved in this interaction (30).

The interaction of viruses with polarized epithelial cells may be an important determinant of certain aspects of viral pathogenesis. For example, following initial infection of epithelial cell layers, basolateral maturation may be important for viruses to gain access to the circulatory system for subsequent spread to target tissues. A mutant of Sendai virus was recently described that produces a systemic infection and exhibits bidirectional release from epithelial cells, in contrast to the wild-type virus, which is released apically and produces an infection localized to the respiratory tract (31). Virus maturation at restricted membrane domains may also limit the access of components of the immune system to virus-coded antigens. HIV-1 has been detected *in vivo* in intestinal epithelial cells (32), and several epithelial cell lines have also been reported to be susceptible to HIV infection *in vitro* (33). Infection of male and female rhesus macaques with simian immunodeficiency virus has been demonstrated to occur efficiently upon application of cell-free virus to the vaginal mucosa without damage to the epithelium (34), suggesting that the virus is able to infect epithelial tissues. Thus, the process of release of HIV from infected epithelial tissues may play a role in multiple aspects of the pathogenesis of HIV infections.

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