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The Intracytoplasmic Domain of gp41 Mediates Polarized Budding of Human Immunodeficiency Virus Type 1 in MDCK Cells

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Human immunodeficiency virus type 1 (HIV-1) has been shown to exhibit a specific basolateral release in polarized epithelial cells. Previous investigators have used vaccinia virus recombinants expressing HIV proteins to demonstrate that virus release is nonpolarized in the absence of viral envelope glycoproteins. In this study, we developed a transient expression system which allows the use of Madin-Darby canine kidney polarized epithelial cells directly grown on semipermeable membranes. This procedure allowed us to investigate polarized HIV viral budding following introduction of proviral DNA constructs. Expression of *env* gene products in *trans* demonstrated the ability to polarize *env*-negative viruses in a dose-dependent manner. The targeting signal for polarized virus release was shown to be present in the envelope gp41 transmembrane protein and absent from the gp120 portion of *env*. At least part of this signal is within the gp41 intracytoplasmic domain. Mutants of the p17^{gag} matrix protein were shown to be nonpolarized only when unable to interact with the envelope glycoproteins. Together, these data are consistent with a model of polarized virus budding in which capsid proteins, lacking a targeting signal, are targeted for specific basolateral release via an interaction of p17 with the envelope glycoprotein containing the polarization signal in its intracytoplasmic domain.

Contact between epithelial cells induces the formation of tight junctions subdividing the membrane surface into two domains. The apical domain faces the lumen of the organ (kidney or intestine), whereas the basolateral side of the cell is in contact with the blood circulation (9, 26). In polarized epithelial cells, such as Madin-Darby canine kidney (MDCK) cells, membrane-associated proteins are specifically sorted to one or the other plasma membrane domain. This targeting of membrane proteins leads to a nonhomogeneous or polarized distribution of surface proteins and allows each domain to develop a specific structure and function (2, 10, 18, 30).

The budding of enveloped viruses in epithelial cells is polarized, with different viruses maturing through specific cell surfaces (25, 36). In most cases, the polarized maturation and budding of enveloped viruses is dependent on the site of transport of their viral envelope glycoproteins. The glycoproteins are targeted to their specific membrane in the absence of all other viral proteins, suggesting that they may harbor the sole signal necessary for polarized viral maturation. The targeting signals for polarized sorting are probably similar in cellular and viral proteins, but no general consensus sequence or structure for apical or basolateral sorting has yet been conclusively identified (2, 10, 18, 30).

Human immunodeficiency virus type 1 (HIV-1) has been shown to exhibit a specific basolateral release in polarized epithelial cells (22, 23), similar to the maturation of other retroviruses in polarized cells (13, 27). Retrovirus envelope glycoproteins are not required for the formation and release of mature virions with a lipid envelope (7, 28, 29, 31). However, the viruses which are produced in the absence of envelope glycoproteins are noninfectious. Interestingly, it has been reported that such envelope-negative viruses are released in a nonpolarized fashion (23). The *gag* gene products alone are thus apparently devoid of targeting signals and require association with the envelope glycoproteins for their polarized transport.

The envelope glycoproteins of HIV-1 are processed and transported to the cell surface by the same mechanism as other membrane glycoproteins. The gp160^{env} precursor polypeptide matures into two functional subunits through cleavage by the cellular protease furin (8). The gp120 extracellular subunit is involved in recognition of the cellular receptor CD4. The gp41 transmembrane subunit interacts noncovalently with gp120 to retain it at the membrane surface and is involved in membrane fusion (14).

We developed a transient expression system which allows the use of polarized epithelial MDCK cells directly grown on semipermeable membranes to investigate HIV-1 polarized budding following introduction of proviral DNA constructs. Virus particle production was detected by using a sensitive p24^{gag} protein assay on virion pellets from the medium bathing either membrane domain. The expression of Env glycoproteins in *trans* was shown to polarize the budding of *env*-negative viruses to the basolateral domain. To define the targeting domain in the viral envelope glycoproteins, we studied the effects of mutations in these proteins on the sorting of budding virions. These experiments also identified a region in the carboxyl terminus of the gp41 intracytoplasmic domain which is required for polarized viral budding.

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MATERIALS AND METHODS

Plasmid constructs. The HXBH10 (34) proviral construct which contains a stop codon at the initiation site of the *env* gene and a frameshift at the *Kpn*I site (nucleotide position 5934; +1 is the transcription initiation site) in *env* has been previously described (37). This envelope-negative construct is referred to ATG− in this study. The *Sal*I-*Bam*HI (HXBc2 nucleotide positions 5331 to 8017) fragment of a *tat* truncated HXBc2 plasmid which contains a stop codon at the end of the gp120 sequence of *env* was subcloned into the envelope expressor pSVIIIenv (6) to create a gp120 expression plasmid. The plasmid encoding gp41 (BRU-derived pTRenv [24]) contains a deletion in most of gp120 but retains the signal peptide required for translocation to the endoplasmic reticulum, allowing correct export to the cell surface (gift of Lautaro Perez, University of Minnesota). Plasmid pEnv83, encoding glycoproteins retained in the endoplasmic reticulum as a result of a glutamic acid-to-arginine substitution at amino acid position 83, was provided by Joseph Sodroski and Udy Olshevsky (Dana Farber Cancer Institute, Boston, Mass.). Other constructs expressing truncated forms of the glycoproteins have been described elsewhere (6). Proviral plasmids expressing mutant forms of the matrix p17 protein have been also described elsewhere (4).

Cell culture. MDCK cells were seeded at 10^6 cells per 24.5-mm-diameter chamber and grown for 2 to 3 days on semipermeable 0.45-μm-pore-diameter membranes (Costar no. 3425) and maintained in Dulbecco modified Eagle medium supplemented with 8% fetal calf serum and 1% antibiotics (penicillin and streptomycin) at 37°C under a 5% CO_2 atmosphere. MDCK cells were used at a passage lower than 100, and the polarization of the cell monolayer was tested by monitoring development of electrical resistance between upper and lower chambers. Resistance across the monolayer was constantly above 550 ohms · cm^2 at the time of DNA introduction (Millicell-ERS resistance system; Millipore).

Lipofection and transient expression. Cells were washed with phosphate-buffered saline, and the lower chamber was filled with Opti-MEM (Gibco/BRL), supplemented with 1% antibiotics. A total of 5 μg of DNA was mixed thoroughly with 750 μl of Opti-MEM, while 30 μl of Lipofectin (Gibco/BRL) was mixed into 750 μl of Opti-MEM in a separate tube. Diluted DNA and Lipofectin were then combined and mixed well prior to pouring into the upper chamber over the cell monolayer. Cells were then incubated as described above. Medium was removed 24 h after lipofection, and the cells were maintained in Dulbecco modified Eagle medium supplemented with serum and antibiotics.

Virus quantitation. Media in the upper and lower chambers were harvested 48 h after lipofection, cellular debris were removed by filtering through a 0.45-μm-pore-diameter filter (Millipore), and the filtrate was ultracentrifuged at 30,000 rpm in a Beckman Ti 50.4 rotor for 1.5 h at 4°C. The virus pellet was resuspended in 200 μl of Dulbecco modified Eagle medium and p24 antigen was detected by using an enzyme-linked immunosorbent assay (ELISA) HIVAG-1 kit (Abbott Laboratories) according to the manufacturer's instructions. The p24 concentrations (picograms per milliliter) were then converted to total picograms of p24 released through each side of the membrane.

RESULTS

Optimization of the transient expression system in MDCK cells. Preliminary experiments were performed to establish a simple transient expression system that would allow rapid analysis of polarized viral budding upon introduction of proviral DNA constructs. The use of Lipofectin proved to be slightly more efficient than other transfection procedures (DEAE-dextran, calcium phosphate precipitation) when DNA was introduced into MDCK cells grown to confluency on semipermeable membranes. The lipofection method was thus used for subsequent studies since this method also appeared to be less disruptive to the integrity of the cell monolayer. The p24 ELISA assay was sufficiently sensitive to measure virus in the pellet obtained following ultracentrifugation of media bathing the transfected cells 48 h after introduction of plasmids.

Effect of gp160 on viral budding. The role of the viral envelope protein gp160 in targeting of viral budding was examined by using this transient expression system in MDCK cells. A plasmid containing complete proviral DNA (ATG+) or a proviral mutant defective in gp160 synthesis (ATG−) was transfected into MDCK cell monolayers, and the release of p24 in the medium bathing either the apical or basolateral domain was measured. The amounts of p24 were similar in the apical and basolateral chamber sides when the cells expressed *env*-negative viruses. In contrast, the amount of p24 detected in the basolateral medium of cells expressing wild-type viruses was more than 95% of the total amount of p24 detected (Fig. 1A). We then examined whether this polarization phenotype could be achieved by providing the gp160 protein in *trans*. Cotransfection of increasing amounts of an expression vector encoding gp160 with the *env*-negative proviral construct restored polarization of viral budding (Fig. 1B). As relative amounts of introduced envelope expressor decreased, apical release of virions increased until reaching close to 50% of total virus release. These data indicate a loss of polarized budding at *env*/*gag* ratios approaching 1:100. It was thus clearly established that gp160 is responsible for targeted HIV-1 budding to the basolateral surface of MDCK cells when expressed in *cis* or in *trans*. This effect was observed even when the molar ratio between *env* and *gag* was much lower than expected for wild-type virus.

Identification of the *env* moiety harboring the basolateral targeting signal. The *trans*-complementing assay was further used to localize the signal in gp160 responsible for basolateral transport of virions. The *env*-negative proviral construct was cotransfected with either a gp120 or gp41 expression vector. When the plasmid expressing only the gp120 portion of gp160 was used, the ratio of apical to basolateral virus release was similar to that observed with the *env*-negative provirus alone (Fig. 2). Conversely, expression of gp41 was sufficient to restore normal polarized budding of virions. Some recent data (15) indicate that, in contrast with earlier claims (24), the gp41 construct might not give rise to authentic gp41 but rather might give rise to uncleaved precursor retaining a small part (about 1/10) of gp120. However, since complete gp120 is not sufficient for polarization of virus release, it is most likely that the polarization signal responsible for basolateral targeting resides in gp41. The total amount of p24 released was greater when the gp120 expressor was introduced than when the gp41 expressor was introduced (Fig. 2). This observation is also consistent with previous data (Fig. 1) showing an apparent increase in total virus release in the absence of polarization. The exact reason for this phenomenon was not further investigated.

Similar experiments were performed with a plasmid encoding a mutant HIV-1 Env protein which is retained in the endoplasmic reticulum to gain further insight into intracellular events involved in polarized basolateral transport mediated by viral glycoproteins. Expression of this mutant HIV-1 envelope protein (Env83) did not result in polarized virus budding, suggesting that the intracellular expression of envelope per se is not sufficient for polarization.

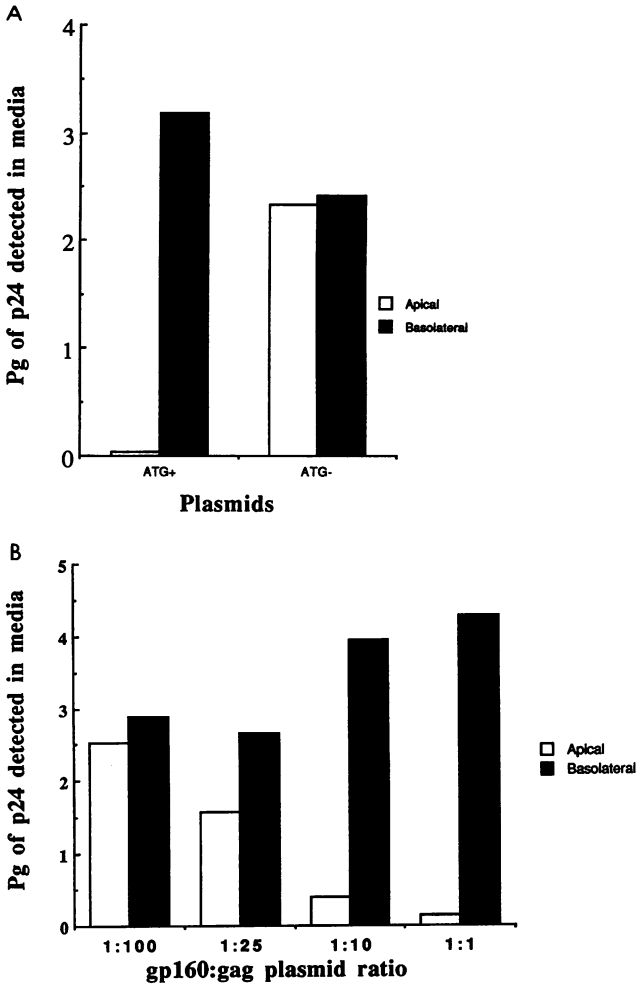

FIG. 1. Effect of gp160 on polarized virus release. (A) Proviral DNAs (5 μg) expressing (ATG+) or lacking (ATG−) the envelope glycoproteins were transfected into MDCK cells. Apical and basolateral supernatants were harvested 48 h after lipofection, and virus release was detected by p24 ELISA as described in Materials and Methods. (B) A proviral construct lacking Env glycoproteins was cotransfected with a $gp160^{env}$ expression plasmid, and viral release was detected as described for panel A. Increasing molar ratios of the *env* expressor plasmid relative to the amount of proviral DNA were used.

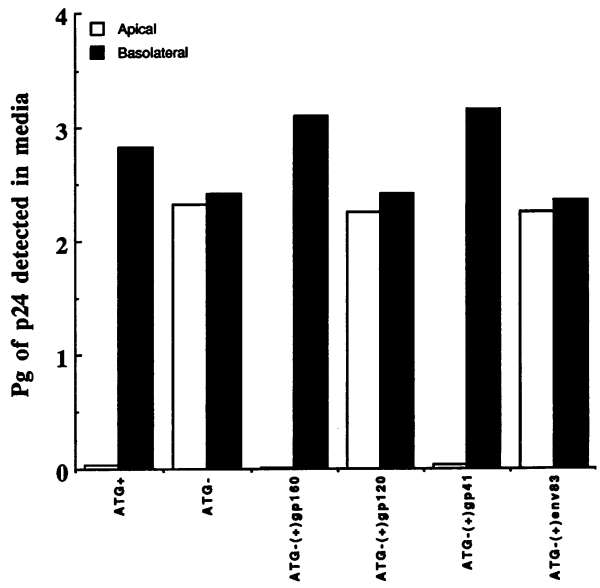

FIG. 2. Effect of *env* expression on polarized virus release. The *env*-negative proviral DNA was cotransfected with equimolar amounts of plasmids encoding either gp160, gp120, gp41, or a mutant gp160 retained in the endoplasmic reticulum (Env83) (total of 5 μg of DNA). Apical and basolateral supernatants were harvested, and p24 assays were performed as described in Materials and Methods.

Effects of mutations in the gp41 intracytoplasmic domain. The intracytoplasmic domain has been shown to encompass the basolateral targeting signal of other viral and cellular proteins (1, 3, 11, 17, 20, 32, 35). On the basis of this observation, experiments were performed to determine if the basolateral targeting signal, apparently present in gp41, can be assigned to the intracytoplasmic domain. Three envelope mutants which encode for gp160 proteins containing a deletion of 131, 103, or 43 amino acids at the carboxyl terminus of gp41 did not restore polarized budding of virions (Fig. 3). In contrast, a gp41 protein containing a deletion of only six amino acids at the carboxyl terminus restored polarized budding, with more than 95% of the virus released at the basolateral side, similar to that observed with the wild-type Env protein.

Other mutations in the gp41 C-terminal end gave rise to intermediate phenotypes. Deletion of 11, 17, or 43 amino acids gradually decreased the ability of gp41 to promote polarized virus release. These results indicate that the signal necessary for basolateral transport of glycoproteins and polarized budding of virions is, at least in part, located within the intracytoplasmic domain of gp41. The boundary of the sequence or structure acting as a signal appears to be close to the carboxy-terminal end, since deletion of as few as 11 amino acids is sufficient to significantly reduce basolateral targeting. Most of these gp41 mutants exhibited almost normal amounts of Env incorporation into the viral particle, as determined in a previous study (6) (Fig. 3). The slightly reduced amounts of virion-associated Env glycoproteins associated with these C-terminal truncations in gp41 expressed in COS cells were previously shown to result from decreased levels of cell-associated Env glycoproteins rather than to a specific defect in virion incorporation (6). These small decreases are not likely to cause the lack of targeting, especially since both the Δ846–856 and Δ851–856 gp41 mutants exhibited different polarization phenotypes and yet were shown to be similarly incorporated into the virions. Furthermore, our previous data suggested that even a 10-fold reduction in the level of Env glycoproteins should not significantly affect the extent of polarization (Fig. 1B). It is most likely that the effect of small deletions on the polarization phenotype is due not to an indirect effect on envelope incorporation but rather to a loss of a polarization signal located in the intracytoplasmic region of gp41. This polarization signal thus appears to be distinct from sequences required for Env incorporation into viral particles.

Effect of alterations in the p17 matrix protein on polarized virus release. It was previously shown that sequences within the p17 matrix protein domain of the HIV-1 *gag* precursor are essential for Env glycoprotein incorporation into assembling virions (4, 38). To determine whether a specific interaction between Env and Gag proteins is required for polarized virus release, the effects of mutations in the p17 matrix protein were examined. Without exception, substitutions and deletions in the p17 matrix protein previously shown to prevent the incorporation of Env glycoprotein (4) caused a nonpolarized distri-

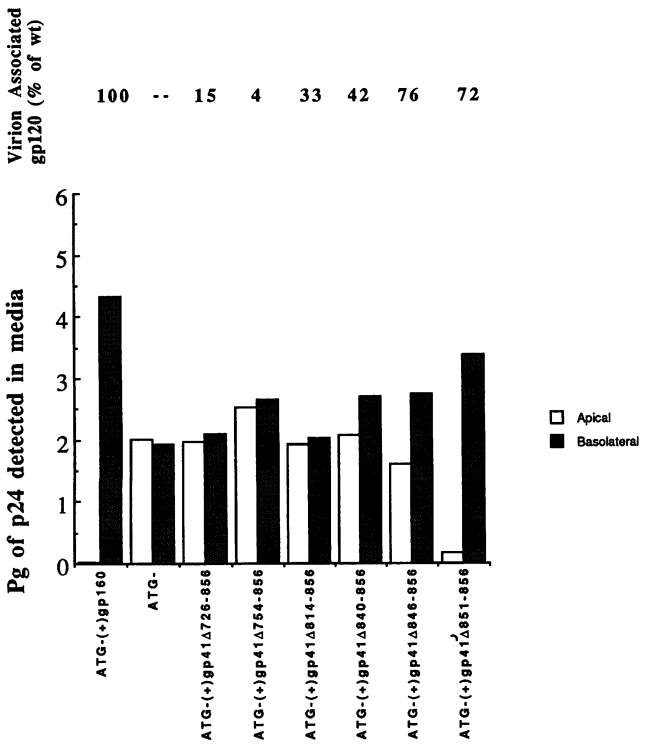

FIG. 3. Effect of C-terminal truncations in gp41 on polarized virus release. Plasmids encoding different Env proteins truncated in their gp41 intracytoplasmic domains were cotransfected in equimolar amounts with the *env*-negative proviral plasmid (total of 5 μg of DNA). Apical and basolateral media were harvested, and viral p24 was quantitated as described in Materials and Methods. Numbers refer to the positions of deleted amino acids in each mutant. The amino acids are numbered relative to the initial methionine of the *env* gene. The amounts of envelope proteins associated with virus particles relative to wild-type level have been determined in a previous study (6) and are shown at the top.

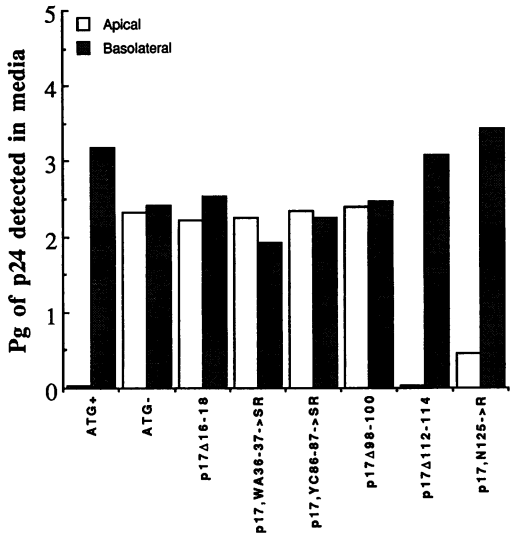

FIG. 4. Effects of mutations in the matrix $p17^{gag}$ protein on polarized virus release. Proviral constructs which contain mutations in the p17 matrix protein were introduced by lipofection into MDCK cells, supernatants were harvested, and viral p24 was quantitated as described in Materials and Methods. Numbers refer to the positions of the altered amino acids relative to the initial methionine of Gag.

bution of p24 (Fig. 4). By contrast, two mutants with alterations near the C-terminal end of the p17 matrix protein did not affect Env glycoprotein incorporation (4) (mutants Δ112–114 and N125R) and also retained a normal polarized phenotype (Fig. 4). These results demonstrate a clear correlation between the ability of mutant $p17^{gag}$ to interact with Env glycoprotein and polarized Gag protein release.

DISCUSSION

This report describes a method to investigate polarized HIV-1 viral budding by using MDCK cells without disturbing their differentiated state. This experimental system has the additional advantage of using HIV-1 proviral constructs, thus simulating conditions which are likely to be closer to that of natural HIV-1 infections than previously used vaccinia virus vectors.

Using this approach, we have confirmed previous findings which demonstrated that HIV-1 glycoproteins are responsible for viral polarized budding from the basolateral surface of epithelial cells (22, 23). The *trans*-complementation system in which an *env*-negative provirus and an envelope expressor plasmid were coexpressed confirmed that the Env glycoproteins are responsible for polarized virus release. However, virions were still released in a polarized fashion when lesser amounts of the glycoproteins were incorporated. It is possible that these virions harbor Env molecules but at a level below a threshold required for correct transport to the basolateral surface.

The intracytoplasmic domain of several glycoproteins has been shown to harbor a signal responsible for their proper targeting to the basolateral surface (1, 3, 11, 17, 20, 32, 35). Accordingly, we have demonstrated that small carboxy-terminal deletions of the intracytoplasmic domain of gp41 were sufficient to significantly alter polarized transport, indicating that this region is important in the targeting process.

Results obtained with a mutant Env glycoprotein incorrectly transported to the cell surface initially suggested that the polarized transport of Env, and presumably its incorporation into the virion, is required for polarized budding. To further examine this possibility, we analyzed the effect of mutations in the p17 matrix protein. The results of this study demonstrated that mutations, previously shown to affect the incorporation of envelope glycoproteins (4), also precluded the polarization of viral budding. Conversely, mutations which do not affect the incorporation did not alter the polarization phenotype. These data support the view that an interaction between the envelope and the rest of the capsid, through the amino-terminal region of p17, is required for polarization of virus release.

Altogether, the results of this study demonstrate that the gp41 protein, harboring an intracytoplasmic signal for polarized virus release, must interact with the p17 matrix protein in order for basolateral targeting of virus budding to occur. These data are consistent with a mechanism by which viral capsids can be targeted to the cell surface by the same cellular machinery responsible for the transport of membrane proteins. An association of capsids with transport vesicles has been previously suggested for other retroviruses (12). The association of capsids with specific vesicles destined for the basolateral membrane domain might be favored by the presence of viral envelope proteins interacting with p17. Additional studies are required to specifically demonstrate whether such a phenomenon is actually involved.

Several previous studies have stressed the potential importance of epithelial cell infection in HIV pathogenesis (5, 16, 19,

21, 33). The polarized release of viruses is likely to be important for their dissemination in the host as well as for their pathogenic potential. The studies in this report describe a method which may lead to a better understanding of mechanisms involved in polarized virus budding in epithelial cells.

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