

Two Distinct Oncornaviruses Harbor an Intracytoplasmic Tyrosine-Based Basolateral Targeting Signal in Their Viral Envelope Glycoprotein

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It has been clearly established that the budding of the human immunodeficiency virus (HIV-1), a lentivirus, occurs specifically through the basolateral membrane in polarized epithelial cells. More recently, the signal was assigned to a tyrosine-based motif located in the intracytoplasmic domain of the envelope glycoprotein, as previously observed on various other viral and cellular basolateral proteins. In the present study, expression of human T-cell leukemia virus type 1 (HTLV-1) or Moloney murine leukemia virus envelope glycoproteins was used for *trans*-complementation of an envelope-negative HIV-1. This demonstrated the potential of oncornaviral retrovirus envelope glycoproteins to confer polarized basolateral budding in epithelial Madin-Darby canine kidney cells (MDCK cells). Site-directed mutagenesis confirmed the importance of a common motif encompassing at least one crucial membrane-proximal intracytoplasmic tyrosine residue. The conservation of a similar basolateral maturation signal in different retroviruses further supports its importance in the biology of this group of viruses.

Release of enveloped viruses often occurs at specific membrane domains in polarized epithelial cells exhibiting clear differentiation of their membrane surfaces into apical and basolateral domains. It has been observed that retroviral envelope glycoproteins are targeted to the basolateral membrane domain in such cells; as a result, viral release is also restricted to this cell surface (9, 21, 36, 37, 48). Initial observations have been made with murine retroviruses, but detailed analysis has been performed only in the last few years with human immunodeficiency virus type 1 (HIV-1), which belongs to the *Lentivirinae* subfamily of retroviruses (29, 30). In those studies, it has been established that only the viral envelope glycoprotein (Env) harbors the targeting signal. Furthermore, incorporation of the envelope glycoprotein into the virions is essential to ensure targeting of viral budding; mutations of the viral matrix protein preventing envelope incorporation abolish targeting of viral budding (29). Furthermore, it was clearly shown that alteration of basolateral targeting of the viral envelope glycoprotein does not necessarily preclude its incorporation to the viral particle (30). Retroviruses are peculiar in their budding mechanism in the sense that the presence of envelope glycoprotein is not required for viral budding and release; however, viruses harboring a lipid envelope devoid of envelope glycoprotein are noninfectious (13, 49, 52, 56).

In the last few years, basolateral targeting signals involving crucial tyrosine residues have been found in intracytoplasmic regions of proteins destined for the basolateral domain of epithelial cells. Tyrosine-based basolateral signals were most frequently reported for different viral and cellular proteins, although alternative signals, including dileucine motifs, have also been observed (4, 18, 20, 32, 34, 61, 62). The intracyto-

plasmic tyrosine-based basolateral signals are also clearly related to endocytosis signals; the neighboring structure may well affect the ability of otherwise similar signals to act either as endocytic or basolateral signals (19, 42, 43, 63). The most common consensus motif observed in basolateral and endocytosis signals is Y-X-X-aliphatic/aromatic and, accordingly, the membrane-proximal intracytoplasmic tyrosine residue in the HIV envelope glycoprotein is found in such a consensus (30).

The polarization signal of the membrane glycoprotein of HIV-1 was found to be part of the intracytoplasmic domain, and the membrane-proximal tyrosine was recently shown to be essential (30). Different amino acid substitutions for this crucial tyrosine residue eliminate the basolateral targeting (non-conservative substitutions) or reduce its efficiency (a conservative tyrosine-to-phenylalanine substitution); as a result, viruses are released bidirectionally from both the apical and the basolateral membrane surfaces as observed in the complete absence of viral envelope glycoprotein. The three other tyrosines in the intracytoplasmic region of the protein were clearly dispensable for basolateral polarized targeting (29, 30); their non-conservative replacement with a serine had no effect on the polarization phenotype. Local structural determinants are also likely to be involved since replacement of a proline residue near the membrane-proximal tyrosine abolishes the polarized budding of HIV-1.

Important amino acid residues or motifs involved in protein function are often revealed through sequence comparisons of proteins exhibiting similar biological properties. Envelope glycoproteins from other retroviruses, in addition to HIV-1, were also examined to determine if they harbor putative tyrosine-based basolateral targeting signals. This point also presents a further interest since most retroviruses possess much shorter intracytoplasmic domains than HIV-1 (30). Interestingly, sequence comparisons between intracytoplasmic domains of many different retroviruses showed the presence of at least one tyrosine residue in an adequate Y-X-X-aliphatic/aromatic con-

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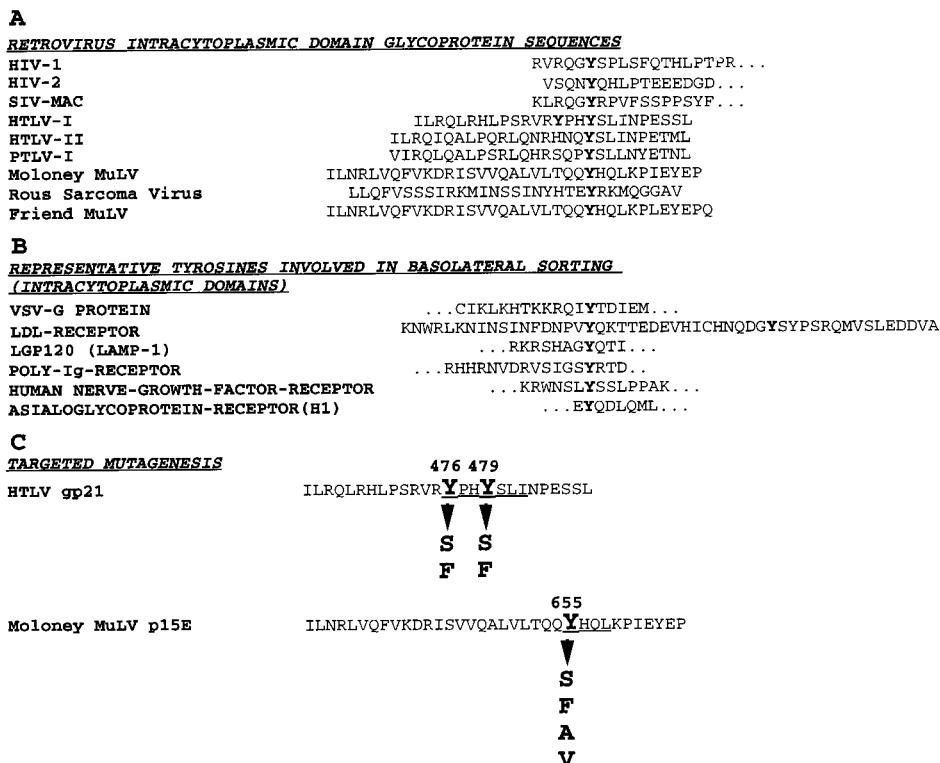


FIG. 1. (A) Intracytoplasmic domains of different retroviral envelope glycoproteins. The sequences are aligned according to their intracytoplasmic tyrosine residue most likely to be involved in a targeting signal (15, 23, 30, 38, 50, 51, 53, 55). (B) Representative examples of well-established basolateral targeting signals in the intracytoplasmic domains of different proteins (reviewed in references 33 and 62). (C) Schematic representation of the targeted mutagenesis performed on the HTLV-1 and Mo-MuLV glycoprotein intracytoplasmic domains. Amino acids are numbered from the first methionine of the envelope glycoproteins. Putative sorting signals (Y-X-X-aliphatic/aromatic) are underlined. SIV, simian immunodeficiency virus; PTLV, primate T-lymphotropic virus; VSV, vesicular stomatitis virus; LDL, low-density lipoprotein; LGP, rat lysosomal glycoprotein; Ig, immunoglobulin.

text (Fig. 1A). For comparative purposes, a short list of well-established basolateral proteins in which the signal was clearly shown to depend on a critical intracytoplasmic tyrosine residue is presented (Fig. 1B).

It was thus of importance to examine the importance of intracytoplasmic tyrosine residues in two different retroviruses belonging to the *Oncornavirinae* subfamily. Human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus whose budding site in epithelial cells has never been reported. Its intracytoplasmic domain possesses two tyrosine residues located very close to each other, and this domain is also much shorter than in HIV (24). The budding of Moloney murine leukemia virus (Mo-MuLV) was previously suggested to occur predominantly through the basolateral surfaces of polarized epithelial cells (48). This virus has a simple, basic genome organization common to all retroviruses, with the genes *gag*, *pol*, and *env* encoding capsid proteins, enzymatic functions, and envelope glycoproteins, respectively. The intracytoplasmic domain of Mo-MuLV envelope glycoprotein is also short although it harbors two tyrosines, one of them being located very close to the carboxy-terminal end of the protein.

The capability of heterologous retroviral envelope glycoproteins to be incorporated into the HIV virion (24, 31, 57) was thus used to determine if these oncornaviral glycoproteins can confer polarized basolateral budding. An HIV-1 envelope-negative proviral DNA was previously described and was constructed by introducing both a stop codon and a frameshift in the *Env*-encoding sequence (29). This proviral DNA can be *trans*-complemented with an HIV-1 envelope expression vec-

tor. The DNA constructs were introduced by lipofection directly onto confluent Madin-Darby canine kidney epithelial cells (MDCK cells) grown on semipermeable filters. This allows separate access to both the media bathing the apical and basolateral cell surfaces and quantitation of viral release by a sensitive enzyme-linked immunosorbent assay (ELISA) technique allowing immunodetection of the HIV p24 capsid protein, as previously described (29). The high sensitivity of the ELISA assay, combined with the capability of HIV capsids to incorporate heterologous *Env* glycoproteins, has made possible the study of polarized budding of these pseudotyped viruses. As shown in Fig. 2A, gradual increase in the amount of the *trans*-complementing envelope expression plasmid resulted in gradual establishment of the basolateral polarization phenotype; at larger envelope amounts essentially all the virus was released from the basolateral membrane. A similar phenomenon was observed upon gradual *trans*-complementation by addition of either HTLV-1 or Mo-MuLV envelope expression vector (Fig. 2B and C); these HTLV-1 and Mo-MuLV envelope glycoprotein vectors have been previously described (6, 28). However, the amount of *Env*-encoding plasmid required to achieve polarization was greater than that of the homologous HIV-1 envelope. This could not be due to differences in promoter efficiency since we verified that in MDCK cells, both the cytomegalovirus (CMV) promoter and the Mo-MuLV long terminal repeat (LTR) promoter used in these two expression vectors are as strong as or stronger than the LTR promoter used in the HIV envelope expression vector, as found in a transient chloramphenicol acetyltransferase (CAT) activity ex-

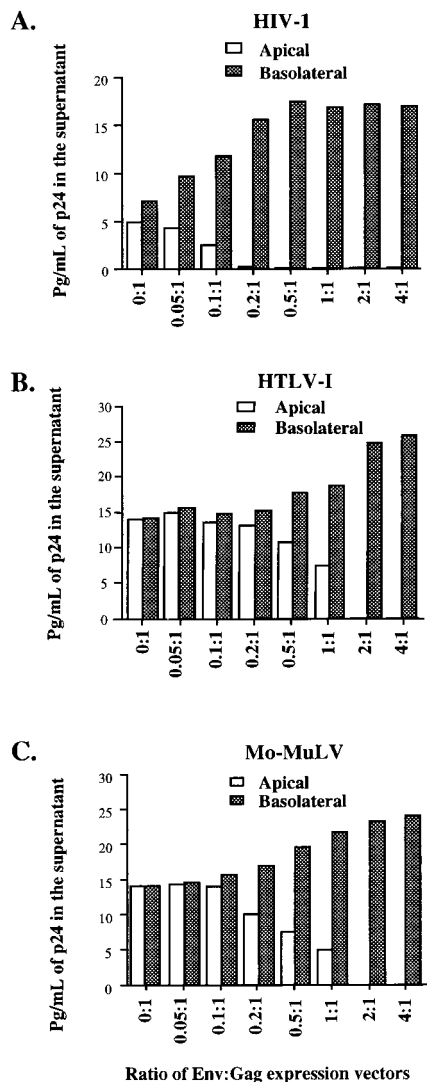


FIG. 2. Effect of retroviral glycoproteins on polarized HIV release. MDCK cells were grown on semipermeable filters (2.5-cm diameter; 1- μ m pore size) and then cotransfected with Env-negative HIV proviral DNA (0.5 μ g) and various amounts of plasmids encoding the envelope glycoprotein from either HIV-1 (A), HTLV-1 (B), or Mo-MuLV (C). In each case, the total amount of DNA was kept constant at 5 μ g by addition of vector plasmid DNA. Transfections were performed by lipofection as previously described (30), and supernatants were harvested from the apical and basolateral compartments 48 h after DNA introduction. Virus release was quantitated by p24 ELISA. Ratios refer to the amount, in each cotransfection, of Env-encoding plasmid relative to that of HIV Env-negative proviral DNA encoding Gag. Results are the means of two independent experiments, which differed by less than 10%.

pression assay (results not shown). We rather suspect a lower efficiency of heterologous Env incorporation into the viral particle. This may be due to lower efficiency of incorporation per se or indirectly to lower translational efficiency or stability of these glycoproteins, although this point was not further examined. However, it is quite clear that both HTLV-1 and Mo-MuLV envelope glycoproteins possess a basolateral targeting signal that can allow them to substitute for the HIV-1 envelope and promote basolateral maturation of the pseudotyped viruses.

Mutant envelope glycoproteins were next examined in order to confirm the presence of a distinct basolateral targeting sig-

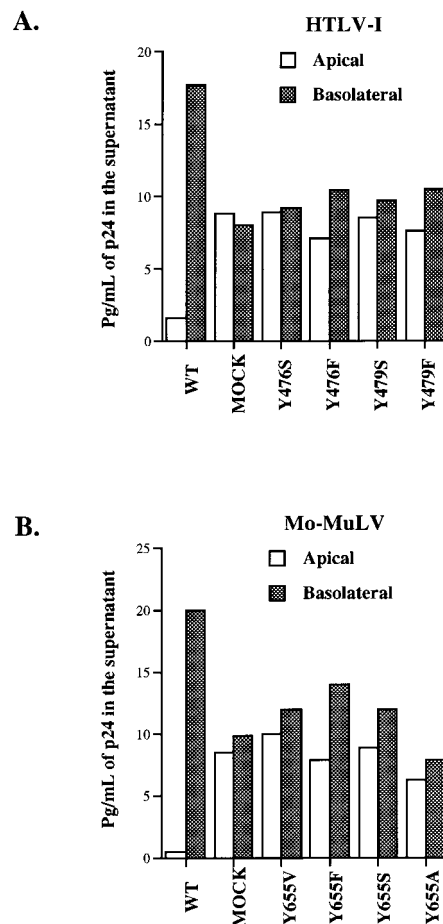


FIG. 3. Effect of HTLV-1 or Mo-MuLV glycoprotein intracytoplasmic tyrosine substitutions on HIV polarized release. Env-negative proviral DNA (0.5 μ g) was transfected in filter-grown MDCK cells with an eightfold excess of plasmids encoding the wild-type (WT) or the mutated envelope glycoprotein from either virus. Transfections were performed by lipofection as previously described, and supernatants were harvested from the apical and basolateral compartments 48 h after DNA introduction. Virus release was quantitated by p24 ELISA. Mock transfections were done with HIV Env-negative proviral DNA alone. Results are the means of two independent experiments, which differed by less than 10%.

nal on these glycoproteins. Basolateral tyrosine-based signals are often found in the intracytoplasmic domain, and this was shown to be the case for HIV (30). Since preliminary data obtained with deletion mutants also supported the importance of the intracytoplasmic domain in both HTLV-1 and Mo-MuLV (data not shown), we targeted this region for site-directed mutagenesis analysis. Tyrosine residues were thus individually examined in both the HTLV-1 and Mo-MuLV intracytoplasmic envelope glycoprotein region. Briefly, for the HTLV-1 mutants, the HTLV-1 fragment of the CR strain (35) was cloned in phagemid pGEM7ZF+, oligonucleotide-directed mutagenesis was performed (22), and the mutated DNA was cloned back into the Env-expressing plasmid (5, 6). Mo-MuLV Env substitutions were introduced by using degenerate double-stranded oligonucleotides cloned into the Mo-MuLV pLTRSDSA Env expression vector (28).

In HTLV-1, replacement of either tyrosine by serine (Y476S or Y479S) drastically affected HTLV-1 envelope-mediated polarized release of pseudotyped virions (Fig. 3A); similar amounts of viruses were found to bud from both the apical and the basolateral plasma membrane domains. An eightfold ex-

cess of the mutant Env-encoding plasmid, more than sufficient for optimal polarization with the wild-type protein, was used in these experiments. Conservative replacement of tyrosine by phenylalanine produced somewhat unexpected results, since neither substitution (Y476F or Y479F) resulted in the partial polarization phenotype previously observed with a similar substitution in the HIV glycoprotein. In the Mo-MuLV envelope, a deletion eliminating the tyrosine closest to the carboxy-terminal end did not affect the polarization phenotype (results not shown). In contrast, replacement of the membrane-proximal tyrosine by either valine, serine, or alanine essentially abolished the polarization. The phenylalanine substitution produced a somewhat intermediate phenotype as previously observed in HIV-1 (30).

Altogether, these data suggested that in both HTLV-1 and Mo-MuLV, as also previously described for HIV, intracytoplasmic tyrosine residues play a crucial role as basolateral targeting determinants. Further characterization was required to better support this idea, since the intracytoplasmic tail has been shown to modulate various functions of retroviral glycoproteins: viral envelope incorporation, glycoprotein cell surface expression, stability, and infectious potential have all been shown to be altered by certain substitutions in the intracytoplasmic domain (7, 8, 11, 12, 16, 23, 41, 44–46, 54, 58). To reach clear conclusions concerning the mutant glycoproteins, it was therefore necessary to ensure that these molecules are incorporated into the budding viral particles at a level comparable to that of the wild-type protein. This is especially important since, as previously mentioned, actual incorporation of the envelope glycoprotein into the budding virion is required for the polarized release of the virus (29). In order to clarify this point for the different mutants, an infectivity assay was performed using a reporter *CAT* gene. The envelope-negative HIV proviral construct was modified in order to replace the nonessential *nef* gene with the *CAT* gene (17, 60). This proviral construct was cotransfected into MDCK cells with an expression plasmid for wild-type glycoprotein or any of the mutant glycoproteins. The amount of recovered virus was then determined by a reverse transcriptase assay (26), the same amount of virus was used to infect susceptible Jurkat-TA lymphocytes (for HIV or HTLV-1 pseudotypes) or NIH 3T3 mouse fibroblasts (for Mo-MuLV pseudotypes), and the resulting *CAT* activity was measured in the cell lysates (14, 25). This assay can be used quantitatively since increasing or decreasing amounts of virus applied to the cells resulted in corresponding changes in *CAT* activity levels (data not shown). Cotransfections with various amounts of the plasmid used for expression of wild-type HIV-1 envelope glycoprotein were then performed. This revealed that viral infectivity begins to drop when less than half of the amount of HIV envelope expression vector is used relative to the Env-negative *CAT* proviral construct (Fig. 4A). Similar experiments performed with the heterologous glycoproteins revealed that the larger amounts of HTLV or Mo-MuLV envelope plasmid expression vector needed to achieve polarization do reflect the larger amount of these heterologous glycoprotein-expressing plasmids required for optimal transfer of *CAT* activity. Therefore, there exists a direct correlation between the level of heterologous Env required for infectivity and polarized budding. This may be due to reduced stability or differences in the maturation process but most likely reflects differences in the efficiency of heterologous envelope glycoprotein incorporation into the HIV capsid.

The same procedure was used to analyze the infectivity of the virions pseudotyped with the various envelope mutants using equimolar amounts of envelope-negative HIV proviral DNA and mutant envelope expression vectors. For Mo-MuLV,

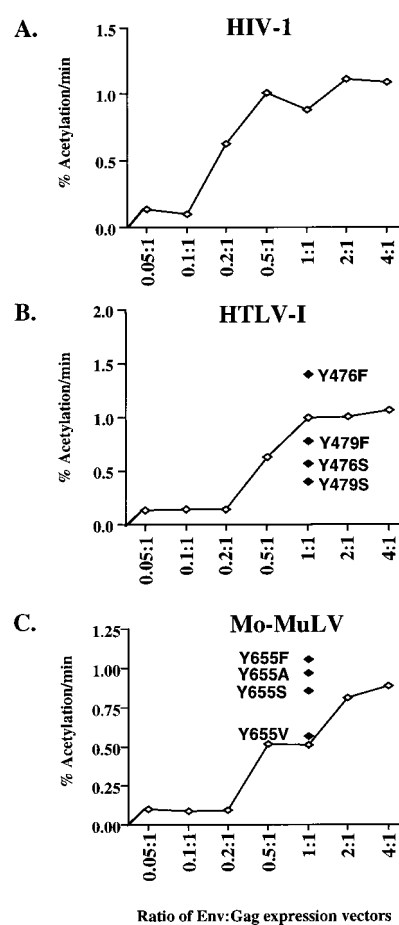


FIG. 4. The envelope-negative *CAT*-encoding proviral DNA was cotransduced into MDCK cells (10^6 cells in 75-cm^2 petri dishes) with various amounts of plasmids encoding the envelope glycoprotein from either HIV-1, HTLV-1, or Mo-MuLV. In each case, the total amount of DNA was kept constant at $20\ \mu\text{g}$ by addition of vector plasmid DNA containing the HIV-1 LTR (for HIV-1), CMV (for HTLV-1), or Mo-MuLV LTR (for Mo-MuLV) promoters. Equivalent amounts of the different viruses were then used to infect human Jurkat-TA cells (A and B) or murine NIH 3T3 cells (C). *CAT* assays with cell lysates were then performed, and the percentage of chloramphenicol conversion to acetylated forms was determined by scintillation counting. Results are presented as *CAT* activity relative to the ratios of Env-encoding plasmid to HIV Env-negative proviral DNA in each initial MDCK cell cotransfection. The mean *CAT* activity was calculated from two different incubation periods in the linear range of the assay. The results obtained at a 1:1 ratio for the different mutants described in the text are presented as closed symbols.

the different tyrosine mutants were able to confer infectivity as efficiently as or even better than the wild-type protein (Fig. 4C). The situation is somewhat more complex for HTLV-1. The conservative phenylalanine replacement of the membrane-proximal tyrosine (Y476F) increased the capacity to confer infectious potential compared to wild-type HTLV-1 envelope (Fig. 4B). However, similar replacement of the carboxy-proximal tyrosine (Y479F) had a very different effect. The conservative Y479F mutation reduced the infectivity of pseudotyped virus to approximately two-thirds of that obtained with the wild-type HTLV-1 glycoprotein (Fig. 4B). Both tyrosine-to-serine substitutions (Y476S and Y479S) had an even more negative effect on the infectivity of the pseudotyped virus. Similar results were also obtained when comparisons with the wild-type were made at different ratios of envelope

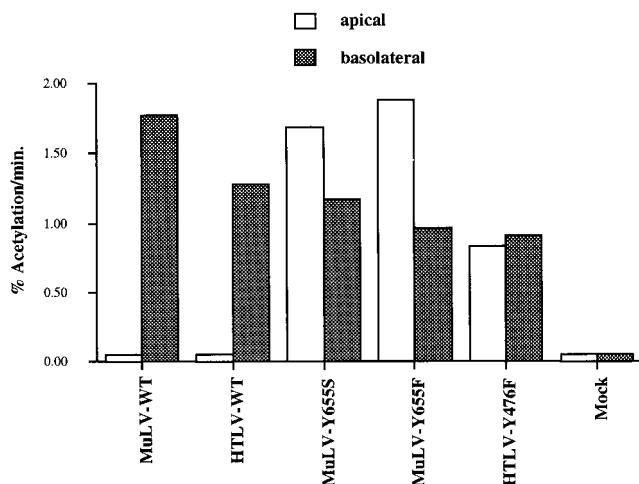


FIG. 5. Effect of HTLV-1 (Y476F) or Mo-MuLV (Y655S or Y655F) glycoprotein intracytoplasmic tyrosine substitutions on the infectivity of apically or basolaterally released pseudotyped viruses analyzed by using the CAT trans-complementation assay. Env-negative CAT-encoding proviral DNA (0.5 μ g) was transfected in filter-grown MDCK cells with an eightfold excess of plasmids encoding the wild-type (WT) or the mutated envelope glycoprotein. Transfections were performed by lipofection as previously described, and supernatants were pooled from the three apical or basolateral compartments 48 h after DNA introduction. Viruses released were used to infect Jurkat-TA (HTLV WT and mutant Y476F) or NIH 3T3 (Mo-MuLV WT and mutants) cells. Mock transfections were done with the HIV CAT-encoding Env-negative proviral DNA alone.

expression vector to Env-negative proviral DNA (data not shown).

Since all Mo-MuLV mutants analyzed exhibited an essentially identical or even greater efficiency than the wild-type glycoprotein in their ability to complement the envelope-defective CAT proviral construct, the loss of polarization is unlikely to be due to a difference in stability, processing, or virion incorporation of these proteins but must instead be due to alteration of a polarization signal per se. A conservative phenylalanine substitution at this position gave rise to an intermediate polarization phenotype. The CAT reporter assay was further used to confirm that the viral assembly takes place correctly in polarized cells lipofected with representative mutants already shown to possess normal infectivity while being affected in their polarized budding phenotype: Mo-MuLV mutants Y655S and Y655F and the HTLV-1 mutant Y476F. Confluent MDCK cells grown on semipermeable membranes were colipofected with the HIV CAT provirus and envelope expression vectors. Supernatants were then recovered from either the apical or the basolateral surface and tested for the presence of infectious virus by infection of susceptible cells followed by measurement of CAT activity. Results presented in Fig. 5 confirmed that glycoprotein envelope mutants lacking the ability to be targeted specifically at the basolateral surface are still normally incorporated into infectious virions released from either the apical or the basolateral membrane domain.

The effect of tyrosine substitutions observed in the Mo-MuLV glycoprotein is similar to our previous observations with the HIV glycoprotein, in which case it was also possible to demonstrate the presence of tyrosine-based basolateral targeting signals, independently of sequences mediating envelope incorporation and required for the infectious potential of the virus (30). In HTLV-1, the relative importance of the two tyrosines was more difficult to evaluate since replacement of tyrosine 479 significantly disrupts virus infectious potential.

However, viruses harboring a substitution of tyrosine 476 to phenylalanine are as infectious as, if not more infectious than, the wild-type HTLV-1 Env pseudotyped viruses. The lack of polarized budding observed with the Y476F mutant is therefore directly linked to the mutation. In contrast to the important distance between tyrosine residues in HIV-1, tyrosine 479 is very close to the membrane-proximal tyrosine 476 in HTLV-1. Replacement of a proline residue located close to the membrane-proximal tyrosine in HIV was previously shown to affect polarization, probably due to an alteration of a putative β -turn in this region (30). Neighboring β -turns are suspected to have an important role in tyrosine-based endocytosis and polarization sorting signals (19, 42, 43, 63). It is possible that replacement of the tyrosine at position 479 in HTLV-1 somehow affects the local structure in a similar manner. Furthermore, tyrosine 476 cannot be solely required, since it is not conserved in HTLV-2 (Fig. 1A). It should also be stressed that it is quite likely that the different functions are more uniformly distributed throughout the length of the much longer HIV glycoprotein intracytoplasmic domain while these different signals may be closely packed in the shorter intracytoplasmic domain of other retroviral glycoproteins, rendering it more difficult to dissociate these different functions. Nevertheless, these experiments clearly established that basolateral targeting is a phenomenon dependent on the envelope glycoproteins and is common to the envelope glycoproteins of members of both the *Oncornavirinae* and the *Lentivirinae*. The shorter intracytoplasmic tails of the two members of the *Oncornavirinae* examined in this study appear to encompass the required information for polarized viral budding which, as in HIV, is dependent on one or more critical membrane-proximal tyrosine residues located in the first 30 amino acid residues extending from the cytoplasmic face of the plasma membrane. The procedure used in this study took advantage of HIV pseudotyping with oncornavirus glycoproteins. However, incorporation of wild-type or mutant Mo-MuLV envelope glycoproteins into homologous virions using cotransfection with a MuLV-based retroviral vector harboring a puromycin resistance gene has confirmed the results obtained with the heterologous system (data not shown).

One of the intriguing aspects of the results is the ability of oncornavirus envelope glycoproteins to direct the site of lentivirus budding in polarized cells. There is no detectable sequence similarity between the membrane-spanning and cytoplasmic domains of the oncornavirus and lentivirus envelope glycoproteins, yet the oncornavirus proteins can efficiently prevent the budding of lentivirus particles from the apical membrane of polarized epithelial cells. The recovery of pseudotyped HIV virions with infectivity similar to that of wild-type or Y655-substituted Mo-MuLV envelope glycoproteins indicates that the tyrosine-based polarization signal is affecting the sorting of envelope glycoproteins rather than the association between glycoproteins and the retroviral particle. The data suggest a model in which Gag molecules, or a yet-unknown protein required for retroviral budding, are sorted together with the envelope glycoprotein to basolateral membranes through an association mediated by a structure of the envelope glycoprotein, a structure not readily recognized from sequence analysis. Further study of the incorporation of short intracytoplasmic Env glycoproteins into the HIV particle and of their ability to polarize the budding viruses may help elucidate the mechanisms involved in the recognition of Env by Gag in virus assembly and morphogenesis.

Tyrosines in Y-X-X-L motifs found in the cytoplasmic domain of Env proteins have been found in other retroviruses to be involved in viral pathogenesis. In bovine leukemia virus,

replacement of the tyrosine affects the capability of the virus to disseminate in the organism (64). In simian immunodeficiency virus, mutation of the membrane-proximal tyrosine results in the protein's more uniform distribution at the plasma membrane of infected lymphocytes (23).

Several studies have discussed the importance of epithelial-cell infection in HIV pathogenesis (1, 10, 39, 40, 59). A polarized release of viruses in these cells may be important for dissemination in the host as well as individual-to-individual transmission, which has been shown to occur mostly through infected cells rather than by free virus. It is well established that cell-to-cell transmission of retroviruses is more efficient than infection by cell-free virus, and this is especially true for HTLV-1, for which cell-free transmission is especially inefficient (3, 27). The conservation of basolateral maturation in different retroviruses may well reflect the importance of the phenomenon that could also have importance in other cell types, in addition to epithelial cells. It is generally thought that sites of intercellular contact are somehow analogous to basolateral surfaces, as far as transport of plasma membrane proteins is concerned; accordingly, budding of basolaterally targeted viruses tends to occur at these intercellular contact zones (47). Elegant electron-microscopic studies have shown the preferential budding of HIV virions at contact sites between infected lymphocytes and cultured epithelial cells (2, 39, 40). This, and the observation that virions tend to bud from a specific pole in infected lymphocytes, suggests the importance of polarization in cell-to-cell transmission of retroviruses, and this point will certainly deserve further study. Since replacement of the membrane-proximal tyrosine residue has an effect on the polarization phenotype, independently of any effect on envelope incorporation or viral infectivity, this raises the possibility of using the murine model for studying the importance of polarized budding in retroviral pathogenesis.

In conclusion, despite the large body of work that has been devoted to the topic, the identification of polarization signals on various additional proteins is still required in order to get a better overall understanding of polarized transport and sorting. Our demonstration of the polarization phenomenon, and identification of signals involved, is clearly an essential prerequisite for a better understanding of the interactions between retroviruses and epithelial cells; this can also have important consequences at the level of cell-to-cell transmission in other cell types.

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