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The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells

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Budding of retroviruses from polarized epithelial Madin–Darby canine kidney cells (MDCK) takes place specifically at the basolateral membrane surface. This sorting event is suspected to require a specific signal harbored by the viral envelope glycoprotein and it was previously shown that, as for most basolateral proteins, the intracytoplasmic domain plays a crucial role in this targeting phenomenon. It is well known that tyrosine-based motifs are a central element in basolateral targeting signals. In the present study, site-directed mutagenesis was used to generate conservative or non-conservative substitutions of each four intracytoplasmic tyrosines of the human immunodeficiency virus (HIV-1) envelope glycoprotein. This approach revealed that the membrane-proximal tyrosine is essential to ensure both the basolateral localization of envelope glycoprotein and the basolateral targeting of HIV-1 virions. Substitutions of the membrane-proximal tyrosine did not appear to affect incorporation of envelope glycoprotein into the virions, as assayed by virion infectivity and protein content, nor its capability to ensure its role in viral infection, as determined by viral multiplication kinetics. Altogether, these results indicate that the intracytoplasmic domain of the HIV-1 envelope glycoprotein harbors a unique, tyrosine-based, basolateral targeting signal. Such a tyrosine-based targeting signal may play a fundamental role in HIV transmission and pathogenesis.

Keywords: basolateral signal/epithelial cells/human immunodeficiency virus/lentiviruses/protein targeting

Introduction

Maintaining the integrity of cellular architecture, through an asymmetrical distribution of surface and intracellular proteins, is essential to ensure that different cell types assume their role in tissue organization. Perhaps the best example of a specific subcellular distribution of plasma membrane proteins is observed at the surface of polarized epithelial cells (Simons and Fuller, 1985; Compans and Srinivas, 1991; Mellman *et al.*, 1993; Matter and Mellman, 1994). These cells are best known as essential building blocks of the intestinal wall lining, kidney tubules and various mucosal surfaces. In epithelial cells, two plasma membrane domains are clearly differentiated and exhibit a distinct lipid and protein composition (Sargiacomo *et al.*, 1989; van Meer, 1993). The apical domain, often abundant with microvilli, faces the lumen of the organ; its surface harbors proteases and glycolytic enzymes, and houses most of the proteins involved in specialized functions. The basolateral domain is found below the tight junctions that link cells together to form a tight epithelium; in mucosal surfaces, this membrane is thus oriented toward the underlying extracellular matrix, cells or blood flow. The basolateral membrane mostly harbors proteins associated with cellular adsorption and housekeeping functions common to all cells.

The generation of different plasma membrane domains with the specific distribution of their proteins involves the sorting of these different proteins at the exit of the Golgi apparatus (Simons and Wandinger-Ness, 1990; Rodriguez-Boulan and Zurzolo, 1993). The exact nature of the hypothetical sorting signals still remains largely unknown. However, accumulating evidence points to the existence of distinct targeting signals on proteins destined to either the apical or basolateral membrane domain; in the absence of such signals, a roughly uniform distribution of the protein is generally observed at the cell surface (Wandinger-Ness *et al.*, 1990). In the last few years, tyrosine-based signals, especially in Y-X-X-aliphatic/aromatic consensus motifs, have been found in the intracytoplasmic domain of basolateral membrane proteins and associated with targeted protein delivery (Hunziker *et al.*, 1991; Matter *et al.*, 1992; Dagermont *et al.*, 1993; Thomas *et al.*, 1993; Thomas and Roth, 1994; Höning and Hunziker, 1995; Ohno *et al.*, 1995). Interestingly, these tyrosine-based signals have also been found to be used as endocytosis signals (Hopkins, 1992; Prill *et al.*, 1993; Trowbridge *et al.*, 1993; Pytowski *et al.*, 1995).

Viral envelope glycoproteins are synthesized and transported by the normal cellular machinery, and it has long been recognized that they are good representative models for the study of intracellular protein transport pathways (Rodriguez-Boulan and Sabatini, 1978; Rodriguez-Boulan, 1983; Rodriguez-Boulan *et al.*, 1983; Tucker and Compans, 1993). Accordingly, different viruses have evolved mechanisms for the specific delivery of their envelope glycoproteins to one or the other membrane domains of polarized epithelial cells. Classical examples include the vesicular stomatitis virus G glycoprotein that harbors a critical intracytoplasmic tyrosine residue required for polarized basolateral targeting (Thomas *et al.*, 1993; Thomas and Roth, 1994). Likewise, addition of a tyrosine residue to the intracytoplasmic domain of the normally apical hemagglutinin envelope glycoprotein of influenza virus redirects this protein to the basolateral surface (Brewer and Roth, 1991). The targeting of viral envelope glycoproteins has important consequences in viral maturation and release; although capsid proteins

generally lack membrane domain targeting signals themselves, viral budding resulting in release of enveloped viruses specifically occurs through the membrane domain harboring an adequate envelope glycoprotein (Tucker and Compans, 1993).

In the case of human immunodeficiency virus (HIV-1), targeted viral release was shown to occur mainly through the basolateral membrane surface (Owens and Compans, 1989; Fantini *et al.*, 1991; Owens *et al.*, 1991). A specific basolateral release has also been previously suggested for another retrovirus, namely murine leukemia virus (Roth *et al.*, 1983; Kilpatrick *et al.*, 1988). More recently, we showed that targeting of HIV budding requires the intracytoplasmic portion of the envelope glycoprotein transmembrane moiety, gp41. Large carboxy-terminal deletions of this intracytoplasmic region severely impair polarized release of the virus, whereas smaller deletions have little or no effect (Gabuzda *et al.*, 1992b; Lodge *et al.*, 1994). The actual incorporation of the glycoproteins into the budding virions was also found to be essential, since alterations of the matrix protein precluding their incorporation into the viral particles also prevented targeting of viral budding (Dorfman *et al.*, 1994; Lodge *et al.*, 1994).

In the present study, we performed site-directed mutagenesis, individually substituting each of the intracytoplasmic tyrosine residues of the envelope glycoprotein of HIV-1. Non-conservative substitutions of the membrane-proximal tyrosine residue abolished basolateral targeting of both envelope glycoproteins and viral budding, but had no apparent effect on *in vitro* infectious potential of the resulting viruses. Such tyrosine-based basolateral signals may play a fundamental role in efficient urogenital transmission and pathogenesis of lentiviruses (Phillips and Bourinbaiar, 1992; Phillips *et al.*, 1994), promoting viral dissemination in the host and access to various sensitive cell types.

Results

Rationale for mutagenesis

Several viral and cellular membrane proteins have been shown to harbor tyrosine-based basolateral targeting determinants in their intracytoplasmic domains (Hunziker *et al.*, 1991; Matter *et al.*, 1992; Thomas *et al.*, 1993; Thomas and Roth, 1994). The HIV-1 gp41 transmembrane glycoprotein harbors four tyrosine residues in its 150 amino acid-long intracytoplasmic region, as shown in Figure 1A. These four residues are highly conserved between HIV-1 isolates; of the four, residues 712 and 768 are found in a Y-X-X-L context. Interestingly, different retroviral envelope glycoproteins also harbor one or more intracytoplasmic tyrosine residues in a similar context; and this is despite the fact that other retroviruses possess much shorter intracytoplasmic domains compared with HIV-1 (Figure 1B). We thus undertook this site-directed mutagenesis study in order to clarify the possible occurrence of a tyrosine-based basolateral targeting signal in HIV-1 envelope glycoproteins.

Substitution mutagenesis of the HIV envelope intracytoplasmic tyrosines

We used a technique of polymerase chain reaction (PCR) for the mutagenesis of an HIV-1 envelope gp160 plasmid expression vector as described in Materials and methods. Using this technique, each tyrosine was substituted for a serine; such a substitution maintains the presence of an hydroxyl side chain at this position, but eliminates the aromatic ring and should thus be considered as a non-conservative substitution. The plasmid expression vectors encoding wild-type or mutant envelope glycoproteins were then introduced into polarized monolayers of MDCK cells in order to complement an envelope-defective proviral DNA as described in Materials and methods. This *trans*-complementation assay, performed by lipofection of confluent cells forming a tight monolayer on semi-permeable filters, was described previously (Lodge *et al.*, 1994). Released viral particles were then recovered in the upper chamber medium bathing the apical membrane or lower chamber medium bathing the basolateral membrane attached onto the semi-permeable filter. Recovered viral particles were then quantitated by a sensitive immunodetection method using an ELISA directed against the p24 viral capsid protein.

Substitution of the membrane-proximal tyrosine (Y712S) completely abolished the polarized basolateral release. The ratio of apically to basolaterally released virions was similar to the levels obtained with envelope-negative viruses. In contrast, substitution of either of the three other carboxy-terminal tyrosines (Y768S, Y795S, Y802S) maintained viral release exclusively basolateral (Figure 2A).

Further analysis of the tyrosine-dependent basolateral viral budding signal

To establish further the importance of tyrosine 712, we examined the effect of another non-conservative substitution to alanine (Y712A) as well as a conservative substitution for the aromatic amino acid, phenylalanine (Y712F). Furthermore, a proline residue found close to the tyrosine and well conserved among HIV viral isolates was changed for an alanine (P714A). This amino acid was chosen as a target for site-directed mutagenesis since it is known to promote the formation of β-turns and such a structure is suspected to play an important role as part of tyrosine-based targeting signals (Collawn *et al.*, 1990; Hopkins, 1992; Prill *et al.*, 1993; Trowbridge *et al.*, 1993; Pytowski *et al.*, 1995). When co-transfected in MDCK cells with the envelope-negative HIV proviral construct, the expression vector encoding the non-conservative Y712A substitution mutant did not rescue polarized budding of the virus. A significant amount of virus could also be detected in the apical-bathing medium of cells encoding the conservative Y712F mutant; however, viral release at the basolateral surface was still favored and it thus seems that this mutation does not completely eliminate the polarization signal (Figure 2B). Alanine substitution of the proline 714 residue abolished basolateral targeting and similar amounts of virus were released in both apical- and basolateral-bathing media. Taken together, these results indicate a critical role for an aromatic amino acid, preferably a tyrosine, in a membrane-proximal position and also suggest a role for a local secondary structure, namely a putative β-turn, in the targeting signal.

Plasma membrane distribution of the mutant envelope glycoprotein

The membrane targeting of envelope glycoproteins, rather than the polarized budding of viruses, was examined to

Fig. 1. (**A**) Conservation of tyrosine residues in the intracytoplasmic domain of various HIV or SIV Env glycoprotein isolates. Sequences surrounding the tyrosines are shown; gaps between the sequences are represented by dots. Predicted protein secondary structures are also included and amino acids are numbered from the first Met of gp160 (HXBc2). Single amino acid deletions are represented by a dash. (**B**) Positions of intracytoplasmic tyrosines in various retrovirus transmembrane (TM) glycoproteins. For comparative purposes, some known viral and cellular tyrosine-based basolateral signals are also included.

establish further the importance of the membrane-proximal intracytoplasmic tyrosine in basolateral targeting of Env. Since the lipofection procedure on confluent MDCK cells was not sufficiently efficient to detect envelope protein expression *per se*, an alternate method was devised using virions pseudotyped with the vesicular stomatitis virus G envelope glycoprotein (VSV-G), since VSV is able to infect MDCK cells. Viruses having incorporated a mosaic of HIV-Env and VSV-G glycoproteins, or representing a VSV-G-containing and HIV-Env-containing heterogeneous viral population, were generated in transfected COS cells as described in Materials and methods. Immunoprecipitation of labeled pseudotyped viral particles with both a rabbit anti-VSV serum and a HIV-positive human serum clearly show the presence of gp120 and VSV-G glycoproteins (Figure 3A, lane 4). VSV-G-mediated infection allowed the transduction of wild-type or mutant HIV viral genomes by infection of MDCK cells with the pseudotyped viruses, allowing HIV-Env expression in a large amount of cells. Radiolabeling and surface immunoprecipitation were then performed by applying a monoclonal anti-gp120 antibody to either the apical or basolateral membrane compartments of filter-grown cells. The results of this experiment, shown in Figure 3B, confirmed that the wild-type glycoprotein is restricted to the basolateral surface, where virus budding occurs (compare lanes 5 and 8). Similarly, the presence of the Y712S mutant glycoprotein at both membrane surfaces (lanes 6 and 9) correlates with equivalent viral release from both plasma membrane domains observed with this mutant. Immunoprecipitation of intracellular proteins with the same antibodies clearly shows that expression of wild-type Env and Y712S mutant glycoproteins was equivalent (lanes 2 and 3).

Effects of amino acid substitutions on glycoprotein incorporation into the virions

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 mutations in the intracytoplasmic domain of HIV gp41 (Dubay *et al.*, 1992; Gabuzda *et al.*, 1992b; Shimuzu *et al.*, 1992; Ritter *et al.*, 1993; Spies and Compans, 1994; Freed and Martin, 1995; LaBranche *et al.*, 1995). To achieve 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 the wild-type protein. This is especially important since it was previously demonstrated that actual incorporation of the envelope glycoprotein into the budding virion is required for the polarized release of the virus (Lodge *et al.*, 1994). In order to clarify this point for the different mutants, a *trans*-complementation infectivity assay was first performed using a reporter CAT gene (Helseth *et al.*, 1990). The envelope-negative proviral construct was modified to replace the non-essential *nef* gene by the CAT gene. This proviral construct was co-introduced with an expression plasmid for wild-type or either of the different mutant

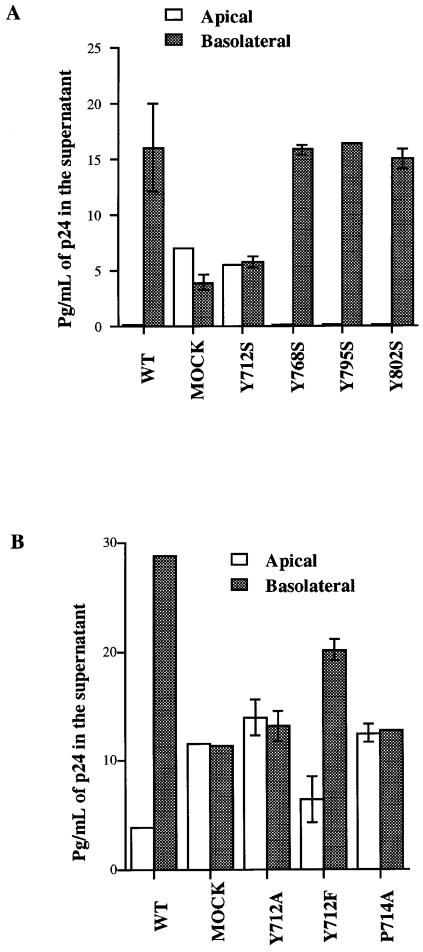

Fig. 2. (**A**). Effect of tyrosine-to-serine mutations in the gp41 intracytoplasmic domain on polarized virus release. The *env*-negative HXBH10 proviral construct was co-transfected in equimolar amounts with plasmids encoding HIV wild-type (WT) or mutant (Y712S, Y768S, Y795S, Y802S) gp160 as described in the text. Apical and basolateral supernatants were harvested 48 h after lipofection, and p24 ELISA were performed as described in Materials and methods. (**B**) Effect of various substitutions (Y712A, Y712F) of the membrane-proximal gp41 intracytoplasmic tyrosine or a nearby proline (P714A). Viral release was detected as in (A). The mock results were obtained following introduction of *env*-negative proviral plasmid DNA alone and all results are presented as the average of separate values obtained from two separate lipofections plus or minus SD.

glycoproteins; MDCK cells grown in Petri dishes being used in these experiments. Identical amounts of recovered virus, as determined by RT (viral reverse transcriptase) measurement, was then used to infect susceptible CD4+ Jurkat lymphocytes and the resulting CAT activity was measured in the cell lysates, as described in Materials and methods. All HIV-1 glycoproteins harboring substitution mutations did confer the infectious potential to the envelope-negative CAT provirus (Figure 4). The capacity of the different envelope proteins to promote viral-mediated transfer of CAT activity is thus indicative of normal envelope incorporation into the virion and the capability to interact with the viral receptor to initiate infection. These results were also observed when viruses harboring mutant Y712S glycoprotein were recovered from apical or basolateral supernatants of cells grown on semi-permeable membranes. Viruses from both apical and basolateral supernatants displayed similar ability to mediate CAT transfer, indicating that the Env glycoproteins incorporated into these viruses were functional (Figure 5A). This, again, clearly demonstrates that transport of the mutant glycoproteins themselves was no longer polarized and that virions can acquire the mutant Y712S envelope glycoprotein from either apical or basolateral cell surfaces. Furthermore, we directly confirmed that wild-type amounts of viral glycoproteins were present on virions released from both membrane surfaces in MDCK cells transfected with the Y712S mutant as determined by Western blot analysis (Figure 5B) and radioimmunoprecipitation of labeled viral particles (data not shown). Probing of the same nitrocellulose filter with the human HIV-positive serum detected similar amounts of major viral capsid protein p24 in the apical and basolateral supernatants of cells expressing the Y712S mutant, demonstrating that virions were recovered from both surfaces, while only basolateral virion-associated proteins were detected in the presence of wild-type envelope glycoprotein (data not shown).

Replication kinetics of mutant HIV viruses

In order to establish further that the mutant HIV envelope glycoproteins possess normal functional properties, appropriate mutations were introduced into an infectious proviral clone. The different proviral DNA constructs were then transfected into Jurkat CD4+ T cells and the kinetics of viral multiplication were followed through the measurement of reverse transcriptase activity in the supernatant (Figure 6B). Virus production was maximum at day 12 for the positive wild-type provirus control HXBc2. Similarly, all proviruses containing a substitution at tyrosine 712 (Y712S, Y712A, Y712F) or tyrosine 768 (Y768S, used as a control) exhibited equivalent levels of reverse transcriptase activity at day 12. These results indicate that there is no apparent loss of function in the *in vitro* replicative capacity of these proviruses containing substitutions in the tyrosine-based basolateral targeting signal. Furthermore, as determined by radioimmunoprecipitation of transfected Jurkat lymphocytes just before the peak of viral replication (day 12), expression levels of mutant viral glycoproteins, as well as their incorporation into the virion, were shown to be similar to the wild-type glycoprotein. This further establishes that defects in polarized release are not due to a deficient processing or incorporation of the mutant glycoproteins (Figure 6A).

Discussion

The identification of targeting signals resulting in polarized basolateral budding of HIV-1 is clearly an essential prerequisite for a better understanding of its interactions with epithelial cells. The intracytoplasmic domain of several basolateral membrane proteins has been shown to encompass the molecular determinants involved in their polarized transport (Hunziker *et al.*, 1991; Matter *et al.*, 1992; Geffen *et al.*, 1993). More recently, tyrosine-based basolateral targeting signals have been identified in the cellular polymeric immunoglobulin and LDL receptors as well as in the intracytoplasmic tail of the VSV-G (Figure 1B) (Casanova *et al.*, 1991; Aroeti *et al.*, 1993; Thomas *et al.*, 1993; Thomas and Roth, 1994). The significance of tyrosine-based signals in polarized basolateral transport

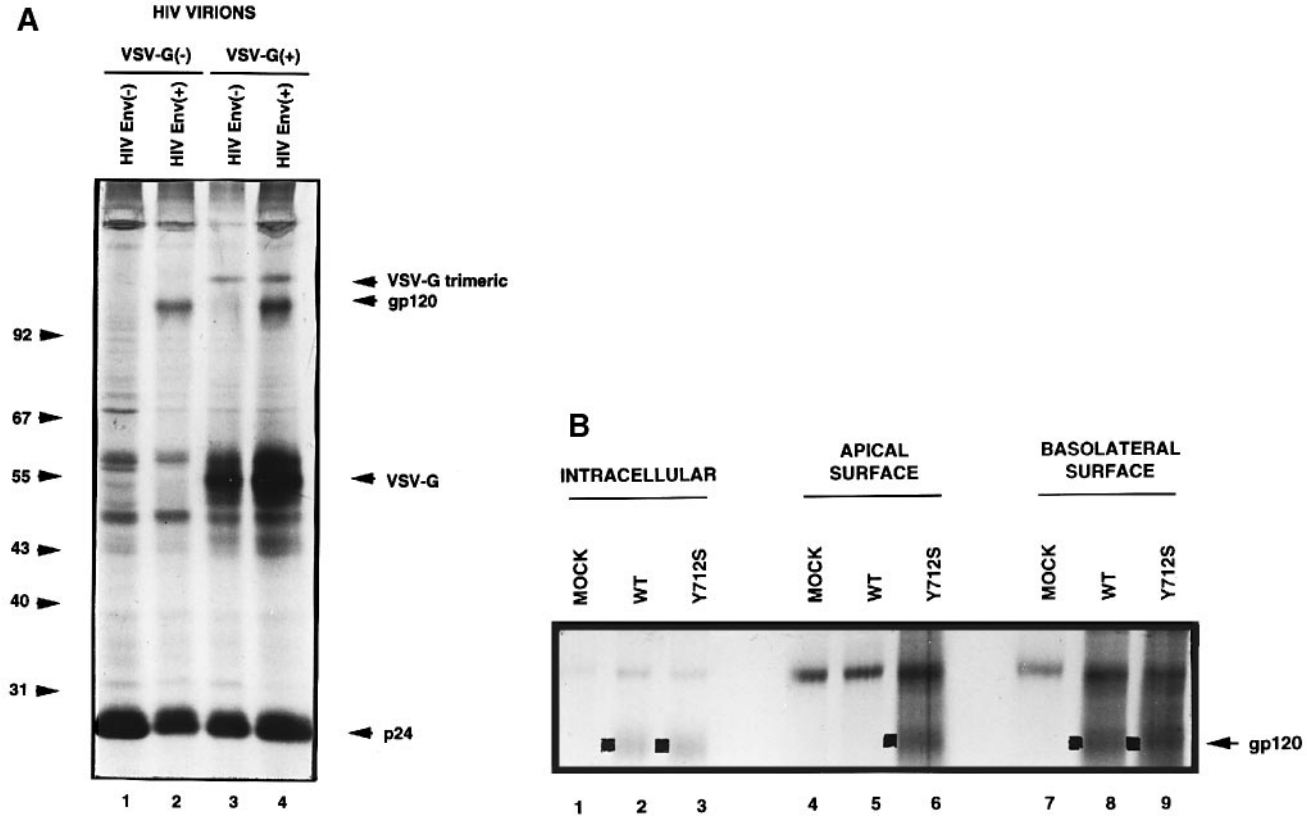

Fig. 3. (**A**) Incorporation of VSV-G glycoprotein in HIV virions. 10^6 COS cells were transfected with 10 μg of either proviral construct with or without 10 μg of SVCMV-VSV-G. Cells were labeled 40 h after transfection with 150 μCi/ml of [^{35}S]methionine for 8 h. Cell-free supernatants were collected and ultracentrifuged, and viral pellets were then resuspended in RIPA lysis buffer. Viral proteins were immunoprecipitated with the HIV-positive human serum combined with the rabbit anti-VSV serum and loaded on a 10% SDS–PAGE gel as described in Materials and methods. Labeled proteins were then revealed by autoradiography. Detection of p24, VSV-G, gp120 and VSV-G trimeric forms is indicated by arrows. Positions of molecular weight markers are indicated. (**B**) MDCK cell surface expression of Env. Identical volumes of transfected COS cell-free supernatants containing the G-protein chimeric wild-type or mutant Env HIV viruses were added onto 10^6 MDCK cells grown in 100 mm diameter Petri dishes for 8 h. MDCK cells were then trypsinized and grown for 1–2 days on semi-permeable 1 μm pore diameter filter membranes. After an 8 h labeling with 150 μCi/ml of [^{35}S]methionine, cells were washed with PBS and monoclonal anti-gp120 antibody was added to either their apical or basolateral surface. Cells were then washed, lysed with RIPA, and added to protein A–Sepharose beads. Intracellular proteins were obtained by immunoprecipitating the cell lysate supernatants of the surface protein immunoprecipitates. Immunocomplexes were separated by 10% SDS–PAGE and revealed by autoradiography as described in Materials and methods. The position of gp120 is indicated by an arrow and squares.

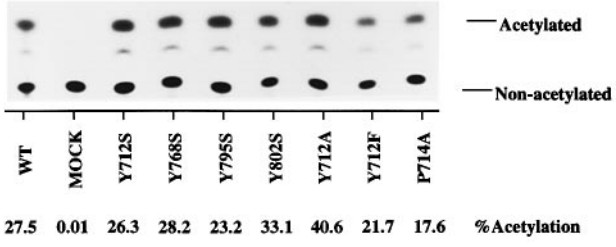

Fig. 4. Effect of intracytoplasmic tyrosine or proline substitutions on HIV Env glycoprotein incorporation and virus infectivity. The modified *env*-negative HIV proviral construct encompassing a reporter CAT gene was co-transfected in MDCK cells with an equimolar amount of wild-type or mutant *env*-expressing plasmids as described in Materials and methods. Viruses were harvested from transfected MDCK cell supernatants and used to infect Jurkat cells. CAT activity was measured 48 h after infection as described in Materials and methods, and analyzed by TLC followed by autoradiography. Positions of acetylated products and non-acetylated chloramphenicol substrate are indicated. Results shown represent the mean of two independent assays.

was further supported when it was reported that a single substitution incorporating a tyrosine in the intracytoplasmic domain of influenza virus hemagglutinin, a naturally apical glycoprotein, can redirect its transport to the basolateral domain in MDCK cells (Brewer and Roth, 1991). Since then, several basolateral tyrosine-based signals in viral or cellular proteins have been identified, although not all known basolateral determinants are associated with tyrosines (Hunziker and Fumey, 1994). The comprehensive study of basolateral and apical targeting signals on additional proteins is thus still required in order to get a better overall understanding of the phenomenon.

In the present study, we pursued the identification of the signal responsible for basolateral budding of lentiviruses, and more specifically HIV-1. Having demonstrated that the intracytoplasmic domain of HIV gp41 mediates the basolateral budding of the virus (Lodge *et al.*, 1994), we thus substituted the four tyrosines of the intracytoplasmic tail of gp41. Using this approach, we clearly identified the membrane-proximal tyrosine as the sole tyrosine involved in the signal required for specific basolateral budding of viral particles. Radioimmunoprecipitation analysis confirmed the presence of the mutant glycoprotein in roughly equal amounts on both cell surfaces, in contrast to the wild-type protein. This again supports the notion that the targeting signal responsible for HIV polarized

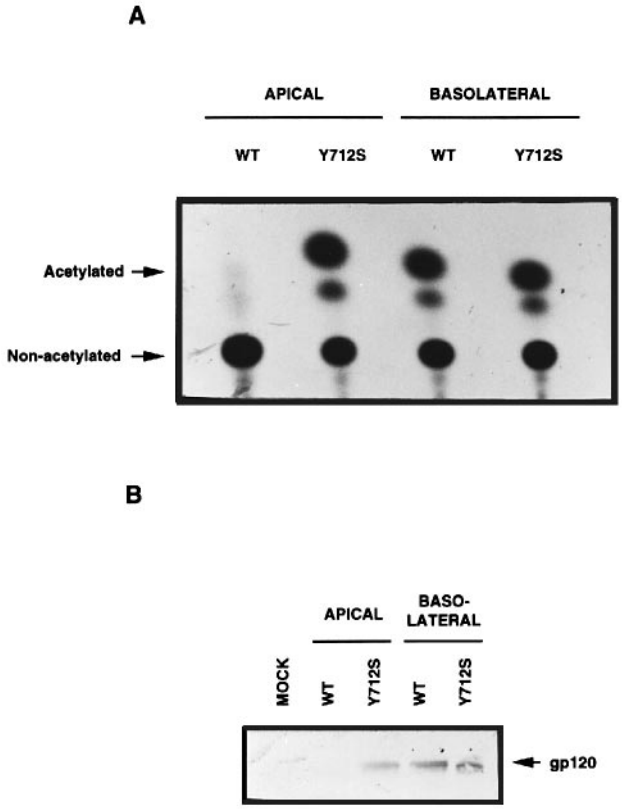

Fig. 5. (**A**) Infectivity of apical and basolateral viruses. The modified *env*-negative HIV proviral construct encompassing a reporter CAT gene was co-transfected in MDCK cells seeded on semi-permeable membranes, with an equimolar amount of wild-type or mutant *env*-expressing plasmids as described in Materials and methods. The apical or basolateral supernatants of three pooled transfected MDCK cell monolayers were used to infect Jurkat cells. CAT activity was measured 48 h after infection as described in Materials and methods, and analyzed by TLC followed by autoradiography. Positions of acetylated products and non-acetylated chloramphenicol substrate are indicated. (**B**) Western blot analysis was performed on viral pellets obtained by ultracentrifugation of either apical or basolateral cell-free pooled supernatants of three MDCK cell monolayers. The cells were transfected and viruses treated as in the viral polarized budding assay described in Materials and methods. Virus pellets were resuspended in RIPA and viral proteins separated on 10% SDS–PAGE, transferred onto nitrocellulose and incubated with the monoclonal antibody against gp120. Bound antibodies were then detected with horseradish peroxidase-linked anti-mouse immunoglobulin and revealed by using an enhanced chemiluminescence detection system, as described in Materials and methods. The position of gp120 is indicated by an arrow.

budding resides in the envelope glycoprotein. These findings also clearly show that, in the presence of envelope glycoprotein, viral budding is restricted to this membrane surface due to Env–Gag protein interactions. Similarly to other published reports of tyrosine-based signals (Hopkins, 1992), conservative substitution for an aromatic phenylalanine residue had a somewhat less pronounced effect on basolateral targeting of viral budding. Also, the substitution of a nearby proline by an alanine impaired basolateral targeting, suggesting that the local conformation surrounding the tyrosine is also important to allow normal function of the signal. Based on similar tyrosine-dependent endocytosis signals (Collawn *et al.*, 1990; Hopkins, 1992; Prill *et al.*, 1993; Trowbridge *et al.*, 1993; Pytowski *et al.*, 1995) and the helix-breaking character of the proximal proline, the best-fitting structure around the tyrosine residue would be similar to that of a β-turn conformation. This would also comply with a previous report indicating a potential overlapping endocytosis signal present in the intracytoplasmic domain of the simian immunodeficiency virus (SIV) and HIV TM glycoproteins and encompassing tyrosine 712 (LaBranche *et al.*, 1994, 1995; Rowell *et al.*, 1995; Sauter *et al.*, 1996). Such an overlapping between endocytosis and basolateral targeting signals was previously shown in other proteins (Hopkins, 1992; Prill *et al.*, 1993; Trowbridge *et al.*, 1993; Pytowski *et al.*, 1995). We showed previously that mutant gp41 glycoproteins harboring large deletions in the intracytoplasmic domain greatly impaired polarized budding of the virus, even though these deleted glycoproteins retained the membrane-proximal tyrosine (Lodge *et al.*, 1994). It is, therefore, most probable that important alterations, carboxy-terminal to the tyrosine-based signal, can also perturb the function of the tyrosine-based polarization signal.

The HIV envelope glycoprotein mutants altered in their polarization phenotype exhibited an identical efficiency in their ability to *trans*-complement the envelope-defective proviral mutant in the CAT infectivity assay. It should also be noted that we were able to obtain a reduction in the amount of CAT activity down to 20% of wild type, by reducing the amount of envelope-expressing plasmid, without any significant effect on the polarization phenotype (data not shown). Radioimmunoprecipitation and Western immunoblotting analysis confirmed the normal levels of mutant envelope glycoprotein incorporation. In addition, using proviral constructs, expression and processing of the mutant glycoproteins as well as their incorporation into virions were shown to be similar to wild type in infected CD4+ Jurkat cells. We can, therefore, clearly demonstrate the presence of the basolateral targeting signal independently from sequences mediating envelope incorporation and required for the infectious potential of the virus.

Although the viral envelope glycoprotein clearly harbors a targeting signal, it could not be excluded that other viral products participate in the phenomenon. HIV and other lentiviruses harbor genes encoding accessory proteins with various roles in viral replication and infectivity (Subbramanian and Cohen, 1994; Trono, 1995). The Nef, Vpr and Vif accessory viral proteins have been reported to be incorporated into the budding viral particle (Cohen *et al.*, 1990; Karczewski and Strebel, 1996; Welker *et al.*, 1996), whereas Vpu and Nef modulate surface expression of the viral envelope glycoprotein or the viral receptor (Willey *et al.*, 1992; Aiken *et al.*, 1994). In order to clarify a possible role of these proteins in HIV polarized budding, isogenic proviral constructs, encoding or lacking either one of the accessory proteins, were examined. None of these four proteins influenced polarized release of HIV, thus excluding any role of accessory viral products in basolateral targeting (results not shown).

Several studies have discussed the importance of epithelial cell infection in HIV pathogenesis (Bourinbaiar and Phillips, 1991; Fantini *et al.*, 1992; Phillips and Bourinbaiar, 1992; Tan *et al.*, 1993; Phillips *et al.*, 1994). Such a polarized release of viruses may be important for their dissemination in the host, as shown for a Sendai virus mutant whose pathology is strongly modified by

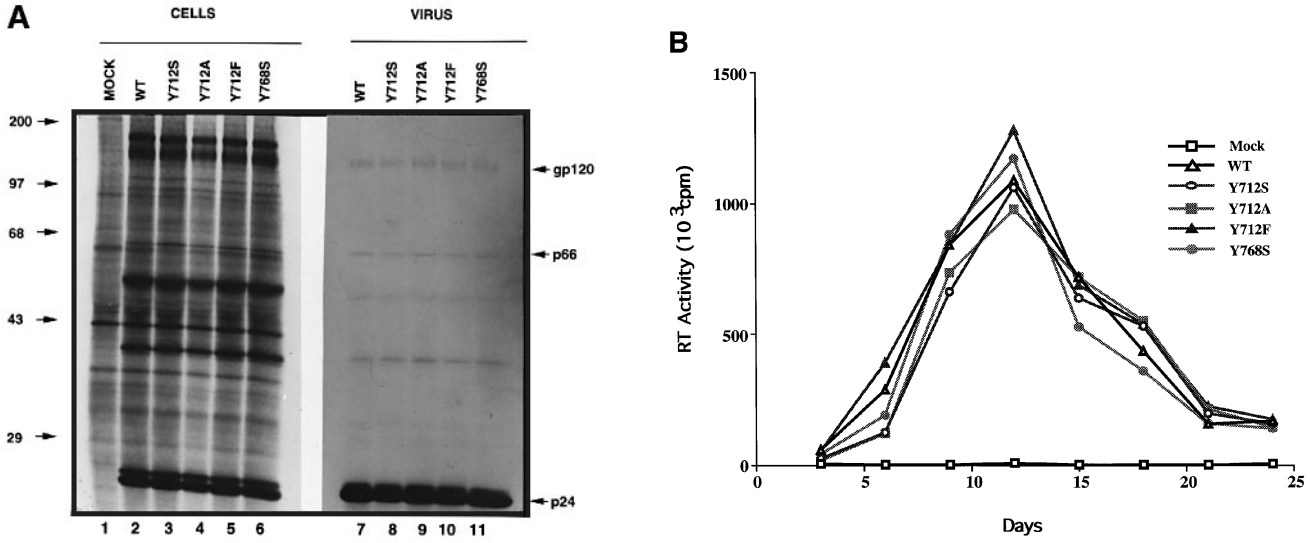

Fig. 6. (**A**) Detection of glycoproteins in the Jurkat-transfected cells and released virions. Jurkat cells were transfected with wild-type or various mutant provirus constructs as described in Materials and methods. Cells were subjected to metabolic labeling 12 days post-transfection with 150 μCi/ml of [^{35}S]methionine for 5 h. Cell-free supernatants were harvested and cleared by ultracentrifugation; cells and viral pellets were then resuspended in RIPA lysis buffer. Viral proteins were immunoprecipitated with the HIV-positive human serum and resolved on a 10% SDS–PAGE gel. Labeled proteins were then revealed by autoradiography. Molecular weight markers are indicated. Virion-associated proteins are indicated by arrows. (**B**) Replication kinetics of mutant viruses. Aliquots of transfected Jurkat cell supernatants were recovered and analyzed for reverse transcriptase activity at different times post-transfection. Results are presented as trichloroacetic acid-precipitable values per 10^6 cells.

alteration of the polarized budding phenotype (Tashiro *et al.*, 1990). Transmission of the HIV virus by direct cell-to-cell contact may increase the efficiency of viral spread and pathogenic potential; such regions of cell-to-cell contacts are considered to be somehow analogous to basolateral membrane surfaces since budding of basolaterally targeted viruses tends to occur at these intercellular contact zones (Rodriguez-Boulan *et al.*, 1983). Accordingly, elegant electron microscope studies have shown a preponderant budding of HIV virions at contact sites between infected lymphocytes and cultured epithelial cells (Bugelski *et al.*, 1995). Budding of HIV has been observed preferentially in pseudopods of monocytes (Perotti *et al.*, 1996). Furthermore, the budding of HIV has been associated with cytoskeletal elements (Pearce-Pratt and Malamud, 1994; Fais *et al.*, 1995); cytoskeletal structures are strongly suspected of an involvement in establishing cellular differentiation in separate poles (Fath *et al.*, 1993).

Mutagenesis of intracytoplasmic tyrosine residues of two other retroviruses has been reported and gave rise to interesting observations consistent with the hypothesis that these residues may be critically involved in viral pathogenesis. In bovine leukemia virus, such a mutation affects the capability of the virus to disseminate in the organism (Willems *et al.*, 1995). In SIV, mutation of the membrane-proximal tyrosine results in the protein's more uniform distribution at the plasma membrane of infected cells (LaBranche *et al.*, 1995). This contrasts with the usual appearance of infected cells in which viral morphogenesis is usually restricted to one pole of the cell and suggests that the SIV membrane-proximal tyrosine may also be involved in a putative polarization signal. Mutations of the tyrosine affecting the overlapping endocytosis signal increase surface expression of the SIV glycoproteins and are associated with accelerated infection kinetics (LaBranche *et al.*, 1994, 1995; Sauter *et al.*, 1996). Interestingly, these effects on viral replication were not observed for the HIV tyrosine substitutions, suggesting that other endocytosis signals may compensate for the loss of the tyrosine-based signal. Accordingly, these other endocytosis signals which are present in a full-length Env cytoplasmic tail would be absent from the much shorter Env cytoplasmic domain encoded by the SIV strain in the studies of Sauter *et al.* and LaBranche *et al.* Finally, although the role of this tyrosine-based sorting signal in *in vivo* infection remains unclear, a recent study reported that a membrane-proximal tyrosine to cysteine substitution may be linked to rapid CD4+ T-cell decline *in vivo*, as found in a HIV viral clone derived from a patient with an unusually high viral load at seroconversion (Gao *et al.*, 1996).

In conclusion, we have established the existence of a tyrosine-based signal in HIV glycoproteins that exhibits properties similar to the already described basolateral signal found on various viral and cellular membrane proteins. Although mutations of the critical membrane-proximal residue do not affect viral multiplication *in vitro*, the basolateral transport signal of the envelope glycoprotein, by mediating specific transport of HIV to distinct membrane domains, could play a critical role in HIV pathogenesis in its human host.

Materials and methods

Plasmid constructs

The HXBH10 proviral construct which contains a stop codon at the initiation site of the *env* gene and a frameshift at the *Kpn*I site (HXBH10 nucleotide position 5934; +1 = site of transcription initiation) in *env* has been described previously (HXBH10*env*–) (Terwilliger *et al.*, 1989a; Yao *et al.*, 1992). The HXBc2 proviral construct and pSVIII*env* plasmid have also been described previously (Fisher *et al.*, 1985; Ratner *et al.*, 1987; Gabuzda *et al.*, 1992b). pSVIII*env* is the *tat*-truncated, *rev* and *env*-expressing plasmid that was used as the HIV-1 wild-type or mutant *env*-encoding plasmid throughout these experiments (Gabuzda *et al.*, 1992b). In order to ensure the total absence of Env protein, as in the *env*-negative proviral construct used in the polarization experiments,

plasmid HXBH10CAT*env*–(ATG-, *Kpn*Ifs) was generated by cloning the *Apa*I–*Bam*HI fragment (nucleotide positions 1560 and 8058; +1 = site of transcription initiation) of HXBH10*env*– into pHXBΔ*env*CAT, a CAT-encoding proviral construct with a CAT gene cloned in *nef* (Terwilliger *et al.*, 1989b; Helseth *et al.*, 1990). The various isogenic proviral constructs were obtained as follows: *vpr*+/–: HxBRU and HxBRUR– with a stop codon at the initiation site of the *vpr* gene of HIV-BRU have been described elsewhere (Lavallée *et al.*, 1994; Yao *et al.*, 1995); *vpu*+/–: HXBH10 and HXBH10*vpu*-, were described previously (Yao *et al.*, 1992); *nef*+/–: pNL4.3-based pNLV102 and pNLV102Δ*Xho*, gifts of Dr S.Venkatesan, and described previously (Ahmad and Venkatesan, 1988); *vif*+/–: HXB2 and HXB2*vif*stop, containing a frameshift in *vif* (Ratner *et al.*, 1987; Gabuzda *et al.*, 1992a), obtained from Dr Dana Gabuzda. Finally, plasmid SVCMV-VSV-G contains the *Eco*RI 1.6 kbp fragment of plasmid pMD.G (obtained from Dr D.Ory) under the early CMV promoter, and the SV40 virus origin of replication.

Site-directed mutagenesis in HIV gp41

Substitution of tyrosine 795 to serine was done using the unique site elimination site-directed mutagenesis procedure of Deng and Nickoloff (1992). The selection primer used to eliminate the unique *Sal*I site in pSVIII*env* was 5′-GCTGCAGATCGATCGTTGCTT-3′ and the mutagenic primer was 5′-CCCTCAAATCTTGGTGGAATC-3′, which also resulted in elimination of a *Ssp*I site. A megaprimer PCR-directed mutagenesis using Pfu polymerase (Stratagene) was used thereafter to generate the other substitution mutants (Upender *et al.*, 1995). Primer 5′-CTCTGTAGGTAGTTTGTCC-3′ recognizes a non-HIV-related sequence 3′ to the mutagenized sites and primer 5′-GCATCAAGCA-GCTCCAAGC-3′ a sequence 5′ to the mutagenized sites, in *env*. Mutagenic primers used were: 5′-GCAGGGATCTTCACCATTATCG-3′ (for Y712S); 5′-CTCTTCAGCTCCCACCGCTTG-3′ (for Y768S); 5′-CTCCTACAGTCTTGGAGTCAG-3′ (for Y802S); 5′-GCAGGG-ATTTTCACCATTATCG-3′ (for Y712F); 5′-GCAGGGAGCTTCACC-ATTATCG-3′ (for Y712A) and 5′-GCAGGGATATTCAGCAT-TATCGTTTCAG-3′ (for P714A). The *Hin*dIII to *Xho*I (respectively HXBc2 nucleotide positions 7683 and 8439; +1 = initiation of transcription) fragment of each PCR product was then cloned into pSVIII*env*. All mutants were sequenced and several clones tested to ensure proper mutagenesis and the absence of second-site mutations. Relevant mutated *env* fragments were recovered from the various pSVIII*env*-based vectors and subcloned into provirus construct HXBc2.

Cell lines and antisera

The canine kidney polarized epithelial MDCK cell line and the SV40-transformed African green monkey kidney COS-7 cell line used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum and 1% antibiotics (penicillin and streptomycin) at 37°C under a 5% CO_2 atmosphere. The Jurkat CD4+ human lymphoid cell line was maintained in RPMI-1640 containing 10% fetal calf serum and 1% antibiotics (Cohen *et al.*, 1990). The HIV-1-positive human serum 162 used in this study has been previously described elsewhere (Lavallée *et al.*, 1994). The monoclonal anti-gp120 antibody 1001 and the rabbit VSV antiserum were obtained from American Bio-Technologies (ABT) and Dr Laurent Poliquin, respectively.

Viral polarized budding assay

Polarized budding of the viruses from MDCK cells was assayed as described previously, with modifications (Lodge *et al.*, 1994). Briefly, 10^6 cells were seeded per 24.5 mm diameter chamber and grown for 2–3 days on semi-permeable 1 μm pore diameter filter membranes (Falcon No. 3102) in DMEM as described above. Polarization of the cell monolayer was tested by monitoring the electrical resistance between upper and lower chambers (Millicell-ERS resistance system, Millipore). Resistance across the monolayer was constantly above 550 ohms·cm^2 at the time of DNA transfection. Cells were washed with DMEM and 750 μl of DMEM containing 30 μl of prepared liposomes (Leventis and Silvius, 1990; Mouland *et al.*, 1994) were thoroughly mixed with 750 μl of DMEM containing 5 μg of DNA before pouring in the upper chamber over the cell monolayer. Medium was removed 24 h after lipofection, and cells were maintained in DMEM supplemented with serum and antibiotics. Media in the upper and lower chambers were harvested 48 h after lipofection, cellular debris removed by filtering through a 0.45 μm pore diameter filter (Millipore), and the filtrate ultracentrifuged at 30 000 r.p.m. in a Beckman 50.4 rotor for 1.5 h at 4°C. The pellet was resuspended in 200 μl DMEM and p24 antigen was quantitated using an ELISA p24 kit (Coulter Diagnostics) according to the manufacturer's instructions.

Incorporation of VSV-G in HIV virions

The lipofection procedure on confluent MDCK cell monolayers was not sufficiently efficient to detect the HIV glycoproteins. Therefore, an alternate method was used using HIV virions containing either wild-type or mutant Env and the G glycoprotein of VSV. In order to ensure that VSV-G is incorporated in HIV virions even in the presence of Env, 10^6 COS cells were transfected with 10 μg of either proviral construct with or without 10 μg of SVCMV-VSV-G by the lipofection method (Leventis and Silvius, 1990; Mouland *et al.*, 1994). Cells were labeled 40 h after transfection with 150 μCi/ml of [^{35}S]methionine for 8 h. Supernatants were harvested and ultracentrifuged (30 000 r.p.m. for 1 h in a Beckman Ti50.4 rotor); viral pellets were then resuspended in RIPA lysis buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.25% sodium deoxycholate, 0.2% phenylmethylsulfonyl fluoride (PMSF)]. Viral proteins were immunoprecipitated with a mix of the HIV-positive human serum combined with the rabbit anti-VSV serum and loaded on a 10% SDS–PAGE gel as described previously (Yao *et al.*, 1995). Labeled proteins were then revealed by autoradiography.

Plasma membrane distribution of envelope glycoprotein

Wild-type or mutant HIV viruses having incorporated the VSV-G glycoprotein were generated as previously described in COS cells and harvested 40 h after transfection in 2 ml of DMEM supplemented with 8% FCS and filtered on 0.45 μm filters as described above. Identical volumes of cell-free supernatant containing the pseudotype viruses were then added for 8 h onto 10^6 MDCK cells grown in 100 mm diameter Petri dishes. MDCK cells were then trypsinized and grown for 1–2 days on semi-permeable 1 μm pore diameter filter membranes as described above. After an 8 h labeling with 150 μCi/ml of [^{35}S]methionine (at the basolateral amino acid uptake surface), cells were extensively washed with cold phosphate-buffered saline (PBS) and treated for 4 h at 4°C, either at their apical or basolateral surface with the monoclonal anti-gp120 antibody. Cells were then washed, lysed with RIPA and added to protein A–Sepharose beads (Pharmacia). Intracellular proteins were obtained by immunoprecipitating the cell lysate supernatants of the surface protein immunoprecipitates with the same antibodies. Immunoprecipitation procedures were performed as previously described (Lavallée *et al.*, 1994). All immunocomplexes were separated on 10% SDS–PAGE and revealed by autoradiography.

Trans-complementation CAT infectivity assay

The *trans*-complementation assay used for determining the infectivity and efficient incorporation of either HIV wild-type or mutant Env glycoproteins has been described previously and was used with modifications (Terwilliger *et al.*, 1989b; Helseth *et al.*, 1990). Briefly, 5 μg of HXBH10CAT*env*–(ATG-, *Kpn*Ifs) and 5 μg of the various HIV *env*-expressing pSVIII*env*-based plasmids were co-transfected by lipofection into MDCK cells grown in 100 mm diameter tissue culture dishes or, alternatively, in cells seeded on semi-permeable membranes as described above. After 48 h transient expression, media were harvested, filtered on a 0.45 μm membrane and a 50 μl aliquot taken for a reverse transcriptase assay (Lee *et al.*, 1987). Typically, the equivalent of 10^5 c.p.m. of the remaining supernatants was used to infect 10^6 CD4+ Jurkat cells, maintained in RPMI-1640 medium supplemented with 10% serum and 1% antibiotics. Alternatively, all of the apical or basolateral supernatants of three transfected MDCK cell monolayers on semi-permeable membranes were used to infect the Jurkat cells. After 48 h, lymphocytes were harvested and lysed; total protein yields were normalized (Bio-Rad Protein Assay) and a CAT assay performed on cell lysates (Gorman *et al.*, 1982; Leahy *et al.*, 1995). Acetylated and non-acetylated forms of [^{14}C]chloramphenicol were separated by TLC and exposed on Kodak-XAR5 films. Quantitation of acetylated chloramphenicol over total chloramphenicol was carried out on a Molecular Dynamics personal laser densitometer.

Western blot analysis

In order to determine further Env protein incorporation in apically or basolaterally released virions, Western blot analysis was performed on viral pellets obtained from ultracentrifugation (as described above) of three pooled MDCK cell monolayer apical or basolateral supernatants. The cells were transfected and viruses treated as in the viral polarized budding assay described above. Virus pellets were resuspended in RIPA and viral proteins separated by 10% SDS–PAGE. Following electrophoresis, proteins were transferred to nitrocellulose (0.45 μm pore size; Schleicher and Schuell) by electroblotting overnight at 20 V in a Bio-Rad Trans Blot Cell. Blots were incubated for 1 h in blocking buffer

[Tris-buffered saline (TBS) containing 0.5% Tween-20 and 2% non-fat dry milk] and incubated with the monoclonal antibody against gp120 for 1 h at a dilution of 1:1500. Bound antibodies were then probed with horseradish peroxidase-linked anti-mouse immunoglobulin (used at 1:1500), washed extensively and developed by using a sensitive enhanced chemiluminescence detection system (ECL detection kit, Amersham Corp.). The blots were swapped with swapping solution [100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl (pH 6.7)], washed extensively and treated first with the HIV-positive human serum (at 1:2000) and then probed with a horseradish peroxidase-linked anti-human immunoglobulin (used at 1:1500) as described previously to detect virion capsid proteins.

Viral replication kinetics

Proviral DNA constructs (15 μg) were transfected in Jurkat cells using the standard DEAE–dextran technique (Terwilliger *et al.*, 1989a; Yao *et al.*, 1995). Cells were recovered by centrifugation 3 days post-transfection and seeded in fresh medium at a concentration of 10^6 viable cells/ml; the same procedure was repeated each 3 days. Cell supernatants were analyzed for the presence of virus by performing standard reverse transcriptase assays on 50 μl aliquots (Lee *et al.*, 1987).

Prior to the peak of viral replication, 5×10^6 cells were labeled on day 12 post-transfection with 150 μCi/ml of [^{35}S]methionine for 5 h. Supernatants were harvested and ultracentrifuged (30 000 r.p.m. for 1 h in a Beckman Ti50.4 rotor); cells and viral pellets were then resuspended in RIPA lysis buffer. Viral proteins were immunoprecipitated with the HIV-positive human serum and loaded on a 10% SDS–PAGE gel as described previously (Yao *et al.*, 1995). Labeled proteins were then revealed by autoradiography. Quantitation of Env glycoproteins bands was performed on a Molecular Dynamics phosphorImager.

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