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Pseudotyping Human Immunodeficiency Virus Type 1 (HIV-1) by the Glycoprotein of Vesicular Stomatitis Virus Targets HIV-1 Entry to an Endocytic Pathway and Suppresses both the Requirement for Nef and the Sensitivity to Cyclosporin A

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Human immunodeficiency virus type 1 (HIV-1) normally enters cells by direct fusion with the plasma membrane. In this report, HIV-1 particles capable of infecting cells through an endocytic pathway are described. Chimeric viruses composed of the HIV-1 core and the envelope glycoprotein of vesicular stomatitis virus (VSV-G) were constructed and are herein termed HIV-1(VSV) pseudotypes. HIV-1(VSV) pseudotypes were 20- to 130-fold more infectious than nonpseudotyped HIV-1. Infection by HIV-1(VSV) pseudotypes was markedly diminished by ammonium chloride and concanamycin A, a selective inhibitor of vacuolar H^+ ATPases, demonstrating that these viruses require endosomal acidification to achieve productive infection. HIV-1 is thus capable of performing all of the viral functions necessary for infection when entry is targeted to an endocytic route. Maximal HIV-1 infectivity requires the presence of the viral Nef protein and the cellular protein cyclophilin A (CyPA) during virus assembly. Pseudotyping by VSV-G markedly suppressed the requirement for Nef. HIV-1(VSV) particles were also resistant to inhibition by cyclosporin A; however, the deleterious effect of a *gag* mutation inhibiting CyPA incorporation was not relieved by VSV-G. These results suggest that Nef acts at a step of the HIV-1 life cycle that is either circumvented or facilitated by targeting virus entry to an endocytic pathway. The findings also support the hypothesis that Nef and CyPA enhance HIV-1 infectivity through independent processes and demonstrate a mechanistic difference between reduction of HIV-1 infectivity by cyclosporin A and *gag* mutations that decrease HIV-1 incorporation of CyPA.

Both the viral factor Nef and the cellular protein cyclophilin A (CyPA) enhance human immunodeficiency virus type 1 (HIV-1) infectivity through mechanisms that are not completely understood. Nef-defective virions are 4- to 40-fold less infectious than wild-type HIV-1 (13, 32). HIV-1 Δ*nef* particles bind to and enter cells efficiently but fail to undergo efficient reverse transcription in target cells (5, 12, 40). The reduced infectivity of *nef*-defective virions is not due to an intrinsic defect in reverse transcriptase, since purified particles exhibit normal levels of activity when assayed with either the endogenous viral RNA or an added synthetic primer-template as the substrate. Because the Nef defect is apparent at even the earliest stage of reverse transcription, it is likely that Nef facilitates an early step in the viral life cycle, including uncoating or initiation of reverse transcription. The requirement for Nef expression is manifested at the time of virus particle formation, since a *nef*-defective provirus can be complemented by coexpression of *nef* in the virus producer cell but not in the target cell (5, 33, 35).

A similar phenotype has been observed for HIV-1 particles produced in the presence of the immunosuppressive drug cyclosporin A (CsA) and its analogs. These compounds inhibit the binding of CyPA to the HIV-1 Gag precursor and its subsequent incorporation into virions (17, 43). Viruses produced in the presence of CsA, and mutant viruses that fail to incorporate CyPA, exhibit reduced infectivity due to a block prior to reverse transcription (8). Substitution of the envelope glycoproteins of the amphotropic murine leukemia virus (A-MLV) for the HIV-1 envelope proteins does not bypass the CyPA requirement, demonstrating that the defect is independent of CD4-mediated entry. The similarity between the phenotypes of CyPA-deficient and *nef*-defective virions suggests that Nef and CyPA may enhance HIV-1 infectivity by similar mechanisms; indeed, both proteins have been suggested to facilitate HIV-1 uncoating (5, 8, 12, 24, 40).

Enveloped viruses enter cells through two main pathways: direct fusion between the viral envelope and the plasma membrane and receptor-mediated endocytosis. In the latter case, the fusion activity of the viral envelope protein is activated by the altered environment of endosomes. The best-understood example of this pathway is the hemagglutinin (HA) protein of influenza virus (45). The decreased pH of the endosome triggers a dramatic conformational change in the HA protein, resulting in exposure of the fusion domain in the proximity of the target cell membrane. Fusion of the viral and endosomal membranes results in the escape of the viral core from the endosome, allowing subsequent viral replicative steps to occur. The classical test for viruses that require acid activation for entry is the sensitivity of infection to weak bases, such as ammonium chloride, that act by inhibiting endosomal acidification (29).

The route of HIV-1 entry into cells has been the subject of some controversy. Early morphologic analyses of virus-cell interactions revealed HIV-1 particles present in endosomes, sug-

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gesting but not proving that the virus enters cells through an endocytic pathway (20, 36). Furthermore, one study reported that HIV-1 infection is sensitive to ammonium chloride (27), supporting the hypothesis that HIV-1 entry requires exposure to low pH. Later studies found that both HIV-1 and vesicular stomatitis virus (VSV) pseudotyped by the HIV-1 envelope glycoproteins were resistant to the weak bases ammonium chloride and amantadine and to the carboxylic ionophore monensin (31, 42). Fusion of HIV at the plasma membrane was also observed (20, 42). Finally, studies of mutant CD4 molecules exhibiting altered rates of endocytosis demonstrated that CD4 internalization is not required for HIV-1 infection (28, 37). As a result of these findings, HIV-1 infection is now generally thought to occur through direct fusion with the plasma membrane in a pH-independent manner (29).

HIV-1 virions appear to be relatively indiscriminant toward incorporation of non-HIV membrane proteins. Purified virions have been shown to contain several cellular proteins, including CD4 and molecules of the class II major histocompatibility complex (6, 21). In addition, HIV-1 particles can be pseudotyped by envelope glycoproteins from a variety of other viruses, including MLVs, the human T-cell leukemia virus type 1 (25), and VSV (34, 39). In the last case, HIV-1 virions carrying VSV-G are predicted to enter cells through an endocytic pathway, since this is the normal route of VSV infection (30). These chimeric viruses, termed HIV-1(VSV) pseudotypes, are capable of infecting a wide variety of cell types and were recently used to enhance the utility of HIV-1-based gene delivery vectors (34). They may also prove useful for understanding postentry steps in HIV-1 infection, as they should bypass the normal entry pathway taken by incoming HIV-1 virions.

In order to produce high titers of viruses for biochemical analysis of the mechanism by which Nef enhances HIV-1 infectivity, wild-type and *nef*-defective HIV-1(VSV) pseudotypes were generated. Single-cycle infection assays were employed to measure the relative infectivities of pseudotyped and nonpseudotyped virions. Surprisingly, HIV-1(VSV) exhibited a markedly reduced requirement for Nef. However, the requirement for the CyPA-Gag interaction was not suppressed by pseudotyping with VSV-G. Unlike infection by nonpseudotyped HIV-1 or HIV-1 pseudotyped by the A-MLV envelope proteins, infection by HIV-1(VSV) was inhibited by compounds that block endosomal acidification. These viruses therefore enter cells through a low-pH-dependent endocytic pathway. The results suggest that the requirement for Nef is significantly suppressed by targeting HIV-1 entry to an endocytic route and further demonstrate the mechanistic independence of Nef- and CyPA-mediated HIV-1 infectivity enhancement.

MATERIALS AND METHODS

Cells and viruses. 293T cells (obtained from I. Verma, Salk Institute) and P4-2 cells, the generous gift of F. Clavel (11), were cultured at 37°C in 5% CO_2 and Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%), penicillin (50 IU/ml), and streptomycin (50 μg/ml). The full-length proviral DNA constructs used for production of HIV-1 were as follows: R7, wild-type HIV-1; R7ΔN, *nef*-defective HIV-1; R7.221, HIV-1 containing a mutation of Gly221 to Ala; R7.222, HIV-1 containing a mutation of Pro222 to Ala. The construction and characterization of these mutant proviruses will be described elsewhere (2). Envelope-defective variants of the viruses were created by transferring a *Sal*I-*Bam*HI fragment carrying a frameshift mutation in *env* (44) into the corresponding proviral construct. R9 and R9ΔN are full-length plasmid clones of HIV-1 that were created by replacing a *Bss*HII-to-*Bam*HI fragment of R7 or R7ΔN, respectively, with the corresponding fragment from pNL4-3 (1), as previously described (19). In these viruses, the *gag*, *pol*, and gp120 regions of *env* are derived from NL4-3 while the long terminal repeats and *nef* are from HXB2. These viruses therefore encode functional *vpu* and *vpr* genes. The VSV-G expression construct pHCMV-G was kindly provided by J. Burns (University of California, San Diego). Plasmid SV-A-MLV-*env* was provided by N. Landau (Aaron Diamond AIDS Research Institute).

TABLE 1. Infectivities of HIV-1 and HIV-1(VSV) pseudotypes[a]

Virus	RT activity (cpm of ^{3}H/10 μl)	IU/ml	Mean infectivity (IU/cpm of RT) ± SD
R9	23,302	1.68×10^6	0.72 ± 0.046
R9 + pHCMV-G	22,860	3.97×10^7	17.4 ± 1.36
R7ΔE + SV-A-MLV-*env*	52,426	8.71×10^6	1.66 ± 0.14
R7ΔE + pHCMV-G	26,491	4.19×10^7	15.8 ± 0.45

[a] IU, infectious units; RT, reverse transcriptase; R7ΔE, *env*-defective HIV-1.

For production of viruses, 293T cells were transfected in 10-cm-diameter dishes with 20 μg of plasmid vectors as previously described (4). Twelve hours after transfection, the cells were washed with 5 ml of phosphate-buffered saline and 4 ml of complete medium was added. For CsA inhibition experiments, CsA (Sigma) was added as a 1 mM stock solution in absolute ethanol to a final concentration of 10 μM in the culture medium. Control viruses were produced in the presence of equivalent concentrations of ethanol (1%, vol/vol). Cells were cultured for 36 to 48 h after transfection. Virus stocks were prepared by collecting culture supernatants and passing them through 0.45-μm-pore-size cellulose acetate syringe filters (Gelman Sciences, Inc.). Samples were removed for reverse transcriptase and infectivity assays prior to storage of virus stocks at −75°C. Pseudotyped virions were produced by cotransfecting 20 μg each of proviral DNA and an *env*-expression plasmid.

Assays of HIV-1 infectivity. Reverse transcriptase assays were performed as described previously (5). Virus infectivity was measured by infecting P4-2 indicator cells in triplicate with 10-fold serial dilutions of virus stocks as previously described (23). For inhibition experiments, infections were performed in the presence of ammonium chloride (10 mM) or concanamycin A (25 nM). Two days after infection, cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and the number of infectious units was determined by counting the blue-stained foci under a light microscope. Wells containing between 30 and 500 infectious units were used for counting; within this range, the assay was linear, as values obtained for virus dilutions agreed well. Uninfected wells consistently exhibited fewer than five blue cells per well. The results of triplicate infections consistently varied by less than 20%. Infectivity values were calculated by dividing the number of infectious units per well by the reverse transcriptase activity in the inocula.

RESULTS

Pseudotyping by VSV-G dramatically enhances HIV-1 infectivity. It was previously reported that production of MLV particles in the presence of VSV-G results in the generation of retroviruses containing an expanded host range (16). These pseudotyped viruses are stable during ultracentrifugation, making them useful for the generation of retrovirus stocks with very high titers (10). In order to produce high-titer stocks of HIV-1 for the mechanistic analysis of Nef action, HIV-1 expression vectors were cotransfected with the VSV-G expression vector pHCMV-G into human 293T cells. The resulting viral supernatants were assayed for their reverse transcriptase contents and titrated on the HeLa-CD4-derived indicator cell line P4-2. These cells contain an integrated copy of the HIV-1 promoter directing expression of *lacZ*; upon infection with HIV-1 and subsequent production of Tat, these cells express β-galactosidase. Cotransfection of HIV-1 constructs with pHCMV-G resulted in no reduction in the amount of reverse transcriptase activity produced; however, a 24-fold increase in the number of infected cells per unit of reverse transcriptase activity was observed (Table 1). In other experiments where the viral stocks were normalized for p24 content by enzyme-linked immunosorbent assay, a similar enhancement in virus infectivity was observed (data not shown). In several independent experiments, the degree of enhancement of infectivity ranged from 19- to 37-fold over the level of infectivity of nonpseudotyped HIV-1. Similar differences in levels of infectivity were obtained with a CD4-negative cell line as the target, indicating that the infectivity increase conferred by VSV-G was due to CD4-independent entry (data not shown). Because

these observations were made with virions presumably containing both the HIV-1 envelope protein and VSV-G, VSV-G was tested for its ability to enhance the infectivity of virions produced from an envelope-defective HIV-1 construct. For comparative purposes, HIV-1(A-MLV) pseudotypes were also produced by cotransfecting the *env*-defective HIV-1 proviral construct with the envelope expression vector SV-A-MLV-*env*. This construct produces pseudotyped HIV-1 virions with values of infectivity equal to or slightly higher than that of wild-type HIV-1 (5, 25). A 10-fold enhancement of HIV-1 infectivity by VSV-G relative to that of the A-MLV envelope was also observed with the HIV-1 Δ*env* construct (Table 1). This result demonstrates that the increased infectivity of HIV-1 virions conferred by VSV-G does not involve a cooperative interaction between this protein and HIV-1 envelope glycoproteins.

Pseudotyping VSV-G targets HIV-1 entry to a low-pH-dependent endocytic route. VSV enters cells through receptor-mediated endocytosis (30). To determine whether VSV-G alters the pathway of HIV-1 entry to an endocytic route, P4-2 target cells were exposed to HIV-1(VSV) virions in the presence of two inhibitors of endosomal acidification: ammonium chloride and concanamycin A. Concanamycin A is a specific inhibitor of vacuolar H^+ ATPases and does not produce the side effects on cellular processes that weak bases such as ammonium chloride can exhibit (41). Virus stocks were diluted to an appropriate concentration for conveniently scoring infected cells, and cultures were maintained in the presence of the drugs for 2 days. As a control, nonpseudotyped HIV-1 stocks were used in parallel infections. Both ammonium chloride and concanamycin A potently and specifically inhibited infection by HIV-1(VSV) (Fig. 1A). In contrast, neither compound inhibited infection by HIV-1. Interestingly, a slight enhancement of HIV-1 infection was observed in the presence of either compound. As an additional control, we performed infections with HIV-1(A-MLV) stocks in the presence and absence of 10 mM ammonium chloride (Fig. 1B). HIV-1(A-MLV) was also resistant to this compound. These results demonstrate that VSV-G alters the pathway of virus entry to a compartment which requires endosomal acidification and are consistent with the known mechanism of VSV entry (30).

Pseudotyping HIV-1 particles with VSV-G suppresses the requirement for Nef. Previous studies have shown that HIV-1 (A-MLV) pseudotypes exhibit a strong requirement for Nef (5, 33), suggesting that infectivity enhancement by the viral protein is independent of the envelope glycoprotein. To quantify the ability of Nef to enhance the infectivity of HIV-1(VSV) pseudotypes, viruses were produced by cotransfecting wild-type and *nef*-defective HIV-1 proviral constructs with pHCMV-G into 293T cells. The supernatants were assayed for reverse transcriptase activity, and their titers were determined in single-cycle infection assays using P4-2 cells as targets. For nonpseudotyped HIV-1, Nef enhanced virus infectivity by approximately 10-fold (Fig. 2). This result is consistent with those previously reported for single-cycle assays of infectivity enhancement by Nef (5, 32, 40). Surprisingly, HIV-1(VSV) exhibited only a twofold infectivity difference between the wild type and the Δ*nef* virus (Fig. 2). The reduced ability of Nef to enhance viral infectivity was not due to an increased multiplicity of infection by HIV-1(VSV), since the assays were performed in parallel with numbers of infectious units similar to those used for assaying the nonpseudotyped HIV-1 stocks. However, VSV-G could have been masking the Nef infectivity effect by simply increasing the overall infectivity of HIV-1. To test whether the Nef phenotype would be restored by generating less infectious HIV-1(VSV) pseudotypes, wild-type and Δ*nef* HIV-1(VSV) pseudotypes were produced in the presence

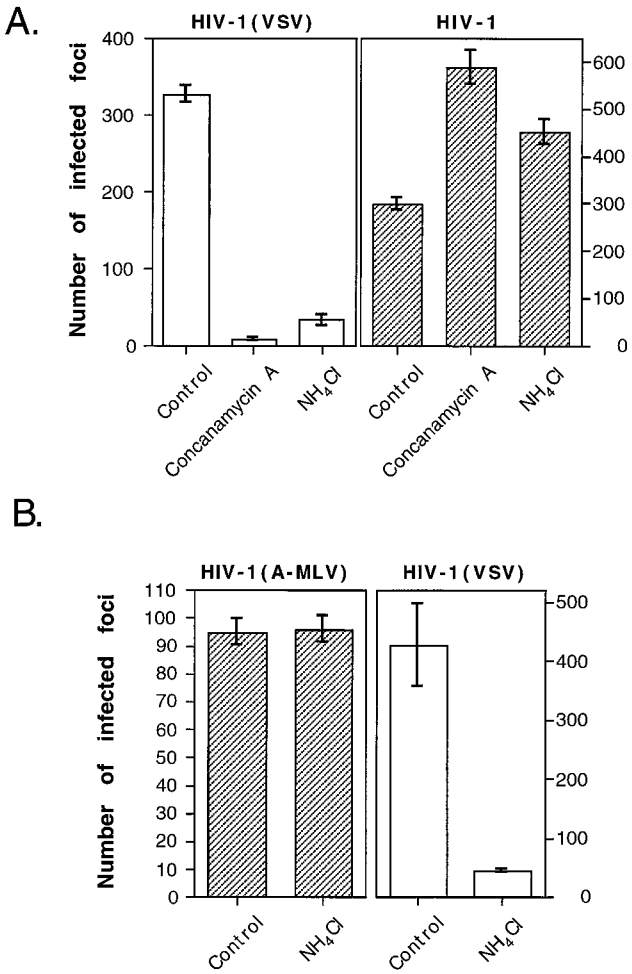

FIG. 1. Reduction of HIV-1(VSV) infection by inhibitors of endosomal acidification. Infections of P4-2 cells were performed in medium containing 10 mM ammonium chloride (NH_4Cl), 25 nM concanamycin A, or neither compound (control). Concentrations of viruses were chosen to produce numbers of infected foci that could readily be counted. (A) Viruses tested were produced by transfection of an envelope-competent HIV-1 proviral DNA construct with and without the VSV-G expression vector pHCMV-G. (B) Viruses tested were produced from an envelope-defective HIV-1 construct with pSV-A-MLV-*env* or pHCMV-G. Blue foci were detected by staining cultures with X-Gal 2 days after infection. Shown are the mean values of triplicate infections; error bars represent 1 standard deviation. The results are representative of at least two independent experiments.

of decreased amounts of VSV-G and were assayed for infectivity. For these experiments, *env*-defective proviral constructs were used to eliminate expression of the HIV-1 envelope glycoproteins to avoid the generation of mixed pseudotypes. As a control, the A-MLV envelope was used. The infectiousness of both wild-type and *nef*-defective HIV-1(VSV) was dependent on the amount of envelope plasmid provided in the transfections; however, the decreased Nef phenotype remained regardless of the amount of VSV-G plasmid used (Table 2). Thus, it is likely that the decreased requirement for Nef for infection by HIV-1(VSV) is not a general effect of the increase of infectivity conferred by VSV-G.

Infection by HIV-1 Δ*nef* virions does not require exposure to low pH. Though Nef is required for efficient HIV-1 infection, it is unclear why HIV-1 Δ*nef* virions retain partial infectivity. Uptake of HIV-1 particles by endocytosis has been previously reported (20, 36), though this is not generally believed to be

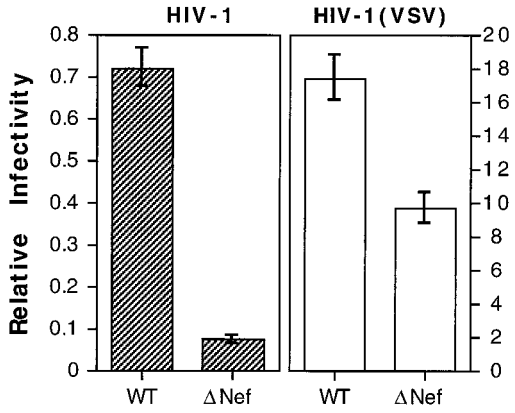

FIG. 2. Infectivity of *nef*$^+$ and *nef* mutant HIV-1 and HIV-1(VSV) pseudotypes. Viruses were produced by transfection of 293T cells with wild-type (WT) and *nef*-defective (ΔNef) HIV-1 proviral constructs with and without pHCMV-G. Infectivity values (ordinate) were determined by titration of the viruses on P4-2 cells and dividing the titers of virus by the levels of reverse transcriptase activity in the inocula. The measured values are the means of triplicate infections, with error bars representing 1 standard deviation. The results are representative of at least three independent experiments.

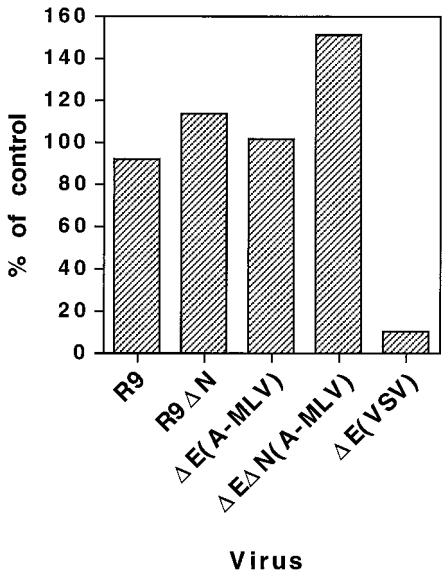

FIG. 3. Ammonium chloride sensitivities of *nef*$^+$ and *nef* mutant HIV-1 and HIV-1(A-MLV) pseudotypes. Viruses were produced by cotransfection of 293T cells with HIV-1 proviral DNA constructs and *env*-expression vectors and assayed for infection on P4-2 cells in the presence and absence of 10 mM ammonium chloride. Shown on the ordinate is the infectivity of each virus in the presence of ammonium chloride relative to those of control infections. The values are the means of duplicate infections. Note that the infectivities by *nef*-defective HIV-1 and HIV-1(A-MLV) are slightly enhanced by ammonium chloride. ΔE, *env* defective; ΔN, *nef* defective.

the major pathway for HIV infection (29, 37). Still, infection by a fraction of HIV-1 particles through an endocytic pathway may occur. Pseudotyping HIV-1 by the VSV-G protein appears to target HIV-1 entry to an endocytic route that is relatively insensitive to Nef. Therefore, it is possible that a percentage of HIV-1 Δ*nef* virions infect cells through an endocytic pathway, thereby circumventing the requirement for Nef. To test whether HIV-1 Δ*nef* requires exposure to low pH to achieve infection, wild-type and *nef*-defective HIV-1 stocks were used to infect P4-2 target cells in the presence of 10 mM ammonium chloride. Neither wild-type nor HIV-1 Δ*nef* infection was significantly inhibited by ammonium chloride; indeed, both viruses appeared to be slightly enhanced by the compounds (Fig. 3). As a control, HIV-1(VSV) was tested in parallel and exhibited 90% inhibition of infection in the presence of 10 mM NH_4Cl. Therefore, HIV-1 Δ*nef* particles do not infect cells through a low-pH-dependent pathway. This result does not preclude the possibility that the virions enter cells through an endocytic pathway but demonstrates that infection by HIV-1 Δ*nef* virions does not require endosomal acidification.

Pseudotyping by VSV-G suppresses the sensitivity of HIV-1 particles to CsA. Previous studies have demonstrated that formation of HIV-1 particles in the presence of CsA prevents incorporation of CyPA into virions and decreases HIV-1 infectivity (17, 43). A similar effect is achieved by mutations in the capsid region of the Gag precursor protein that inhibits CyPA binding (17). Mechanistic analyses of Nef- and CyPA-mediated infectivity enhancement have promoted the hypothesis that both proteins facilitate HIV-1 uncoating in target cells (5, 8, 12, 24, 40). To determine whether targeting HIV-1 to an alternate entry pathway influences the requirement for CyPA incorporation, we produced HIV-1(VSV) pseudotypes by culturing transfected 293T cells in the presence of 10 μM CsA. The supernatants were harvested 48 h after transfection, assayed for reverse transcriptase activity, and titrated on P4-2 indicator cells. HIV-1 infectivity was inhibited by 78% when the virus was produced in the presence of CsA; this inhibition was reduced to 9% when HIV-1(VSV) pseudotypes were tested (Fig. 4). To ensure that the decreased CsA sensitivity was not due to the overall infectivity enhancement conferred by VSV-G, virus stocks were prepared by cotransfection of an HIV-1 *env*-defective proviral construct with decreasing amounts of the VSV-G expression construct or with the HIV-1 *env*-expression vector ΔGPVV · his in the presence or absence of 10 μM CsA. While the HIV-1 Env-containing virions were inhibited by 13.6-fold, the degree of inhibition of HIV-1(VSV) was consistently 2-fold or less (Table 3), even when the virions exhibited comparable infectivities. As a control, the nonpseudotyped *env*-competent virus R7 was tested, and it revealed a 10.4-fold reduction of infectivity when it was produced in the presence of CsA. These results demonstrate that HIV-1 particles bearing VSV-G are relatively resistant to inhibition by CsA.

TABLE 2. Infectivities of HIV-1 pseudotypes produced in the presence of decreased amounts of VSV-G

Envelope construct	Amt transfected (μg)	Mean infectivity of virus ± SD		Infectivity ratio (WT/Δ*nef*)
		WT[a]	Δ*nef*	
SV-A-MLV-*env*	5.0	0.44 ± 0.050	0.039 ± 0.0027	11.2
pHCMV-G	5.0	6.0 ± 0.41	3.3 ± 0.069	1.8
pHCMV-G	1.0	3.6 ± 0.082	2.1 ± 0.28	1.7
pHCMV-G	0.2	1.4 ± 0.39	1.1 ± 0.17	1.3
pHCMV-G	0.04	0.17 ± 0.036	0.091 ± 0.0097	1.9

[a] WT, wild type.

Pseudotyping with VSV-G does not bypass the requirement for Gag-CyPA binding. Previous observations in this laboratory have indicated that *nef*-defective HIV-1 is partially resistant to CsA (3), suggesting that CsA may inhibit additional viral functions besides CyPA-Gag binding. It was therefore of interest to determine whether pseudotyping with VSV-G could relieve the inhibitory effects of mutations in the capsid region of *gag*

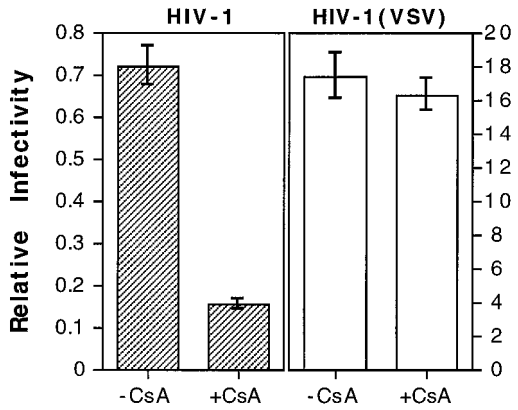

FIG. 4. CsA sensitivities of HIV-1 and HIV-1(VSV) pseudotyped viruses. Viruses were produced by culturing transfected 293T cells in the presence and absence of CsA for 2 days. Virus stocks were assayed for reverse transcriptase activity and titrated on P4-2 cells to determine virus titers. −CsA, control viruses; +CsA, viruses produced in the presence of 10 μM CsA. Shown are the mean values of triplicate infections, with error bars representing 1 standard deviation. The results are representative of at least two independent experiments.

that block CyPA binding and reduce HIV-1 infectivity (8, 17). To address this issue, mutant proviral constructs were cotransfected with pHCMV-G into 293T cells; the supernatants were then assayed for reverse transcriptase activity and infectivity on P4-2 cells. As a control, *nef*-defective HIV-1 was tested in parallel. The decrease in infectivity of HIV-1 containing a substitution of alanine at Gag residue P222 was not relieved by pseudotyping with VSV-G (Fig. 5). These results demonstrate that while VSV-G alleviates both the requirement for Nef and the inhibition of HIV-1 infectivity by CsA, HIV-1(VSV) pseudotypes preserve the effects of mutations rendering the Gag precursor incapable of incorporating CyPA.

DISCUSSION

In early studies of the mechanism of HIV-1 entry into cells, there was controversy over whether virions undergo direct fusion with the plasma membrane or are first internalized via receptor-mediated endocytosis. Although HIV-1 particles can be observed in intracellular vesicles shortly after exposure to cells, the weight of evidence supported direct fusion as the productive pathway of virus infection. First, virions can be observed in electron micrographs undergoing fusion with the cell surface. By itself, this result is not conclusive, since some HIV-1 particles have also been observed in coated vesicles near the plasma membrane (36). However, HIV-1 infection is not blocked by ammonium chloride, a classical test for viruses which require acidic pH to activate membrane fusion. Second, CD4 molecules containing mutations in the cytoplasmic tail that reduce the rate of CD4 internalization, or CD4 molecules whose cytoplasmic domains are replaced by a glycophosphatidylinositol anchor, serve as functional HIV-1 receptors (15, 37). Therefore, although internalization of HIV-1 through endocytosis occurs, entry through this pathway is not required for productive infection.

The results described here demonstrate that HIV-1 entry through receptor-mediated endocytosis can lead to productive infection if the viral core is provided a means of escape from endosomes. This was achieved by pseudotyping HIV-1 particles with VSV-G. VSV entry through an endocytic pathway has been well characterized (30). The virus enters a cell by first

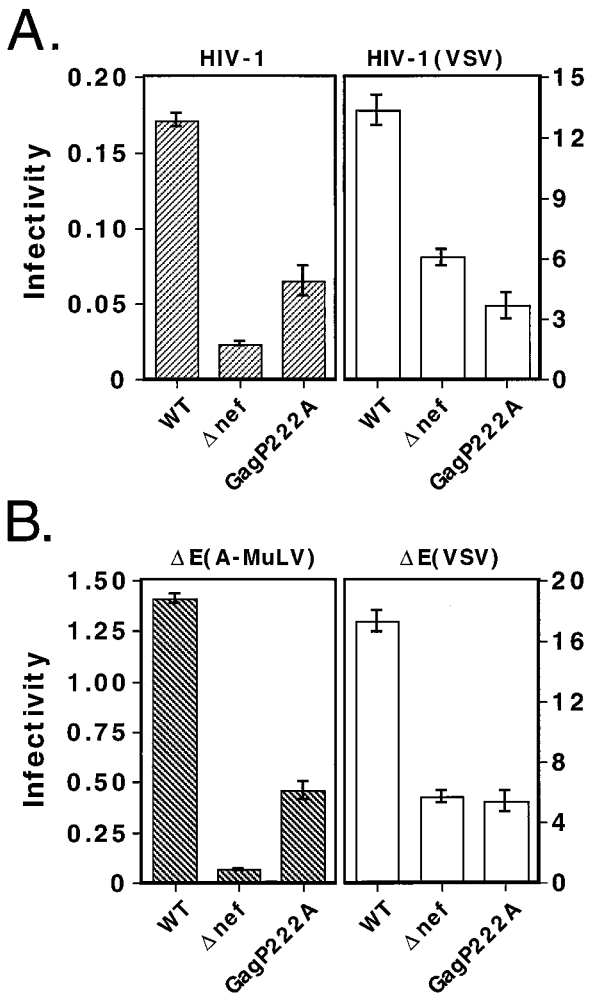

FIG. 5. Effects of VSV-G on the infectivity of an HIV-1 *gag* mutant defective in binding CyPA. Viruses were produced by transfection of 293T cells with proviral constructs with and without pHCMV-G. Virus stocks were assayed for reverse transcriptase activity and titrated on P4-2 cells to measure virus titers. The infectivity values shown are the infectious titers normalized for the reverse transcriptase levels of the virus stocks. (A) Results with viruses produced from *env*-competent HIV-1 proviral constructs; (B) viruses produced from *env*-defective proviral constructs. WT, wild-type control viruses containing functional *nef* and *gag* genes; Δnef, viruses containing a nonfunctional *nef* gene; GagP222A, viruses containing a mutation in *gag* resulting in substitution of Ala for Pro at residue 222; ΔE, *env* defective. Shown are the mean values of triplicate infections, with error bars representing 1 standard deviation. The results are representative of at least two independent experiments.

TABLE 3. CsA sensitivities of HIV-1 pseudotypes produced in the presence of decreased amounts of VSV-G

Envelope construct or R7 control	Amt transfected (μg)	Mean infectivity of virus ± SD		Infectivity ratio (without CsA/with CsA)
		Without CsA	With CsA[a]	
ΔGPVV · his	5.0	0.22 ± 0.0018	0.016 ± 0.0012	13.6
pHCMV-G	5.0	10.0 ± 0.32	6.14 ± 0.095	1.6
pHCMV-G	1.0	6.6 ± 0.77	3.4 ± 0.14	2.0
pHCMV-G	0.2	2.1 ± 0.22	1.4 ± 0.095	1.5
pHCMV-G	0.04	0.37 ± 0.056	0.23 ± 0.0025	1.6
R7	10.0	0.18 ± 0.0048	0.017 ± 0.0017	10.4

[a] Viruses produced in the presence of 10 μM CsA.

binding to its receptor, which is thought to be a phospholipid. After internalization by endocytosis through clathrin-coated pits, the membrane fusion activity of VSV-G is activated by the decreased pH in endosomes, releasing the VSV core into the cytoplasm. Thus, it is not surprising that VSV-G targets HIV-1 infection to this route. Nevertheless, these results indicate that HIV-1 remains competent for the remaining steps of the virus life cycle when it is internalized through an endocytic pathway. These include uncoating and initiation of reverse transcription, events that are normally enhanced by the viral factor Nef and the cellular protein CyPA.

Results presented here demonstrate that HIV-1(VSV) particles exhibit a decreased requirement for Nef for infectivity. The reduced dependence on Nef of these virions is not due to nonspecific enhancement of infectivity by VSV-G, since HIV-1(VSV) virions with a level of infectivity comparable to that of HIV-1 also exhibited a decreased Nef infectivity phenotype. In addition, HIV-1(VSV) failed to suppress the effect of a *gag* mutation inhibiting CyPA incorporation, demonstrating that the suppression was specific for Nef. Because VSV-G alters the pathway of productive infection to an endocytic route, it is likely that entry through the endocytic pathway is the cause of the decreased requirement for Nef. Previous studies have shown that Nef appears to facilitate an early postentry step in the HIV-1 life cycle, perhaps virus uncoating. HIV-1 uncoating is not yet well understood and is difficult to study due to the high percentage of defective HIV-1 particles (26). Nevertheless, dissociation of capsid protein from the viral core is likely to be a prerequisite for subsequent reverse transcription and transport to the nucleus. Two lines of evidence suggest that capsid disassembly occurs rapidly upon infection. First, viral ribonucleoprotein complexes isolated from cells as early as 1 h after infection contain undetectable amounts of capsid protein (9, 18). Second, viral core structures have proven extremely difficult to isolate from HIV-1 virions due to their apparent instability. Nef may stabilize the viral core, allowing for productive disassembly in the proper intracellular compartment; conversely, Nef may destabilize the core, enhancing the efficiency of disassembly. The putative requirement of Nef for efficient HIV-1 uncoating might be partially bypassed by exposure of the viral core to the altered environment of late endosomes, the subcellular compartment from which penetration of HIV-1(VSV) virions probably occurs (30). The altered conditions of this compartment would include acidic pH and the presence of acid proteases, both of which may override blocks to efficient uncoating by destabilizing the core. Nef has recently been detected in significant quantities in purified HIV-1 particles, where it may act in a manner analogous to that of the M2 ion channel of influenza virus (38). In this scenario, Nef would associate with the HIV-1 core, allowing the flow of ions or deoxynucleoside triphosphates into the viral ribonucleoprotein complex and resulting in enhanced reverse transcription in target cells (5, 12, 40).

Alternatively, VSV-G may direct incoming virions to an intracellular site from which subsequent steps required for productive infection occur more efficiently. VSV-G from incoming virions accumulates at the nucleus of an infected cell, and it has been suggested that the glycoprotein targets VSV to a perinuclear region (14). By analogy, VSV-G may transport HIV-1 through cytoplasmic barriers to a central perinuclear region, positioning the preintegration complex for nuclear uptake. This might also allow incoming virions to escape degradation by lysosomes, leading indirectly to more efficient reverse transcription in the target cell, thereby substituting for this function of Nef.

Nef-defective HIV-1 particles are typically 4- to 40-fold less infectious than wild-type HIV-1 but do replicate in culture given ample time. What is the mechanism by which these virions retain their residual infectivity? One possibility is that a fraction of HIV-1 particles enters cells through an alternate pathway that does not require Nef. The observation that HIV-1(VSV) exhibits a reduced requirement for Nef suggested that a fraction of nonpseudotyped HIV-1 Δ*nef* particles might also infect cells through this route. However, HIV-1 Δ*nef* virions were insensitive to ammonium chloride, an agent which effectively inhibited infection by HIV-1(VSV) virions. Ammonium chloride inhibits infection by viruses that infect cells through an endocytic pathway by preventing acidification of endosomes. Nevertheless, this compound does not inhibit endocytosis per se but blocks the activity of viral fusion glycoproteins, such as VSV-G and influenza HA, that require acidic pH for activation. Therefore, it cannot be concluded from the present study that *nef*-defective HIV-1 does not infect cells through an endocytic pathway. Indeed, the slight enhancement of HIV-1 infectivity by ammonium chloride (Fig. 3) suggests that a fraction of HIV-1 virions do, in fact, infect cells through this pathway and that ammonium chloride may prevent degradation of these virions by inhibiting the activity of lysosomal proteases.

Previous results from this laboratory have revealed that Nef and CyPA independently enhance HIV-1 infectivity but that sensitivity to cyclosporins correlates with the presence of a functional *nef* gene (2). This apparent contradiction suggested that the major inhibitory effect of CsA on HIV-1 infectivity in these single-cycle assays was due to inhibition of Nef function. Consistent with this interpretation, pseudotyping with VSV-G suppressed the phenotypes of *nef*-defective HIV-1 and of CsA-treated virions but not that of an HIV-1 *gag* mutant containing decreased CyPA levels. Thus, it is likely that CsA inhibits Nef-dependent HIV-1 infectivity enhancement at the concentrations of CsA employed in these experiments. These findings also indicate that Nef and the CyPA-Gag interaction perform distinct functions and suggest that these two proteins act at different steps in the HIV-1 life cycle. Alternatively, CyPA may be required at two phases of HIV-1 uncoating, one being enhanced by Nef and a later step requiring the CyPA-capsid protein interaction.

The decreased requirement for Nef exhibited by HIV-1 (VSV) pseudotypes has practical implications. The high titers achievable with these viruses, together with their broad host range, make them useful for experiments requiring a majority of target cells to be infected (7). In addition, pseudotyping of HIV-1 with VSV-G has been used to develop HIV-1 vectors for transducing genes in vivo (34). These vectors exhibit a characteristic property of lentiviruses in being able to infect nondividing cells. Although the use of HIV-1-based vectors may be undesirable for use in humans due to even a remote possibility of generating a replication-competent recombinant, the observation that pseudotyping with VSV-G circumvents the requirement for Nef indicates that highly infectious lentiviral vectors may be produced in the absence of Nef. Indeed, HIV-1(VSV) stocks with titers up to 10^7 per ml have been produced in the absence of Vpr, Vpu, and Nef (39). Because *nef* is crucial for efficient simian immunodeficiency virus replication and pathogenesis in vivo (22), this strategy may significantly enhance the safety of these vectors. It will be of interest to determine whether the requirements for other HIV-1 accessory genes, such as *vif*, are also reduced by pseudotyping with VSV-G.

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REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Aiken, C.** Submitted for publication.
3. **Aiken, C.** Unpublished data.
4. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76:**853–864.
5. **Aiken, C., and D. Trono.** 1995. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. J. Virol. **69:**5048–5056.
6. **Arthur, L. O., J. W. Bess, Jr. W., R. C. Sowder II, R. E. Benveniste, D. L. Mann, J.-C. Chermann, and L. E. Henderson.** 1992. Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science **258:**1935–1938.
7. **Bartz, S. R., M. E. Rogel, and M. Emerman.** 1996. Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G2 accumulation by a mechanism which differs from DNA damage checkpoint control. J. Virol. **70:**2324–2331.
8. **Braaten, D., E. K. Franke, and J. Luban.** 1996. Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 before the initiation of reverse transcription. J. Virol. **70:**3551–3560.
9. **Bukrinsky, M. I., N. Sharova, T. L. McDonald, T. Pushkarskaya, W. G. Tarpley, and M. Stevenson.** 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. USA **90:**6125–6129.
10. **Burns, J. C., T. Friedmann, W. Driever, M. Burrascano, and J.-K. Yee.** 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc. Natl. Acad. Sci. USA **90:**8033–8037.
11. **Charneau, P., M. Alizon, and F. Clavel.** 1992. A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. J. Virol. **66:**2814–2820.
12. **Chowers, M. Y., M. W. Pandori, C. A. Spina, D. D. Richman, and J. C. Guatelli.** 1995. The growth advantage conferred by HIV-1 *nef* is determined at the level of viral DNA formation and is independent of CD4 downregulation. Virology **212:**451–457.
13. **Chowers, M. Y., C. A. Spina, T. J. Kwoh, N. J. S. Fitch, D. D. Richman, and J. C. Guatelli.** 1994. Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact *nef* gene. J. Virol. **68:**2906–2914.
14. **Da Poian, A. T., A. M. O. Gomes, R. J. N. Oliveira, and J. L. Silva.** 1996. Migration of vesicular stomatitis virus glycoprotein to the nucleus of infected cells. Proc. Natl. Acad. Sci. USA **93:**8268–8273.
15. **Diamond, D. C., R. Finberg, S. Chaudhuri, B. P. Sleckman, and S. J. Burakoff.** 1990. Human immunodeficiency virus infection is efficiently mediated by a glycolipid-anchored form of CD4. Proc. Natl. Acad. Sci. USA **87:**5001–5005.
16. **Emi, N., T. Friedmann, and J.-K. Yee.** 1991. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. J. Virol. **65:**1202–1207.
17. **Franke, E. K., H. E. H. Yuan, and J. Luban.** 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature **372:**359–362.
18. **Gallay, P., S. Swingler, C. Aiken, and D. Trono.** 1995. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. Cell **80:**379–388.
19. **Gallay, P., S. Swingler, J. Song, F. Bushman, and D. Trono.** 1995. HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. Cell **83:**569–576.
20. **Grewe, C., A. Beck, and H. R. Gelderblom.** 1990. HIV: early virus-cell interactions. J. Acquired Immune Defic. Syndr. **67:**965–974.
21. **Henderson, L. E., R. Sowder, T. D. Copeland, S. Oroszlan, L. O. Arthur, W. G. Robey, and P. J. Fischinger.** 1987. Direct identification of class II histocompatibility DR proteins in preparation of human T-cell lymphotropic virus type III. J. Virol. **61:**629–632.
22. **Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651–662.
23. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66:** 2232–2239.
24. **Klasse, P. J., T. F. Schulz, and K. R. Willison.** 1993. Cyclophilins unfold the Gag? Nature **365:**395–396.
25. **Landau, N. R., K. A. Page, and D. R. Littman.** 1991. Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J. Virol. **65:**162–169.
26. **Layne, S. P., M. J. Merges, M. Dembo, J. L. Spouge, S. R. Conley, J. P. Moore, J. L. Raina, H. Renz, H. R. Gelderblom, and P. L. Nara.** 1992. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. Virology **189:**695–714.
27. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
28. **Maddon, P. J., J. S. McDougal, P. R. Clapham, A. G. Dalgleish, S. Jamal, R. A. Weiss, and R. Axel.** 1988. HIV infection does not require endocytosis of its receptor, CD4. Cell **54:**865–874.
29. **Marsh, M., and A. Pelchen-Matthews.** 1994. The endocytic pathway and virus entry, p. 215–240. *In* E. Wimmer (ed.), Cellular receptors for animal viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Matlin, K. S., H. Reggio, A. Helenius, and K. Simons.** 1982. Pathway of vesicular stomatitis virus entry leading to infection. J. Mol. Biol. **156:**609–631.
31. **McClure, M. O., M. Marsh, and R. A. Weiss.** 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. EMBO J. **7:**513–518.
32. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg.** 1994. The human immunodeficiency virus-1 *nef* gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. **179:**101–113.
33. **Miller, M. D., M. T. Warmerdam, K. A. Page, M. B. Feinberg, and W. C. Greene.** 1995. Expression of the human immunodeficiency virus type 1 (HIV-1) *nef* gene during HIV-1 production increases progeny particle infectivity independently of gp160 or viral entry. J. Virol. **69:**570–584.
34. **Naldini, L., U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono.** 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272:**263–267.
35. **Pandori, M. W., N. J. S. Fitch, H. M. Craig, D. D. Richman, C. A. Spina, and J. C. Guatelli.** 1996. Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. J. Virol. **70:**4283–4290.
36. **Pauza, C. D., and T. M. Price.** 1988. Human immunodeficiency virus infection of T cells and monocytes proceeds via receptor-mediated endocytosis. J. Cell Biol. **107:**959.
37. **Pelchen-Matthews, A., P. Clapham, and M. Marsh.** 1995. Role of CD4 endocytosis in human immunodeficiency virus infection. J. Virol. **69:**8164–8168.
38. **Pinto, L. H., L. J. Holsinger, and R. A. Lamb.** 1992. Influenza virus M2 protein has ion channel activity. Cell **69:**517–528.
39. **Reiser, J., G. Harmison, S. Kluepfel-Stahl, R. O. Brady, S. Karlsson, and M. Schubert.** 1996. Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc. Natl. Acad. Sci. USA **93:**15266–15271.
40. **Schwartz, O., V. Marechal, O. Danos, and J.-M. Heard.** 1995. Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. J. Virol. **69:**4053–4059.
41. **Seglen, P. O.** 1983. Inhibitors of lysosomal function. Methods Enzymol. **96:**737–764.
42. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive cells via virus envelope fusion to the plasma membrane. Cell **49:**659–668.
43. **Thali, M., A. Bukovsky, E. Kondo, B. Rosenwirth, C. T. Walsh, J. Sodroski, and H. G. Gottlinger.** 1994. Functional association of cyclophilin A with HIV-1 virions. Nature **372:**363–365.
44. **Trono, D., M. D. Feinberg, and D. Baltimore.** 1989. HIV-1 Gag mutants can dominantly interfere with the replication of the wild-type virus. Cell **59:**113–120.
45. **White, J. M.** 1994. Fusion of influenza virus in endosomes: role of the hemagglutinin, p. 281–301. *In* E. Wimmer (ed.), Cellular receptors for animal viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.