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Selective infection of human CD4$^+$ cells by simian immunodeficiency virus: Productive infection associated with envelope glycoprotein-induced fusion

(human immunodeficiency virus types 1 and 2/vaccinia virus recombinant/tropism)

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ABSTRACT **Simian immunodeficiency virus (SIV) and human immunodeficiency virus share the property of tropism for CD4-bearing cells. Infection is initiated by a high-affinity interaction between CD4 and conserved domains on the viral envelope glycoprotein. In this report, we demonstrated that SIV had a restricted host range among human CD4$^+$ cells when compared with human immunodeficiency virus type 1 or type 2. This restricted tropism was associated with the inability of the SIV envelope glycoprotein to induce membrane fusion in cells not susceptible to productive exogenous infection by SIV. We conclude that the major route of SIV entry into CD4$^+$ cells is by envelope-mediated direct fusion with the cell and that additional envelope–cell interactions after CD4 binding are required for productive infection.**

The etiologic agents of AIDS, the human immunodeficiency viruses (HIV-1 and HIV-2), are tropic and cytopathic for cells bearing the CD4 molecule (for review, see ref. 1). Several lines of evidence establish CD4 as the cellular receptor for HIV: (*i*) monoclonal antibodies directed against certain CD4 epitopes block HIV binding to and infection of target cells, (*ii*) CD4 and the HIV envelope protein (gp 120) coprecipitate from infected cell lysates incubated with antibodies specific for either protein, (*iii*) soluble CD4 blocks HIV infection *in vitro*, and (*iv*) the introduction of the CD4 gene into human CD4$^-$ cells renders these cells susceptible to HIV infection (1). Regions of the HIV envelope glycoprotein and domains of CD4 required for binding have been delineated (2–4).

The simian immunodeficiency viruses (SIVs) are a family of primate lentiviruses that have been isolated from a number of African primates (for review, see ref. 5). Two SIV isolates, SIV_{mac} from rhesus macaques and SIV_{sm} from sooty mangabey monkeys, share extensive amino acid sequence similarity with HIV-2 and less, but significant, identity with HIV-1 (ref. 6; V.M.H. and P.R.J., unpublished data). Importantly, experimental infection of macaques with SIV induces a clinical syndrome of immunodeficiency similar to AIDS in humans (7), thus providing a valuable animal model for the study of pathogenesis, antiviral drug testing, and vaccine development. The cellular receptor for SIV is CD4 (8). However, several observations suggest that the expression of CD4 alone may not be sufficient to allow a productive infection by SIV. (*i*) Peripheral blood mononuclear cells from chimpanzees can be infected with HIV-1, but not with SIV, despite expression of conserved CD4 epitopes required for HIV infection (8). (*ii*) One human CD4$^+$ tumor cell line (MOLT-3) that supports HIV-1 infection is not permissive for SIV infection (9). The mechanisms underlying these observations are not known.

We have examined a number of well-characterized human CD4$^+$ cell lines for their ability to be infected with HIV-1, HIV-2, or SIV. Only a limited (two of six) number of cell lines could be infected with SIV, whereas all were productively infected with HIV-1 or HIV-2. Furthermore, susceptibility to productive infection of human CD4$^+$ cells correlated with the ability of CD4$^-$ cells infected with recombinant vaccinia viruses expressing the envelope proteins of HIV-1 or SIV to form syncytia with them. Thus, expression of the CD4 molecule alone on the surface of human cells is insufficient to allow for infection by SIV. This fact may be associated with a failure of the SIV envelope glycoprotein to induce fusion of the viral and cell membranes, thereby preventing viral penetration.

MATERIALS AND METHODS

Cell Lines. HeLa and MOLT-4 cell lines were obtained from the American Type Culture Collection. The following CD4$^+$ cell lines were obtained as gifts from other investigators: H9 and MT-4 (B. Fernie, Georgetown University), A3.01 (T. Folks, National Institute of Allergy and Infectious Diseases), Sup-T1 (J. Hoxie, University of Pennsylvania), BHM-23 (E. Max, National Institute of Allergy and Infectious Diseases), and HeLa-T4 (R. Axel, Columbia University). HeLa and HeLa T4 were maintained in Dulbecco's modified Eagle's medium (Microbiological Associates) supplemented with 10% fetal calf serum and gentamicin (20 μg/ml) and passaged twice weekly. All other lymphocyte cell lines were maintained with RPMI 1640 medium (Microbiological Associates) supplemented with 10% fetal calf serum and gentamicin.

Virus Stocks and Infections. Filtered (0.45 μm) supernatants of the A3.01 cell line productively infected with the lymphadenopathy-associated virus isolate of HIV-1 or the ROD isolate of HIV-2 (gift of F. Clavel, National Institute of Allergy and Infectious Diseases) served as HIV-1 or HIV-2 stocks, respectively. SIV stock virus was prepared from the supernatant of H9 cells persistently infected with the SIV_{mac} 251 isolate (provided by N. Letvin, New England Regional Primate Research Center). All viral stocks were stored in liquid nitrogen and had comparable titers of 10^5 tissue culture 50% infective dose per ml. Cells were infected at a multiplicity of infection of 0.01 for 2 hr at 37°C, washed, and resuspended in medium at 5×10^5 cells per ml.

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Abbreviations: SIV, simian immunodeficiency virus; HIV-1 and -2, human immunodeficiency virus type 1 and 2, respectively.

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Transfection Assays. Plasmids containing biologically active molecular clones of HIV-1 (IIIB) or SIV_{mac} 251 (see Table 2) were transfected into cells (1 μg per 10^7 cells) by a modification of the DEAE-dextran method (10). The plasmid pUC-18 was used as a control. Detection of reverse transcriptase activity in culture supernatants was considered evidence for active viral replication. To detect virions produced in a single cycle of replication in cells that were not susceptible to exogenous infection, some transfected cultures were cocultivated with H9 cells 24 hr after primary transfection.

Fusion Assay. Epstein–Barr virus-transformed B cells from an HIV-seronegative human donor were infected with recombinant vaccinia viruses (10 plaque-forming units/cell) for 10–14 hr, washed, and then cocultured with each target cell line in a ratio of 1:10. Cultures were monitored for a period of 4–16 hr for syncytium formation. Recombinant vaccinia viruses used included: VPE-7 (designated here as vacc–HIVenv), expressing the envelope of HIV-1 (IIIB) (11); VSC-40 (designated vacc–HIVgag), expressing the gag proteins of HIV-1 (12); and WR194 (designated vacc–SIVenv), described in Fig. 1.

Reverse Transcriptase Assay. Supernatants from cell cultures were harvested and clarified on the days indicated and stored at −20°C. The assay for reverse transcriptase activity was performed as described (11). Briefly, supernatant fluid was incubated with a mixture containing poly(rA), oligo $(dT)_{12-18}$, and [α-^{32}P]thymidine triphosphate in a microtiter

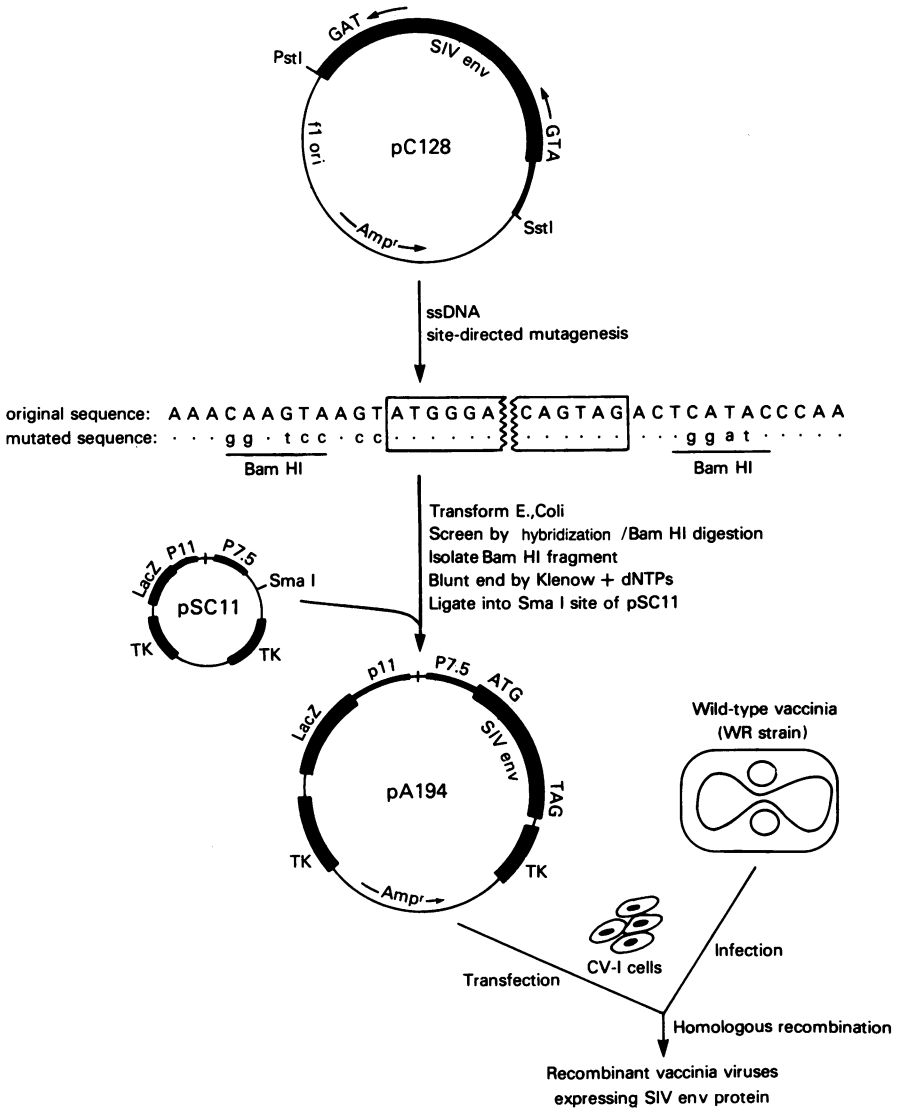

FIG. 1. Construction of a vaccinia virus expressing the SIV envelope protein. A 2870-base-pair (bp) *Sst* I–*Pst* I fragment containing the SIV *env* gene was isolated from a subgenomic λ clone (6; gift of G. Franchini, National Cancer Institute) and then ligated into pTZ18R (Pharmacia) that had been linearized with *Sst* I/*Pst* I (designated pC128). To obtain the precise coding region for expression, unique *Bam*HI restriction endonuclease sites were engineered immediately upstream of the initiating methionine codon and immediately downstream of the termination codon by oligonucleotide directed site-specific mutagenesis (13) of single-stranded template prepared from pC128. In addition, sequences thought to be important for eukaryotic mRNA translation (14) were placed upstream of the initiating methionine codon. Nucleotide sequencing confirmed that the proper sequences had been constructed. The plasmid was digested with *Bam*HI, and the resulting 2235-bp fragment was made blunt-ended with the Klenow fragment of DNA polymerase I in the presence of deoxyribonucleoside triphosphates. This fragment was ligated into the *Sma* I site of the vaccinia virus plasmid coexpression vector pSC11 (15), immediately downstream of the P7.5 (early/late) vaccinia virus promoter. Recombinant vaccinia viruses were obtained as described (15): the resulting coexpression vector (designated pA194) was transfected into vaccinia (WR strain)-infected cells, where homologous recombination yielded live recombinant vaccinia viruses that were identified colorimetrically by the presence of β-galactosidase activity. Expression of the SIV envelope protein was confirmed by indirect immunofluorescence, radioimmunoprecipitation, and Western (immunologic) blot analysis of infected cells. Furthermore, SIV envelope-specific antibodies were induced in mice and primates inoculated with the vaccinia–SIVenv recombinant (data not shown).

plate. Reaction products were dotted onto DEAE-81 paper (Whatman), washed, and exposed to x-ray film. Background activity was monitored by the inclusion in each assay of culture fluids from uninfected cells.

RESULTS

Susceptibility of Human Lymphocyte Cell Lines to Infection with HIV-1, HIV-2, and SIV. We evaluated six human $CD4^+$ lymphocyte cell lines for susceptibility to infection by HIV-1, HIV-2, or SIV. The results are summarized in Table 1, and examples of infection kinetics are shown in Fig. 2. All of the cell lines tested were productively infected by HIV-1 or HIV-2. In contrast, only two lines (H9 and MT-4) were infected by SIV. Analysis of these cell lines with 13 monoclonal antibodies to lymphocyte cell-surface molecules (data not shown) failed to reveal a correlation between infection by SIV and the presence of any particular molecule except for CD4 and the combination of CD4 and HLA-DR. To investigate this observation, we evaluated the cell line BHM-23, a non-Epstein–Barr virus-transformed B-cell line that bears CD4 and HLA-DR. This line was susceptible to HIV-1 and HIV-2 infection but was not infected by SIV. Thus, the presence of HLA-DR did not correlate with infection by SIV. Although these data indicated the necessity of CD4 for infection, CD4 alone was not sufficient to allow a productive infection by SIV. This result suggested that specific interactions subsequent to CD4 binding were required for productive infection by SIV.

SIV Replicates in Cells That Are Not Susceptible to Infection. To further investigate the inability of SIV to infect certain $CD4^+$ lymphocytes, we evaluated the ability of SIV to replicate in cell lines not susceptible to exogenous infection (Table 2). These experiments were designed to bypass viral penetration, thereby evaluating subsequent events in the replicative cycle. Three cell lines were chosen for these experiments: (*i*) $CD4^+$ H9 cells, which are susceptible to HIV-1 and SIV infection; (*ii*) $CD4^+$ Sup-T1 cells, which are susceptible to HIV-1 infection but not SIV infection (see above); and (*iii*) HeLa cells, a $CD4^-$ human epithelial-like cell line that is not susceptible to HIV-1 or SIV infection. A biologically active molecular clone of SIV or HIV-1 was transfected into each of these cell lines, and supernatant fluids were assayed over time for reverse transcriptase activity (Table 2). As expected, H9 cells supported active replication of HIV-1 and SIV. Reverse transcriptase activity was detected in culture fluids 4 to 7 days after transfection, indicating that virions produced by the initial replicative burst had reinfected susceptible H9 cells in the culture. In Sup-T1 cells after transfection with an HIV or SIV molecular clone, HIV-1 established a productive infection, again indicating multiple rounds of replication in susceptible cells; SIV was not detected in Sup-T1 cells for up to 28 days after transfection. This result indicated that either SIV did not replicate in these cells or that virions produced by the initial replicative burst did not reinfect Sup-T1 cells and amplify the infection. To test the latter explanation, Sup-T1 cells were transfected with the SIV molecular clone and then cocultured 24 hr later with H9 cells. Because H9 cells are susceptible to exogenous infection by SIV, infectious virions produced by the transfected Sup-T1 cells would be rescued. As shown in Table 2, SIV was detected in H9 cells that had been cocultured with transfected Sup-T1 cells. This demonstrated that Sup-T1 cells supported SIV replication and confirmed that these cells were not susceptible to exogenous infection by SIV. Cocultivation rescue was required for HIV-1 and SIV in HeLa cells because neither virus is capable of exogenous infection in these cells. Thus, human $CD4^+$ cells, not susceptible to exogenous SIV infection, supported SIV replication when viral penetration was not required.

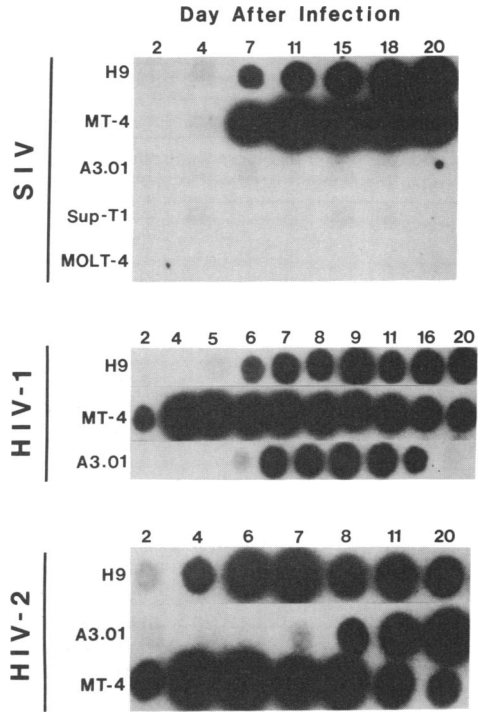

FIG. 2. Infection kinetics of SIV, HIV-1, and HIV-2 in human $CD4^+$ T-cell lines.

Cell Fusion Predicts Infection by SIV. Previously, Hoxie *et al.* (17) demonstrated that CD4 on Sup-T1 cells bound the SIV envelope glycoprotein, but that productive infection did not proceed in the usual manner. These data, considered with our data from the aforementioned transfection experiments, suggested that SIV entry into nonsusceptible cells was defec-

Table 1. Infection of human $CD4^+$ lymphocyte cell lines by HIV-1, HIV-2, and SIV

	Cell surface expression		Productive infection*		
Cell line	CD4	HLA-DR	HIV-1	HIV-2	SIV
H9	+	+	+	+	+
MT-4	+	+	+	+	+
A3.01	+	−	+	+	−
Sup-T1	+	−	+	+	−
MOLT-4	+	−	+	+	−
BHM-23	+	+	+	+	−

*Infection monitored by reverse transcriptase activity in supernatant fluids (see Fig. 2). All cultures positive for reverse transcriptase activity displayed syncytial cytopathic effects.

Table 2. SIV replicates in cells that are not susceptible to exogenous infection

Primary transfection cells	Cocultivation of transfected cells with H9 cells*	Productive infection after transfection with plasmid†		
		pUC	HIV-1	SIV
H9	No	−	+	+
Sup-T1	No	−	+	−
	Yes	−	ND	+
HeLa	No	−	−	−
	Yes	−	+	+

ND, not done.
*Uninfected H9 cells were added to the transfected cells 24 hr after transfection where indicated.
†The plasmids used for transfection were pUC-18 as control, pBK28 for SIV_{mac} 251 (10), and pHXB2 for HIV-1 (IIIB) (16).

Table 3. Cell fusion induced by vaccinia–*env* recombinants predicts productive infection by HIV-1 and SIV

Cell line	Correlation of infection and fusion by HIV-1 and SIV			
	HIV-1		SIV	
	Productive infection	Fusion by vacc–HIVenv	Productive infection	Fusion by vacc–SIVenv*
H9	+	+	+	+
MT-4	+	+	+	+
A3.01	+	+	–	–
Sup-T1	+	+	–	–
BHM-23	+	+	–	–
HeLa-T4	+	+	–	–
HeLa	–	–	–	–

*Syncytia induced in H9 cells were slightly larger and more numerous than syncytia induced in the MT-4 cells.

tive. After binding to CD4, HIV-1 penetrates the cell via fusion with the plasma membrane of the cell at neutral pH (18, 19). To investigate this fusion process for SIV, we constructed a recombinant vaccinia virus that expressed the SIV envelope protein (Fig. 1). We employed the vaccinia–SIV recombinant and a recombinant vaccinia virus expressing the HIV-1 envelope protein (11) in a fusion assay to evaluate the ability of the respective envelope proteins to induce cell fusion in $CD4^+$ human lymphocyte lines. As shown in Table 3 and Fig. 3, all of the six $CD4^+$ cell lines tested were susceptible to syncytium induction by the recombinant vaccinia virus vacc–HIVenv. In contrast, only two of the cell lines (H9 and MT-4) produced syncytia when exposed to the SIV envelope protein expressed by the recombinant vaccinia virus vacc–SIVenv. These two cell lines were also the only ones susceptible to SIV infection. Thus, the ability of the envelope proteins of HIV-1 and SIV to induce cell fusion correlated exactly with the susceptibility of the cell to exogenous infection.

DISCUSSION

Our observations define a major biologic difference between the human AIDS viruses (HIV-1 and HIV-2) and SIV. These viruses have in common (*i*) the use of CD4 as a cellular receptor for the viral envelope glycoprotein and (*ii*) conservation of the region of the envelope glycoprotein that binds to CD4 (2). Despite these important similarities, SIV productively infected relatively few human $CD4^+$ cell lines when compared with HIV-1 or HIV-2. This marked disparity in tropism for $CD4^+$ cells prompted us to investigate the underlying mechanisms. Our data and work by Hoxie *et al.* (17) indicate that the restricted tropism of SIV is associated with the inability of the SIV envelope glycoprotein to induce membrane fusion in certain human $CD4^+$ T-cell lines.

Our results are in general agreement with those of Hoxie *et al.* (17). However, these workers demonstrated that after an extended period of time (>30 days) a small subpopulation of Sup-T1 cells did become infected by SIV (human T-cell lymphotropic virus type IV). The infected cells demonstrated minimal cytopathic effects, minimal down-modulation of CD4, and slight alterations in the size of the SIV transmembrane glycoprotein on Western blots. These data demonstrated that within transformed T-cell lines, subpopulations may be present that have differential susceptibility to infection. Because these cells did not display syncytium formation, viral entry might have occurred by a mechanism other than direct fusion at the target cell surface (see below). Because our experiments were terminated after 4 weeks, we did not observe infection of Sup-T1 cells by SIV. We have seen differences in SIV infection among subclones of the CEM cell line. In our initial experiments, we found that the A3.01 cell line, a clonal derivative of CEM cells, was not susceptible to SIV fusion or infection. However, the CEM_{ss} clonal line (gift of P. Nara, National Cancer Institute), did produce syncytia in our fusion assay and was subsequently shown to be susceptible to infection by SIV. The differences observed between the CEM clones appeared to be explained by the susceptibility of the cells to fusion by the SIV envelope glycoprotein. Thus, within human $CD4^+$ T-cell lines, at least two populations of cells may exist: (*i*) cells that are susceptible to SIV infection by an alternative mechanism of viral entry and (*ii*) cells that differentially express a factor required for virus–cell fusion, the major route of SIV nucleocapsid entry into the host cell.

Most evidence suggests that HIV enters $CD4^+$ cells by direct fusion. The evidence is as follows: (*i*) weak bases do not consistently inhibit HIV infection in a majority of $CD4^+$ cells (19), (*ii*) HIV-infected cells or cells expressing the HIV envelope glycoprotein fuse at neutral pH with uninfected cells expressing $CD4^+$ (19), (*iii*) electron micrographs demonstrate virus–cell fusion at the target cell surface (19), and (*iv*) cells expressing mutant forms of CD4 that are defective in endocytosis can still form syncytia and support productive infection (20). The HIV envelope glycoprotein mediates this direct fusion process (19). The HIV and SIV envelope glycoproteins and other lentivirus envelope glycoproteins share a number of structural similarities with the orthomyxovirus and paramyxovirus fusion proteins. All of these fusion

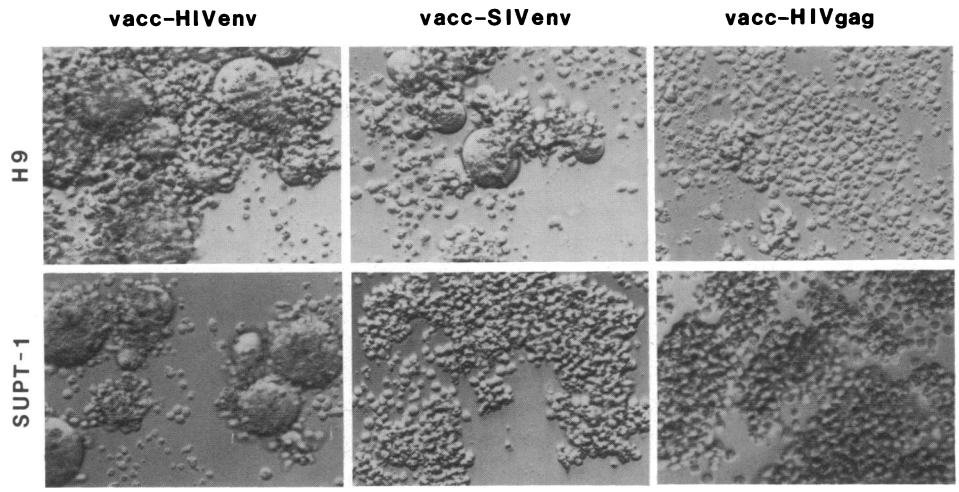

FIG. 3. Syncytia formation in H9 and Sup-T1 cells induced by recombinant vaccinia viruses. The fusion assay was performed as described.

proteins are composed of two subunits generated by post-translational proteolytic (trypsin-like) cleavage. This cleavage is required for infectivity (21) and exposes the characteristic and highly conserved hydrophobic domain (fusogenic peptide) at the amino terminus of the membrane anchoring subunit.

Our data support the concept that HIV and SIV penetrate cells via direct fusion at the plasma membrane. Cells that were not susceptible to SIV infection were resistant to syncytium induction by infectious virions and by the SIV envelope glycoprotein as expressed by a recombinant vaccinia virus. Susceptibility of a cell line to exogenous SIV infection was predicted by the induction of syncytia by the SIV envelope glycoprotein. For HIV-1 and HIV-2, all $CD4^+$ cell lines tested were productively infected and were susceptible to syncytium formation by the HIV-1 envelope. Thus, the typical syncytial cytopathic effect of HIV and SIV reflects the entry mechanism of the virus.

In addition to the viral determinants of fusion, there are requisite cellular factors as well. CD4 is an absolute requirement for virus-induced fusion. However, we have demonstrated that for SIV, CD4 alone is not sufficient for fusion and, therefore, productive infection. A similar observation has been made for HIV-1. Mouse fibroblasts expressing human CD4 bound infectious HIV-1 particles but were not susceptible to fusion or infection (22). Thus, SIV and HIV require, in addition to CD4, another equally important cellular factor for fusion induction and viral entry. Moreover, this cellular factor for SIV may be distinct from the cellular factor for HIV-1 and HIV-2. This observation adds another layer of complexity to the entry mechanism for these viruses.

Theoretically, in a two-step process of binding and fusion, the envelope glycoprotein binds to the cellular receptor and then the fusogenic peptide in the transmembrane glycoprotein binds to a second receptor and induces fusion, perhaps by destabilizing the lipid bilayer. For SIV and HIV-2, the fusogenic peptide is exactly conserved and might be expected to interact with a conserved second receptor on the target cell. However, as we have demonstrated, SIV and HIV-2 probably do not bind to the same second receptor. Therefore, one conclusion might envision the entry process as comprised of at least three steps: (*i*) gp 120 binding to CD4, (*ii*) a second region of the envelope (either in gp 120 or gp 32/41) becomes accessible to and interacts with a second receptor, and (*iii*) this interaction then approximates the fusogenic peptide and the target cell membrane, allowing a nonspecific but finalizing interaction at the plasma membrane surface.

In conclusion, we have demonstrated that the process of viral entry for SIV (and HIV) is a complicated, multistep process that is influenced by viral and cellular factors. The potential for blocking or interrupting these processes at various steps in the entry pathway offers new avenues for prophylactic and therapeutic strategies for AIDS.

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