JOURNAL OF VIROLOGY, Feb. 2002, p. 1588–1599 Vol. 76, No. 4
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.4.1588–1599.2002

Properties of the Surface Envelope Glycoprotein Associated with Virulence of Simian-Human Immunodeficiency Virus $SHIV_{SF33A}$ Molecular Clones

Lisa A. Chakrabarti,† Tijana Ivanovic, and Cecilia Cheng-Mayer*

Aaron Diamond AIDS Research Center, The Rockefeller University, New York, New York 10016

Received 25 July 2001/Accepted 24 October 2001

In vivo adaptation of simian-human immunodeficiency virus (SHIV) clone $SHIV_{SF33}$ resulted in the emergence of pathogenic isolate $SHIV_{SF33A}$, which caused a rapid and severe $CD4^+$ T-cell depletion when inoculated into rhesus macaques. Two molecular clones generated by inserting the *env* V1-to-V5 region amplified from $SHIV_{SF33A}$-infected animals into the parental $SHIV_{SF33}$ genome retained a pathogenic phenotype. The gp120 envelope glycoproteins of pathogenic clones $SHIV_{SF33A2}$ and $SHIV_{SF33A5}$ conferred a threefold increase in viral entry and fusogenicity compared to the parental glycoprotein. Changes in gp120 were also responsible for a higher replication capacity and cytopathicity in primary $CD4^+$ T-cell cultures. Last, gp120 carried the determinants of $SHIV_{SF33A}$ neutralization resistance. Thus, changes in $SHIV_{SF33A}$ gp120 produced a set of properties that could account for the pathogenic phenotype observed in vivo. Measurement of antibody binding to $SHIV_{SF33A}$ viral particles revealed an increased exposure of the CD4-induced epitope recognized by the 17b monoclonal antibody in a region that was shown to contribute to coreceptor binding. Exposure of this epitope occurred in the absence of CD4 binding, suggesting that the envelope glycoprotein of pathogenic $SHIV_{SF33A}$ clones folded in a conformation that was primed for interaction with CXCR4 or for the subsequent step of fusion.

Simian-human immunodeficiency viruses (SHIVs) are chimeric viruses engineered to test the function or immunogenicity of human immunodeficiency virus (HIV) gene products in simian models. Frequently used constructs contain an HIV type 1 (HIV-1) DNA fragment containing the *tat*, *rev*, *vpu*, and *env* genes inserted in the proviral genome of SIVmac239, a molecular clone that causes simian AIDS in rhesus macaques (3, 11, 22, 28, 35, 37, 47, 49). SHIVs play a key role in the development of vaccines that are directed against the HIV-1 envelope glycoprotein and are also valuable tools in the understanding of AIDS pathogenesis (41, 42). Comparative studies of SHIVs that use CXCR4 and the CCR5 coreceptor (X4 and R5 SHIVs, respectively) have revealed the role of tropism in the rate and localization of $CD4^+$ T-cell depletion (19). Pathogenic X4 SHIVs typically cause a severe depletion of $CD4^+$ T lymphocytes in blood and peripheral lymph nodes within 1 month of infection (14, 21, 27, 36). Pathogenic isolates of dualtropic R5X4 SHIVs also cause rapid $CD4^+$ T-cell depletion (29, 46, 50), while pathogenic R5 SHIVs cause a more protracted decrease of peripheral $CD4^+$ T cells similar to that seen in SIVmac infection (18). Rhesus macaques chronically infected with pathogenic X4 or R5X4 SHIVs often maintain a high viral load in spite of an almost complete depletion of $CD4^+$ T lymphocytes, a finding that may be explained by the productive infection of activated macrophages (25).

SHIV constructs do not readily induce disease in rhesus macaques and require in vivo adaptation and often serial in vivo passages to acquire the capacity to replicate at high levels, deplete $CD4^+$ T cells, and induce simian AIDS. Most pathogenic SHIV isolates, such as $SHIV_{KU-1}$, SHIV-89.6P, and $SHIV_{162P3}$, have been recovered after serial transfer of bone marrow or of blood from infected animals (19, 28, 46). Administration of an anti-CD8 monoclonal antibody (MAb) during primary infection with clone $SHIV_{DH-12}$ resulted in $CD4^+$ T-cell depletion in one rhesus macaque, indicating that immunosuppression could favor the emergence of pathogenic SHIVs (26). $SHIV_{SF33}$ differs from other isolates in that it evolved into a pathogenic virus without serial in vivo passages or immunosuppressive treatment. The original virus replicated to intermediate levels in rhesus macaques and did not cause $CD4^+$ T-cell depletion, except in one juvenile animal which developed signs of simian AIDS 2 years after inoculation. The isolate recovered from this animal, designated $SHIV_{SF33A}$ (A, adapted), replicated to high titers, induced a rapid and profound depletion of peripheral $CD4^+$ T cells, and caused an AIDS-like syndrome when passaged intravenously in rhesus macaques (36, 37). In addition, $SHIV_{SF33A}$ could be transmitted by the vaginal route even though it used exclusively the CXCR4 coreceptor for entry (21).

Comparison of original SHIV constructs with their in vivo-adapted counterparts provides a system to map the viral determinants of SHIV virulence (29, 56). Genetic analyses of pathogenic SHIV molecular clones have demonstrated that virulence is primarily modulated by adaptive changes in the two subunits of the envelope glycoprotein (2, 15, 16, 30, 33). We showed previously that, in the context of the $SHIV_{SF33}$ genome, replacing the V1-to-V5 region of the *env* gene with corresponding sequences amplified from $SHIV_{SF33A}$-infected

* Corresponding author. Mailing address: Aaron Diamond AIDS Research Center, 455 First Ave., 6th Floor, New York, NY 10016. Phone: (212) 448-5080. Fax: (212) 448-5159. E-mail: cmayer@adarc.org.

† Present address: Unité d'Immunologie Virale, Institut Pasteur, 75724 Paris Cédex 15, France.

animals was sufficient to confer pathogenicity (20). The extent of $CD4^+$ T-cell depletion and the disease course in animals infected with recombinant molecular clone $SHIV_{SF33A2}$ were similar to those caused by the $SHIV_{SF33A}$ isolate, demonstrating that pathogenic determinants mapped to the V1-to-V5 region of gp120. The aim of the present study was to characterize the properties conferred by the envelope glycoprotein of pathogenic $SHIV_{SF33A}$ molecular clones. We found that changes in gp120 were responsible for increases in fusogenicity, cytopathicity, replication capacity, and neutralization resistance of $SHIV_{SF33A}$ and thus conferred a set of properties that could account for the pathogenic phenotype observed in vivo. Furthermore, a conserved CD4-induced epitope that overlaps with the coreceptor binding site was spontaneously exposed at the surfaces of $SHIV_{SF33A}$ virions, even in the absence of CD4 binding. This finding provided a structural basis for the enhanced fusogenicity and replication capacity of pathogenic $SHIV_{SF33A}$.

MATERIALS AND METHODS

Cells. Human osteosarcoma cells transduced with the chemokine receptor CXCR4 with or without CD4 (HOS-CD4-CXCR4 and HOS-CXCR4, respectively) and human astroglioma cells transduced with CD4 (U87-CD4-pBABE) were gifts from N. R. Landau (Salk Institute, La Jolla, Calif.). These cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1 μg of puromycin/ml. 293T cells used for transfection were cultured in DMEM with 10% FCS. Lymphoid cell line CEMx174 5.25, also a gift from N. R. Landau, was stably transduced with an HIV-1–long terminal repeat (LTR)-green fluorescent protein (GFP) reporter construct and with CCR5. These cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 1 μg of puromycin/ml, and 200 μg of G418/ml. Human peripheral blood mononuclear cells (PBMC) were depleted of $CD8^+$ cells with M450 CD8 Dynabeads according to the manufacturer's recommendation (Dynal AS, Oslo, Norway) and cultured in RPMI 1640 medium supplemented with 10% FCS and 20 U of recombinant interleukin-2/ml.

Construction of the envelope expression vectors. The *Eco*RI-to-*Eco*RV fragment of $HIV\text{-}1_{SF33}$ (nucleotides 1 to 3362 in GenBank sequence M38427), which contains the *tat*, *rev*, *vpu*, and *env* genes, was inserted into mammalian expression vector pCAGGS as described previously (4, 5). The resulting plasmid, pEnv33, expressed the full-length gp160 envelope glycoprotein and did not require the cotransfection of a Rev expression vector. A 1.05-kb *Dra*III-to-*Mun*I fragment encompassing the V1-to-V5 regions of the $SHIV_{SF33A2}$ and $SHIV_{SF33A5}$ clones was used to replace the corresponding sequences in pEnv33 (nucleotides 854 to 1897 in M38427) and generate Env expression vectors pEnvA2 and pEnvA5, respectively. The sequence of the $SHIV_{SF33A2}$ gp120 coding fragment (GenBank accession no. AF373044) has been described previously (20).

Generation of luciferase reporter viruses. Luciferase reporter viruses pseudotyped with the different SHIV envelope glycoproteins were generated by transcomplementation, as described previously (6). The reporter viruses were derived from the HIV-1 pNL4-3 proviral DNA, in which the *env* gene was deleted and a firefly luciferase cassette was inserted in place of the *nef* gene. Because of the lack of a proviral *env* gene, pseudotyped viruses were capable of only a single round of replication. They were generated by lipofection of 1.5 μg each of pNL-Luc-E^-R^- plasmid and of a pEnv expression vector in 293T cells plated at 4×10^5 per well in six-well plates. The lipofection was performed with the DMRIE-C reagent according to the manufacturer's recommendations (Gibco-BRL, Gaithersburg, Md.). Cell culture supernatants were harvested 72 h posttransfection, centrifuged at 800 × *g*, filtered through 0.45-μm-pore-size filters, and stored at −70°C until use. The viral content was quantified by a p24 Gag enzyme-linked immunosorbent assay (ELISA) from Abbott Laboratories (Chicago, Ill.).

Entry assays. For entry assays, HOS-CD4-CXCR4 cells were plated at 7×10^3 per well in 96-well plates and cultured overnight. The cells were pretreated with 2 μg of Polybrene/ml in Hanks balanced salt solution for 30 min and then infected with 0.5 ng of p24 Gag equivalent from each of the pseudotyped viruses for 3 h at 37°C. The viral supernatants were removed, and the cells were further cultured for 72 h before being tested for luciferase activity. Cells were lysed and incubated with the luciferase assay reagents according to the manufacturer's instructions (Promega, Madison, Wis.). The luciferase activity was measured in a Dynex MLX microtiter plate luminometer (Dynex Technologies, Inc., Chantilly, Va.). All infections were done in duplicate. Reporter viruses lacking Env were included as negative controls in each experiment.

For entry assays with cells expressing various amounts of CD4, HOS-CXCR4 cells plated at 6×10^4 cells per well in 24-well plates were lipofected with 0.1 to 600 ng of CD4 expression vector (pCDNAT4 from N. R. Landau). Cells were trypsinized the next day, the content of one well being reseeded in 9 wells of a 96-well plate. Cells were infected with pseudotyped viruses at day 2 posttransfection. Luciferase activity was determined at day 5 as described above. Entry assays with cells expressing various amounts of CXCR4 were performed on U87-CD4-pBABE cells transfected with 0.1 to 300 ng of CXCR4 expression vector (pCDNAlester from N. R. Landau) and 300 ng of CD4 plasmid.

Neutralization assays. Neutralization assays were similar to entry assays except that pseudotyped viruses were preincubated with serial dilutions of antibodies in Hanks balanced salt solution for 30 min at 37°C before being added to target cells. Human MAb immunoglobulin G1b12 (IgG1b12), which recognizes the CD4 binding site, was obtained from D. Burton (Scripps Institute, La Jolla, Calif.) (1). The human IgG-CD4 chimeric protein was obtained from Progenics (Tarrytown, N.Y.), and the soluble form of CD4 (sCD4) was obtained from Chiron (Emeryville, Calif.). Human MAb 17b, which recognizes a discontinuous epitope that is preferentially exposed upon CD4 binding, was provided by J. Robinson (Tulane University, New Orleans, La.) (59, 60).

Fusion assay. Donor cells expressing the different SHIV envelopes and transactivator protein Tat were incubated in the presence of the target cell line CEMx174 5.25, which contained a GFP reporter gene placed under the control of the HIV-1 LTR. Fusion resulted in the diffusion of Tat into the target, which induced GFP expression. To generate the donor cells, 293T cells plated at 4×10^5 per well in six-well plates were lipofected with 1.5 μg of of the pEnv plasmid plus 1.5 μg of the $pTat_{SF13}$ expression vector (34). Two days after transfection, the cells were detached by a 5-min incubation in phosphate-buffered saline (PBS) with 1 mM EDTA and reseeded in a 24-well plate at 2.5×10^5 cells per well in triplicate. CEMx174 5.25 cells were added at a density of 5×10^5 cells per well, and fusion was allowed to proceed for 7 to 10 h in order to obtain detectable levels of GFP expression. Cells were detached by gentle pipetting and counted under a fluorescence microscope. The mean number of GFP-positive syncytia per 1,000 cells is reported for triplicate wells. Donor cells that did not express Env were included as negative controls. Fusion with $CD4^+$-enriched T lymphocytes as target cells was performed similarly except that Tat was not included in the transfection and that the counting of syncytia was done under a light microscope.

Cell surface labeling of envelope glycoproteins. 293T cells were transfected with Env expression vectors and reseeded the next day at 10^5 cells per well in a 24-well plate. Pooled HIV sera were preadsorbed on untransfected 293T cells and diluted 1:10 in PBS. On day 2 posttransfection, each well was incubated with 100 μl of diluted HIV sera for 30 min at room temperature (RT). PBS (900 μl) with 1 mM EDTA was then added to each well. The cells were dissociated by pipetting, transferred into microcentrifuge tubes, and washed three times in PBS-EDTA to remove antibodies that had not attached to the cell surface. Cells were then resuspended in 100 μl of kinase extraction buffer (KEB) containing 50 mM Tris, pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% Nonidet P-40 (NP-40) detergent, and 1 μg of each of the protease inhibitors aprotinin, leupeptin, and Petablock/ml. The protein extracts were incubated for 10 min on ice, clarified by spinning for 10 min at 4°C in a microcentrifuge and immunoprecipitated by overnight incubation at 4°C with 50 μl of protein G-Sepharose (Pharmacia, Uppsala, Sweden). The immunoprecipitates were washed four times in 1 ml of KEB and resuspended in sodium dodecyl sulfate loading buffer for analysis by immunoblotting. Samples were run on a 4 to 12% polyacrylamide gradient gel (Novex, San Diego, Calif.) and immunoblotted with goat anti-gp120 serum Env2-3 (Chiron) as previously described (38). The total envelope glycoprotein content of transfected cells was assessed by direct lysis of 293T cells in 100 μl of KEB followed by protein extraction, incubation of the extracts with 5 μl of preadsorbed pooled HIV sera for 2 h at 4°C, addition of 50 μl of protein G-Sepharose, and overnight incubation at 4°C. The samples were then washed and immunoblotted as described above.

Infections with GFP reporter viruses. The HIV-1 R7/3-EGFP, a proviral HXB2 clone that contains a GFP reporter gene inserted in place of *nef*, was a gift from Mark Muesing (Aaron Diamond AIDS Research Center, New York, N.Y.). This clone was constructed by inserting the enhanced GFP (EGFP) coding sequence (Clontech, Palo Alto, Calif.) between nucleotides 8797 and 9516 of HIV-1 R7/3 so that the *nef* ATG was converted to the ATG of the EGFP gene (numbering is that of HXB2). To replace the HIV-1 R7/3-EGFP *env* gene with that of $HIV\text{-}1_{SF33}$, the *Bbs*I-*Bam*HI fragment of clone R7/3 (nucleotides 6219 to

8475) was exchanged with corresponding sequences in HIV-1_{SF33}. The *Bam*HI site, which was not conserved in HIV-1_{SF33}, first had to be introduced at position 2720 by site-directed mutagenesis (numbering refers to sequence M38427 in GenBank). The mutation was made using the QuickChange kit (Stratagene, San Diego, Calif.) and the complementary mutagenic oligonucleotides SF33 BF (5′-CGA TTA GTG AAC GGA TCC TTA GCA CTG TTC TGG G-3′) and SF33 BR. The resulting proviral construct, HIV-1 R7/3-33, expressed an envelope glycoprotein identical to that of HIV-1_{SF33} except for the last 106 amino acids of the cytoplasmic domain, which originated from the R7/3-EGFP clone. A similar contruct, HIV-1 R7/3-33A2, was obtained by replacing the *Bbs*I-*Bam*HI fragment in R7/3-EGFP with corresponding sequences from $SHIV_{SF33A2}$.

For infections with the GFP reporter viruses, 1.2×10^7 $CD4^+$ enriched human PBMC that had been stimulated with the mitogen phytohemagglutinin for 3 days were infected with 200 ng of p24 Gag equivalent for 3 h at 37°C, washed, distributed in 6 wells of a 24-well plate, and cultivated in 1 ml of RPMI 1640 medium per well supplemented with 10% FCS and 20 U of interleukin-2/ml. The number of infected cells that expressed GFP was determined every 2 to 3 days by flow cytometry. Cells were resuspended in 100 μl of PBA buffer (PBS plus 1% bovine serum albumin and 10 mM NaN_3), incubated for 15 min at RT in the presence of an anti-CD4 allophycocyanin-labeled antibody (Exalpha, Boston, Mass.), washed in 3 ml of PBA, and resuspended in PBA with 1% paraformaldehyde. Analyses were performed using a FACSCalibur flow cytometer with the Cellquest software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Negative controls for GFP labeling consisted of cells infected with mock supernatants. Negative controls for CD4 labeling were obtained by incubating cells with an APC-labeled IgG1 isotypic control antibody.

Antibody binding to the soluble envelope glycoprotein. The binding of human MAbs and of IgG-CD4 to secreted envelope glycoproteins was assessed by ELISA. Supernatants of 293T cells transfected with the pEnv33, pEnvA2, and pEnvA5 vectors were incubated overnight at RT on ELISA plates precoated with D6205, a sheep polyclonal antibody directed against the 15 carboxy-terminal amino acids of gp120 (International Enzymes, Fallbrook, Calif.). Plates were washed in Tris-buffered saline (TBS; 144 mM NaCl, 25 mM Tris pH 7.5) and incubated with serial dilutions of MAbs or IgG-CD4 in TBS-MF (TBS supplemented with 4% dry milk and 10% FCS). After 3 h of incubation at RT, plates were washed in TBS, incubated overnight at 4°C with a 1:10,000 dilution of goat anti-human IgG serum conjugated to alkaline phosphatase (Zymed, San Francisco, Calif.) in TBS-MF, and revealed with the AMPAK kit according to the manufacturer's recommendations (Dako, Cambridgeshire, United Kingdom). Quantitation of the antibodies bound to gp120 was obtained by measuring the optical density at 485 nm (OD_{485}) in an absorbance microassay reader. All the supernatants tested were first normalized for envelope glycoprotein content by measuring their ELISA reactivity against a pool of patient sera that had a broad anti-HIV reactivity (54).

Antibody binding to virions. Virions were purified by ultracentrifugation of SHIV culture supernatants through a 32% sucrose cushion (54). For normalization of envelope glycoprotein content, virion preparations were lysed in 1% NP-40 and tested for their ELISA reactivity against pooled HIV sera, as described above. The binding of MAbs to intact virions was measured as described by Stamatatos and Cheng-Mayer (53). Briefly, increasing concentrations of MAbs diluted in RPMI 1640 with 10% FCS were incubated with the virion preparations for 3 h at RT. The virion-MAb complexes were then separated from unbound MAbs by pelleting the complexes at $15{,}000 \times g$ for 90 min at 4°C. The pellets were resuspended in 100 μl of TBS-MF with 1% NP-40. The detergent lysed the virions but did not disrupt the gp120-MAb complexes, which were then captured on a 96-well plate coated with antibody D6205. The MAbs bound to gp120 were then quantitated by ELISA as described above. Negative controls consisted of mock virion preparations derived from uninfected cell supernatants and of samples incubated in the absence of MAbs. The binding of certain anti-gp120 antibodies can induce the dissociation of gp120 from the virion surface (45, 55), which could lead to an underestimation of the amount of virion-bound antibody in the assay. To control for possible gp120 dissociation, supernatants recovered after the centrifugation of virion-MAb complexes were tested in the same ELISA as the pellets. The shedding of gp120 was minimal, except when virions were incubated with a combination of the 17b MAb and sCD4. In this case, OD values obtained for virion pellets and supernatants were added and reported on Fig. 8D.

Nucleotide sequence accession number. The sequence of the $SHIV_{SF33A5}$ *Dra*III-to-*Mun*I fragment has been deposited in GenBank under accession no. AF401229.

RESULTS

Sequences of $SHIV_{SF33A}$ envelope glycoproteins. The original $SHIV_{SF33}$ construct was obtained by inserting the *env*, *vpu*, *tat*, and *rev* open reading frames from X4 clone HIV-1_{SF33} within the SIVmac239 genetic backbone (37). Two SHIV molecular clones were engineered by replacing the Env V1-to-V5 region of $SHIV_{SF33}$ with the equivalent region amplified from $SHIV_{SF33A}$-infected rhesus macaques. Pathogenic molecular clone $SHIV_{SF33A2}$ has been described previously (20). A second clone, derived from animal Rh1546 and designated $SHIV_{SF33A5}$, also proved to be pathogenic by the intravenous route (J. Harouse and C. Cheng-Mayer, unpublished data). The *env* V1-to-V5 regions of $SHIV_{SF33A2}$ and $SHIV_{SF33A5}$ differed from that of the parental $SHIV_{SF33}$ by 25 and 29 residues, respectively (Fig. 1). The envelopes of the two pathogenic clones were derived from different animals but were similar except for seven residues, four of them clustered in the V5 region. Changes common to both clones included the loss of a potential N-linked glycosylation site in the V1 variable loop, the repositioning of such a site in the V2 loop, and the gain of a third glycosylation site at the base of the V3 loop. We have previously shown that the potential glycosylation changes in V1 and V3 contributed to the neutralization-resistant phenotype of $SHIV_{SF33A}$ (4, 38). Of note, the set of 22 mutations common to clones $SHIV_{SF33A2}$ and $SHIV_{SF33A5}$ were also present in *env* genes amplified directly from Mmu 25814, the animal from which the $SHIV_{SF33A}$ isolate was originally recovered (36). The conservation of this particular set of mutations upon in vivo passage points to their importance in determining the pathogenic phenotype.

$SHIV_{SF33A}$ envelope glycoproteins confer increased viral entry. The properties of the envelope glycoproteins derived from the two pathogenic clones, EnvA2 and EnvA5, were compared to those of the original $SHIV_{SF33}$ envelope glycoprotein, Env33. Pseudotyped viruses obtained by cotransfection of an HIV-1–luciferase reporter genome with different gp160 expression vectors were used to assess viral entry in a single-cycle infection assay. Measurement of luciferase activity showed that EnvA2 and EnvA5 conferred a threefold increase in viral entry over Env33 in HOS-CD4-CXCR4 target cells (Fig. 2). An entry increase of the same magnitude was observed in HeLa-$CD4^+$ cells expressing endogenous CXCR4 (data not shown). None of the envelope glycoproteins could mediate entry of HOS-$CXCR4^+$ cells that did not express CD4, indicating that the pathogenic $SHIV_{SF33A}$ clones retained a CD4-dependent mode of entry. Transfection of increasing amounts of a CD4 expression vector in HOS-CXCR4 cells caused a progressive increase in entry mediated by the three envelope glycoproteins (Fig. 3A). The entry advantage conferred by EnvA2 and EnvA5 was observed at high CD4 concentrations but was more limited when CD4 expression levels were low. This was more apparent when analyzing the ratio of EnvA2- or EnvA5-mediated entry to Env33-mediated entry (Fig. 3B). We verified that transfecting increasing amounts of CD4 plasmid resulted in a higher percentage of cells expressing CD4 and also in a linear increase in the mean CD4 fluorescence intensity per cell (data not shown). In additional experiments, the advantage in entry conferred by EnvA2 and EnvA5 was observed in U87 astroglioma cells that expressed high CD4 and high CXCR4 levels

```
          gp120 →                                                                                    100
SHIVSF33  MRARETRKNYQCLWRWGTMLLGMLMICSAAENLWVTVYYGVPVWKDATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLGNVTENFNMWKNNMV

                                DraIII              V1          *                    V2        *          200
SHIVSF33  DQMHEDIVSLWDQSLKPCVKLTPLCVTLNCTDYLGNATNTNNSSGGTVEKEEIKNCSFNITTGIRDKVQKAYAYFYKLDVVPIDDDNTNTSYRLIHCNSS
SHIVSF33A2                      ..............S......E........H.......A...R...E......H..........S...........>
SHIVSF33A5                      ..............S......E........H.......A...R...E......H..........S...........>

                                                                                                       300
SHIVSF33  VITQTCPKVSFEPIPIHYCAPAGFAILKCNNKKFSGKGQCTNVSTVQCTHGIKPVVSTQLLLNGSLAEEEVVIRSDNFTNNAKTILVQLNVSVEINCTRP
SHIVSF33A2 ....A.........................D.......R.....................................V..................>
SHIVSF33A5 ....A.........................D.......R.....................................V........V..................>

          *          V3                                                                          V4      400
SHIVSF33  NNNRRRRITSGPGKVLYTTGEIIGDIRKAYCNISRAKWNKTLEQVATKLREQFGNKTIVFKQSSGGDPEIVMHSFNCRGEFFYCNTTKLFNSTWNENSTW
SHIVSF33A2 ..YT.K.......R.............H.....................R....E..R...........N...G.....................>
SHIVSF33A5 ..YT.K.......R.............H.....................R....E..R...........N...G.....................>

                                                        *  V5          MunI                            500
SHIVSF33  NATGNDTITLPCRIKQIINMWQEVGKAMYAPPIEGQIRCSSNITGLLLTRDGGGDKNSTTEIFRPAGGNMKDNWRSELYKYKVVKIEPLGVAPTKAKRRV
SHIVSF33A2 ..................................................NNG.................                          >
SHIVSF33A5 .................V.................P.................VGT.N..............                          >
```

FIG. 1. Alignment of surface envelope glycoprotein sequences. Predicted gp120 amino acid sequences are compared. $SHIV_{SF33A2}$ and $SHIV_{SF33A5}$ differ from $SHIV_{SF33A}$ only in the V1-to-V5 region, which is encompassed by the *Dra*III-to-*Mun*I restriction fragment. Dots, amino acid identity; stars, changes in predicted N-linked glycosylation sites.

but was abolished in U87 cells with low CD4 and high CXCR4 expression levels (data not shown). These findings suggested that the original HIV-1_{SF33} envelope protein interacted efficiently with CD4 at limiting receptor concentrations and that the entry advantage conferred by the pathogenic SHIV envelope glycoproteins was not explained by better CD4 utilization.

A U87-CD4 cell line with low endogenous CXCR4 expression was chosen to test the effect of increasing CXCR4 concentration on entry (Fig. 3C). The cells were lipofected with a constant amount of CD4 plasmid (0.3 μg) and with increasing amounts of CXCR4 plasmid (0.0001 to 0.3 μg). The CD4 plasmid was included in the transfection to ensure that CD4 expression levels would not be limiting. Entry of the pseudotyped viruses increased with CXCR4 expression levels and rapidly plateaued, indicating that optimal entry could be obtained at relatively low coreceptor expression levels. The entry advantage conferred by EnvA2 and EnvA5 was observed at all CXCR4 expression levels above baseline (Fig. 3D) and thus did not appear to depend on the concentration of coreceptor molecules at the cell surface. This finding was confirmed by the fact that EnvA2 and EnvA5 conferred threefold-better entry in sorted HOS-CD4-CXCR4 clones that expressed either low or high CXCR4 levels (data not shown).

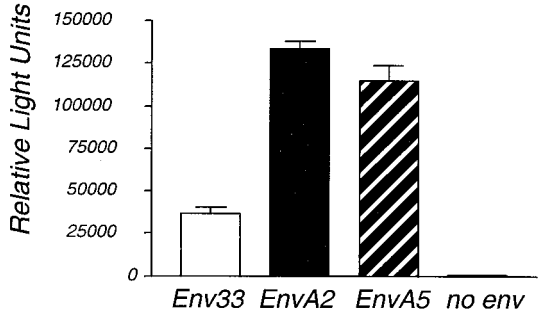

FIG. 2. Comparison of viral entry mediated by the SHIV envelope glycoproteins. Reporter HIV-1 viruses carrying the luciferase gene and pseudotyped with the SHIV envelope glycoproteins Env33, EnvA2, and EnvA5 were assayed for entry into HOS-CD4-CXCR4 cells. Entry is determined by measuring luciferase activity, expressed in relative light units. Error bars, standard errors of the means obtained for triplicate wells.

Fusogenic capacity of $SHIV_{SF33A}$ envelope glycoproteins. Fusion assays were performed by transfecting 293T cells with gp160 expression vectors and overlaying these cells with a CEMx174 indicator cell line transduced with an LTR-GFP reporter cassette. The numbers of GFP^+ syncytia induced by EnvA2 and EnvA5 were three- to fourfold higher than that obtained with Env33 (Fig. 4A). The pathogenic $SHIV_{SF33A}$ envelope proteins also conferred a twofold increase in fusion with primary CD4-enriched human PBMC (Fig. 4B). We verified by Western blotting that the three envelope proteins were expressed to equivalent amounts in 293T cell lysates (Fig. 4C, left). The ratios of gp160 to gp120 were comparable and did not reveal any difference in envelope glycoprotein processing. In addition, comparable amounts of the three envelope glycoproteins were detected at the cell surface (Fig. 4C, right). Thus, increased fusogenicity was an intrinsic property of the $SHIV_{SF33A}$ envelope glycoproteins rather than a consequence of differences in expression or maturation.

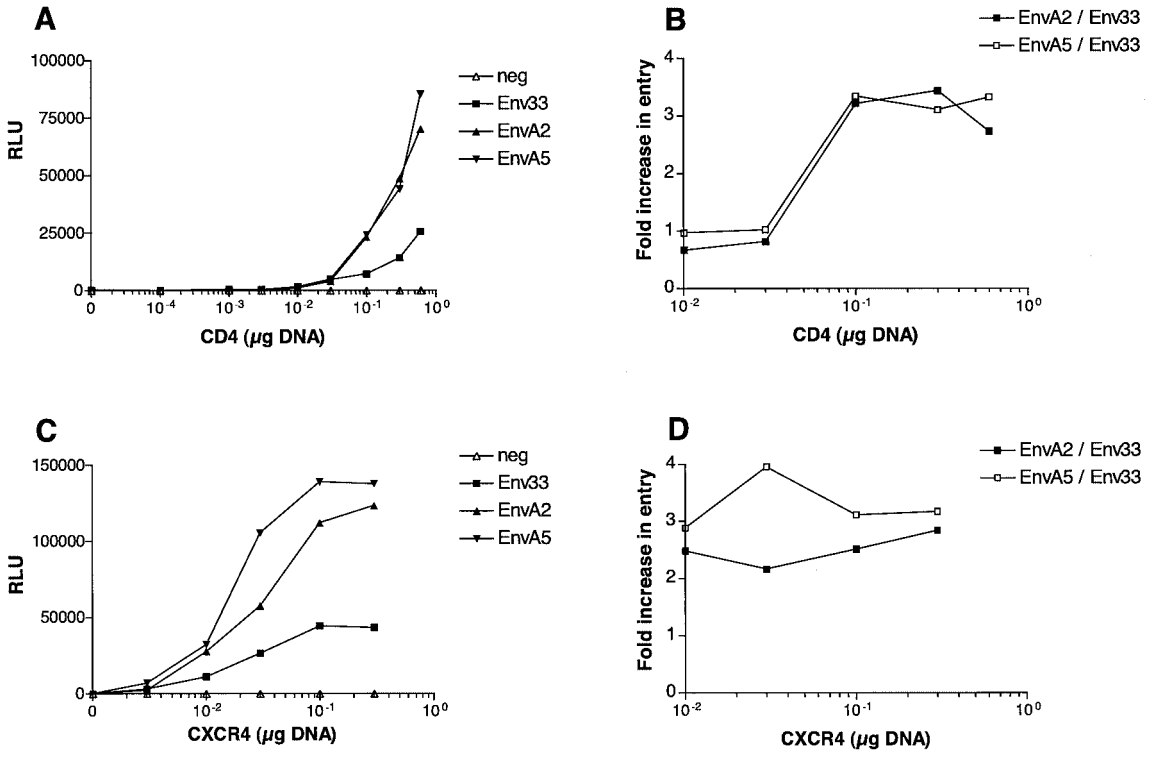

FIG. 3. Dependency of viral entry on CD4 and CXCR4 expression. (A) HOS-CXCR4 cells were transfected with increasing amounts of CD4 expression plasmid before being infected with pseudotyped HIV-1–luciferase viruses. Entry mediated by envelope glycoproteins Env33, EnvA2, and EnvA5 is determined by measuring luciferase activity expressed in relative light units (RLU). (B) Entry advantage conferred by $SHIV_{SF33A}$ envelope glycoproteins as a function of CD4 expression. The data are expressed as the fold increase in entry mediated by EnvA2 or EnvA5 compared to that mediated by Env33. (C) U87-CD4-pBABE cells were transfected with increasing amounts of CXCR4 expression plasmid and a constant amount of CD4 expression plasmid before being infected with pseudotyped HIV-1–luciferase viruses. (D) Entry advantage conferred by $SHIV_{SF33A}$ envelope glycoproteins as a function of CXCR4 expression.

Cytopathic effect induced by $SHIV_{SF33A}$ envelope glycoproteins. The quantitation of dead cells by trypan blue exclusion in the course of fusion assays showed that EnvA2 and EnvA5 were more cytopathic for CD4-enriched human PBMC than Env33 was (Fig. 4D). To analyze the cytopathic effect conferred by the $SHIV_{SF33A}$ envelope glycoproteins in the context of a replication-competent virus, we introduced the EnvA2 and Env33 genes into the genome of HIV-1 R7/3-GFP, an HXB2-based GFP reporter virus. The kinetics of infection of CD4-enriched human PBMC was monitored by determining the percentage of GFP^+ cells by flow cytometry. The clones expressing the $SHIV_{SF33A2}$ envelope glycoprotein (R7/3-33A2) showed a marked replicative advantage over those expressing the $SHIV_{SF33}$ envelope glycoprotein (R7/3-33) (Fig. 5A). In addition, only the R7/3-A2 clones significantly depleted $CD4^+$ T lymphocytes in culture (Fig. 5B). Interestingly, the mean GFP fluorescence was consistently two times lower in R7/3-33A2-infected cells than in R7/3-33-infected cells (Fig. 5C). This suggested that the average level of viral expression per infected cell was lower for R7/3-33A2, possibly because this virus killed its target cell more rapidly. This observation was consistent with the fact that R7/3-33A2 depleted $CD4^+$ cells more efficiently. Measurement of the p24 Gag antigen released in the culture supernatants confirmed the increased replication capacity of the R7/3-33A2 clones (Fig. 5D). The replicative advantage detected by this method was lower than that measured by the percentage of GFP^+ cells (compare Fig. 5A and D), which agreed with the notion that the R7/3-33A2 infection was more cytopathic, a property that would limit the amount of viral antigen produced per infected cell. Taken together, these data showed that expression of EnvA2 led to increased viral replication and cytopathogenicity in $CD4^+$ T lymphocytes.

Neutralization pattern conferred by $SHIV_{SF33A}$ envelope glycoproteins. HIV-1 viruses expressing luciferase and pseudotyped with EnvA2 and EnvA5 were resistant to neutralization by $SHIV_{SF33}$ serum, while viruses carrying Env33 were neutralized at serum dilutions as high as 1:10,000 (Fig. 6A). EnvA2- and EnvA5-expressing viruses were also more resistant to neutralization by heterologous HIV sera (data not shown) and by IgG1b12, a MAb directed against a conserved epitope overlapping with the CD4 binding site (Fig. 6B). The two viruses could still be neutralized by a CD4-IgG fusion protein (Fig. 6C), but the 50% inhibitory concentrations measured for EnvA2 and EnvA5 (1.3 to 1.8 μg/ml) were higher than that obtained with Env33 (0.6 μg/ml). The pattern of increased neutralization resistance conferred by EnvA2 and EnvA5 was also observed when viruses were incubated with 17b, a MAb directed to a CD4-induced (CD4i) epitope that overlaps with the coreceptor binding site (Fig. 6D). Incubation of the pseudotyped viruses with sCD4 markedly increased 17b-medi-

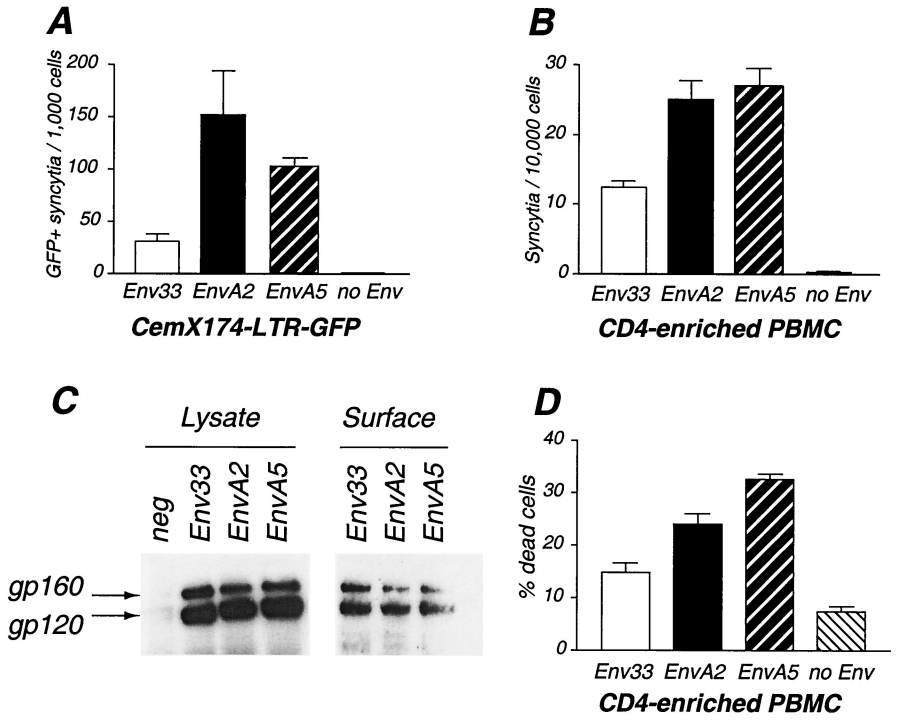

FIG. 4. Fusogenic capacity, cytopathicity, and expression of SHIV envelope glycoproteins. (A) Fusion with CEMx174 cells. 293T cells cotransfected with Env expression vectors and a Tat expression vector were overlaid with indicator cell line CEMx174-LTR-GFP, clone 5.25. Fusion was monitored by counting GFP^+ syncytia per 1,000 cells. (B) Fusion with human PBMC. 293T cells transfected with Env expression vectors were overlaid with CD4-enriched human PBMC. Fusion was monitored by counting the number of syncytia per 10,000 cells. (C) Expression of SHIV envelope glycoproteins in transfected 293T cells. The total amount of Env produced was detected by immunoprecipitation and immunoblotting of cell lysates (left); the expression of Env at the cell surface was detected by incubation of intact cells with pooled HIV sera, followed by extensive washing, immunoprecipitation, and immunoblotting (right). Envelope glycoprotein precursor gp160 and processed surface envelope glycoprotein gp120 are indicated. (D) Cytopathic effect induced by SHIV envelope glycoproteins. 293T cells transfected with Env expression vectors and CD4-enriched human PBMC were allowed to fuse for 16 h. The number of dead cells was determined by the trypan blue exclusion method. Error bars (A, B, and D), standard errors of the means for triplicate wells.

ated neutralization of Env33-carrying virions, consistent with the idea that interaction with CD4 induced envelope conformational changes that exposed the 17b epitope (Fig. 6E). The effect of sCD4 incubation on EnvA2- and EnvA5-carrying virions was much more limited, since >90% neutralization was not achieved at the 17b concentrations tested. These findings suggested that conformational changes triggered by the binding of CD4 to the pathogenic $SHIV_{SF33A}$ envelope glycoproteins were qualitatively different. We noticed that partial neutralization (20 to 50%) of EnvA2- and EnvA5-carrying virions could be achieved at low 17b concentrations (between 0.2 and 0.5 μg/ml) but that the neutralization percentage consistently decreased at intermediate concentrations (ranging between 0.5 and 2 μg/ml), before reincreasing at higher concentrations. This effect was reproducible in three sets of experiments (data not shown) and was also observed when neutralization was performed on target cells that expressed the rhesus macaque CXCR4 molecule instead of the human CXCR4 molecule (Fig. 6F). A possible interpretation was that 17b molecules at low concentration cross-linked gp120 monomers within a trimer and prevented the cooperative process of fusion, while 17b molecules at a saturating concentration independently bound each monomer, avoiding cross-linking and leaving the envelope glycoproteins competent for entry. Taken together, the data showed that $SHIV_{SF33A}$ envelope glycoproteins conferred a broad neutralization resistance similar to that observed for primary HIV-1 isolates. However, partial neutralization at low 17b concentrations suggested that the 17b epitope could be exposed on $SHIV_{SF33A}$ envelope glycoproteins.

Affinity of $SHIV_{SF33A}$ envelope glycoproteins for CD4. The binding of SHIV envelope proteins to the CD4 receptor was evaluated by incubating soluble envelope glycoproteins with the IgG-CD4 fusion protein. Culture supernatants from 293T cells transfected with the different envelope expression vectors were normalized for envelope glycoprotein content and then tested for their reactivity with IgG-CD4 by ELISA. EnvA2, EnvA5, and Env33 gp120 bound IgG-CD4 with similar affinities (half-maximal binding concentration between 0.1 and 0.2 μg/ml), though a limited advantage was detected for Env33 at saturating IgG-CD4 concentrations (Fig. 7A). Since the conformation of soluble Env monomers does not exactly reflect that of native Env oligomers at the surface virions (43, 53, 61), further experiments to determine the affinity of virion-bound $SHIV_{SF33A}$ envelope glycoproteins for CD4 were performed. Viral particle preparations normalized for envelope content were incubated with IgG-CD4, pelleted at high speed to remove excess antibody, and lysed in mild detergent. The resulting envelope–IgG-CD4 complexes were captured on an ELISA

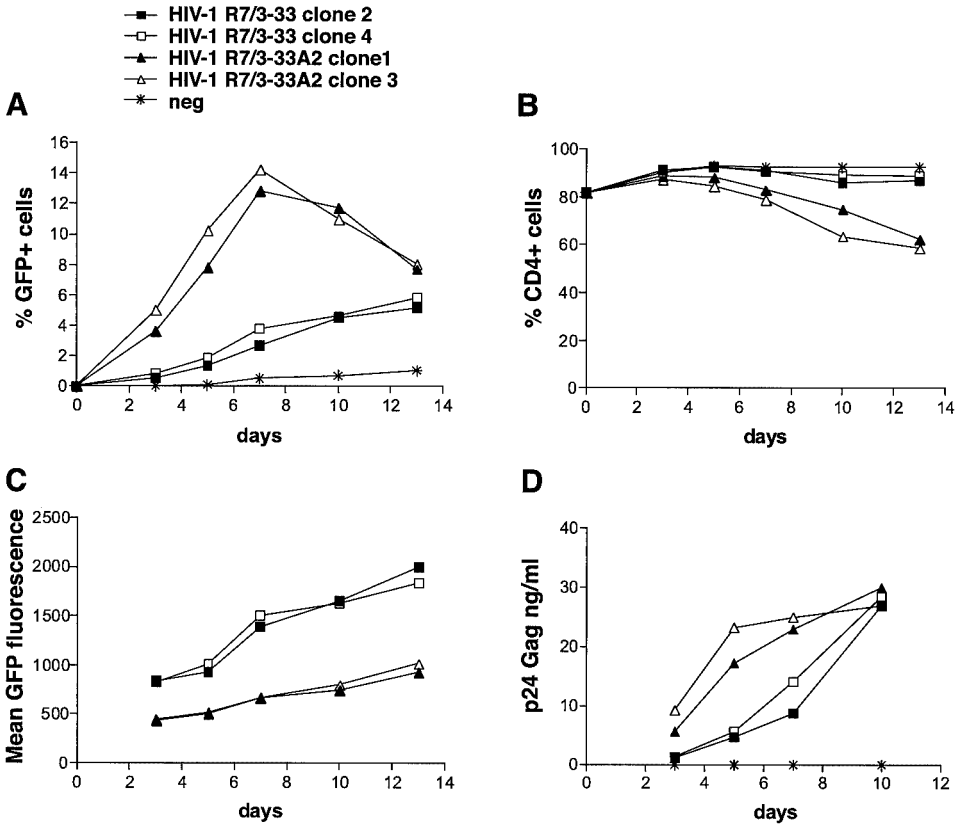

FIG. 5. Infections with HIV-1–GFP reporter viruses expressing SHIV envelope glycoproteins. (A) CD4-enriched human PBMC were infected with the progeny of two HIV-1 clones expressing Env33 (R7/3-33 clones 2 and 4) and two HIV-1 clones expressing EnvA2 (R7/3-33A2 clones 1 and 3). Infection was monitored by measuring the percentage of GFP^+ cells by flow cytometry. (B) The percentage of $CD4^+$ cells in the cultures was monitored by flow cytometry. Only the HIV-1 R7/3-33A2 clones caused a detectable $CD4^+$ cell depletion. (C) The level of GFP expression per infected cell was monitored by measuring the mean fluorescence intensity in the flow cytometer FL-1 channel. (D) The release of viral particles in the culture supernatants was measured with an ELISA that detected the p24 Gag antigen.

plate through a MAb directed against the C terminus of gp120 and quantified with an anti-human IgG antibody. The three SHIV particle preparations were found to bind IgG-CD4 equally well, with 50% binding values of 0.1 to 0.2 μg/ml, which were equivalent to those obtained with soluble envelope proteins (Fig. 7B). Thus, the increased entry conferred by pathogenic $SHIV_{SF33A}$ envelope glycoproteins did not result from a more efficient interaction with CD4.

Binding of $SHIV_{SF33A}$ envelope glycoproteins to the 17b MAb. To further probe the structure of SHIV envelope proteins and determine whether CD4i epitopes could be exposed, we performed binding experiments with the 17b human MAb. The soluble forms of EnvA2 and EnvA5 bound 17b with higher affinity than Env33 did, with half-maximal binding values of 0.05 to 0.07 μg/ml for EnvA2 and EnvA5 versus 0.3 μg/ml for Env33 (Fig. 8A and B). Binding experiments with purified viral particles confirmed the preferential binding of 17b to pathogenic $SHIV_{SF33A}$ envelope glycoproteins, even in the context of native oligomeric envelope spikes (Fig. 8C). Remarkably, 17b could efficiently bind the $SHIV_{SF33A2}$ and $SHIV_{SF33A5}$ particles in the absence of CD4. Adding increasing amounts of soluble CD4 increased only moderately the binding of 17b to $SHIV_{SF33A2}$ and $SHIV_{SF33A5}$ particles but had a marked effect on the binding to $SHIV_{SF33}$ (Fig. 8D). These experiments demonstrated that the 17b epitope was exposed on pathogenic $SHIV_{SF33A}$ particles prior to the conformational change triggered by the Env-CD4 interaction. Thus, $SHIV_{SF33A}$ envelope glycoproteins folded in a particular conformation that exposed conserved regions involved in coreceptor binding.

DISCUSSION

This study showed that changes in the surface envelope glycoprotein were responsible for the increased infectivity, fusion capacity, cytopathicity, and neutralization resistance that characterized $SHIV_{SF33A}$ molecular clones. This set of properties was associated with an altered conformation of the envelope glycoprotein that exposed regions overlapping with the coreceptor binding site. We propose that the particular conformation of the $SHIV_{SF33A}$ envelope glycoprotein facilitates the interaction with the coreceptor or the subsequent triggering of the fusion intermediate, thus explaining the increase in fusion efficiency. A more efficient fusion process can in turn account for the increased viral entry characteristic of the pathogenic clones. Indeed, the $SHIV_{SF33A}$ envelope glycoproteins generated threefold more syncytia than the parental $SHIV_{SF33}$ envelope glycoprotein, an increase of the same magnitude as the one determined for viral entry. A more efficient

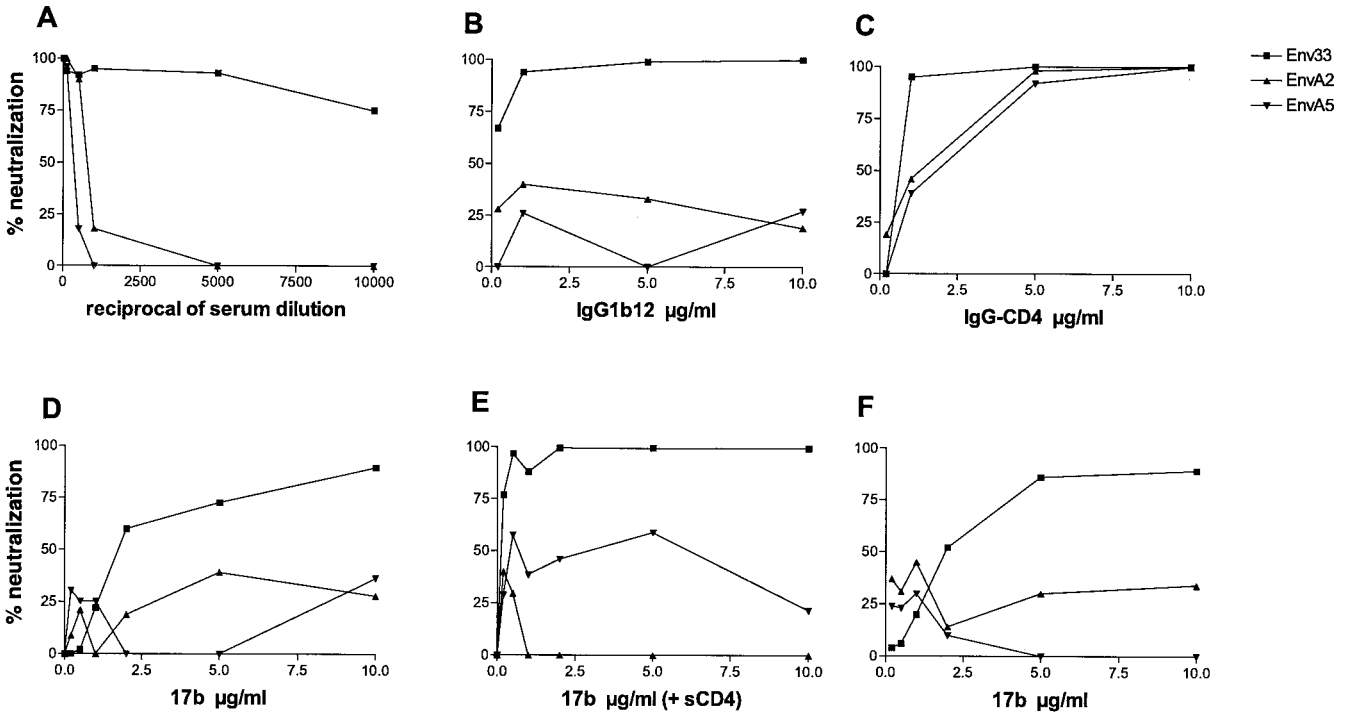

FIG. 6. Neutralization of pseudotyped viruses expressing SHIV envelope glycoproteins. HIV-1–luciferase viruses pseudotyped with Env33, EnvA2, and EnvA5 were incubated with serial dilutions of antibodies before being added to HOS-CD4-CXCR4 target cells. Viral entry was monitored by measuring the luciferase activity at day 3 postinfection. Neutralization was determined by measuring the percentages of entry inhibition at different antibody concentrations. Antibodies used in neutralization assays: $SHIV_{SF33}$ serum from animal 25814 at week 52 (A); MAb IgG1b12, which recognizes the CD4 binding site (B); the IgG-CD4 chimeric protein (C); MAb 17b, which recognizes a CD4i epitope (D); MAb17b in the presence of 0.02 µg of sCD4/ml (E); and MAb 17b (F). For panel F, target cells expressed rhesus macaque CXCR4 instead of human CXCR4.

viral entry translated into an increased replicative capacity in primary T cells, as demonstrated by the use of HIV-GFP repor- ter viruses expressing the different envelope glycoproteins. Though the $SHIV_{SF33A}$ envelope glycoprotein led to higher viral replication, it was also responsible for a lower level of viral expression per target cell, as indicated by a low mean GFP fluorescence of infected cells. A simple interpretation is that more-fusogenic envelope glycoproteins lead to rapid cell

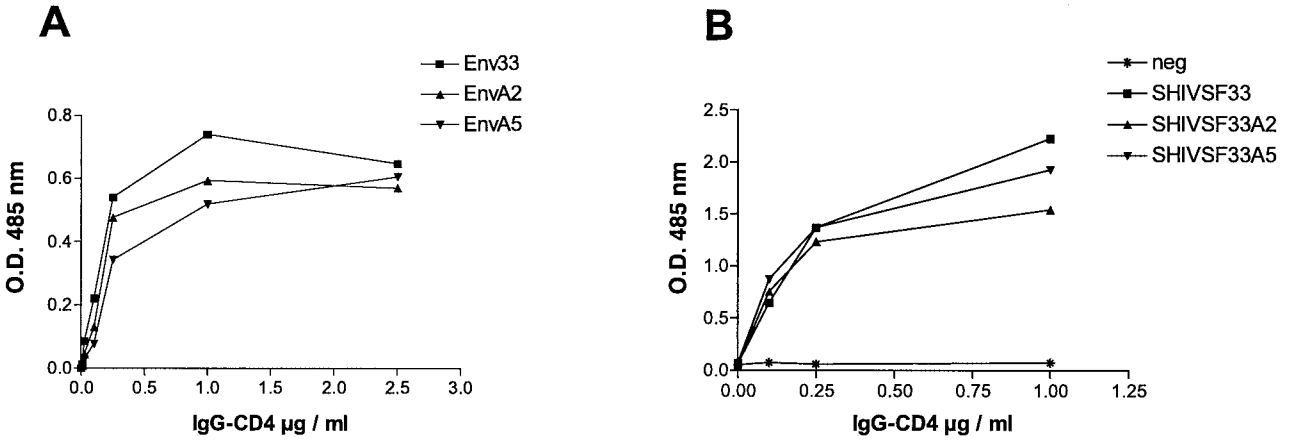

FIG. 7. Binding of IgG-CD4 to soluble and virion-associated SHIV envelope glycoproteins. (A) Binding of chimeric protein IgG-CD4 to soluble SHIV gp120. Supernatants of cells transfected with Env expression vectors were normalized for gp120 content and then incubated with increasing IgG-CD4 concentrations. The gp120–IgG-CD4 complexes were captured on a D6205-coated plate and quantitated by ELISA. (B) Binding of IgG-CD4 to the surface of SHIV virions. Sucrose-purified virion preparations were incubated with increasing concentrations of IgG-CD4. The virion–IgG-CD4 complexes were separated from unbound IgG-CD4 by centrifugation. Pelleted viruses were lysed in 1% NP-40, which did not disrupt the gp120-CD4 complexes. The lysates were added to D6205-coated plates, and the amount of gp120 bound to CD4 was quantitated by ELISA. neg, mock virion preparation. The results are representative of at least three independent experiments.

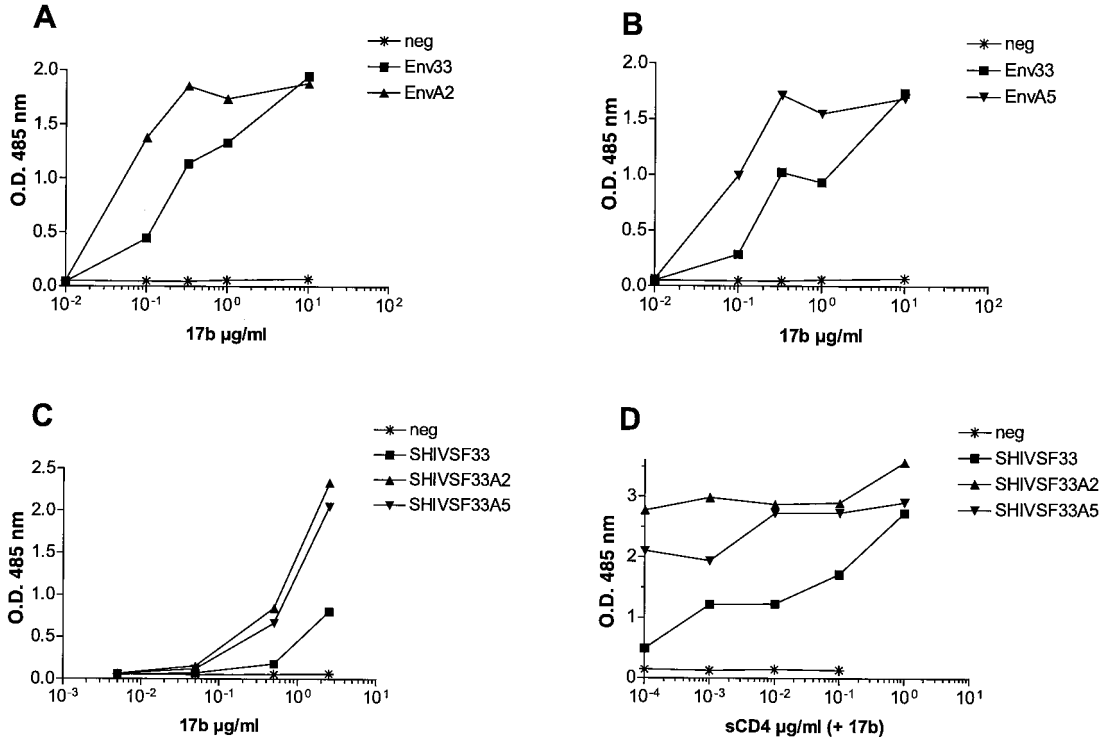

FIG. 8. Binding of MAb 17b to soluble and virion-associated SHIV envelope glycoproteins. (A and B) Binding of MAb 17b, which recognizes a CD4i epitope, to soluble SHIV gp120. Supernatants of cells transfected with Env expression vectors were normalized for gp120 content and then incubated with increasing 17b concentrations. The gp120-17b complexes were captured on a D6205-coated plate and quantitated by ELISA. neg, supernatant from cells transfected with an empty vector (C) Binding of MAb 17b to the surfaces of SHIV virions. Sucrose-purified virion preparations were incubated with increasing concentrations of MAb 17b. The virion-17b complexes were separated from unbound 17b by centrifugation. Pellets were lysed in 1% NP-40 and then added to D6205-coated plates. The amount of gp120 bound to 17b was quantitated by ELISA. (D) Binding of MAb 17b to SHIV virions in the presence of sCD4. Purified virions were incubated with increasing concentrations of sCD4 and 0.5 μg of MAb 17b/ml. Since the combination of sCD4 plus 17b induced shedding of gp120 from the surfaces of virions, results shown are the sums of OD values obtained for pellets and supernatants following the centrifugation of virion-17b complexes (see Materials and Methods). neg (C and D), mock virion preparation. The results are representative of at least three independent experiments.

death, thus preventing the intracellular accumulation of viral products in large quantities. This notion is consistent with the fact that only the viruses expressing the $SHIV_{SF33A}$ envelope glycoprotein depleted $CD4^+$ T lymphocytes in culture. It is also in agreement with studies of SHIV-89.6P and SHIV-HXBc2P 3.2, which demonstrated a correlation between envelope glycoprotein-fusing capacity and the ability of these SHIVs to deplete $CD4^+$ T cells in vitro and in vivo (15, 16, 30, 33). Thus, converging evidence points to envelope glycoprotein-mediated fusion as a key determinant of SHIV virulence. For SHIV-KB9, a pathogenic clone derived from the SHIV-89.6P isolate, changes in the C2 region of gp120 and in the ectodomain of gp41 contributed cooperatively to the increased fusion capacity (16). This is in contrast to $SHIV_{SF33A}$, for which changes restricted to the V1-to-V5 region of gp120 were sufficient to render the envelope glycoprotein more fusogenic. It is possible that the gp41 subunit of the parental clone, $HIV\text{-}1_{SF33}$, which is derived from a tissue culture-adapted isolate, had already evolved toward a fusion-prone conformation during in vitro passage, while the HIV-1–89.6 gp41 had retained a less fusogenic conformation characteristic of primary viruses. This suggests that the critical amino acids that determine SHIV virulence vary depending on the origin of the envelope glycoprotein.

HIV-1 entry is an ordered process in which the envelope glycoprotein interacts sequentially with receptor CD4, coreceptor CCR5 or CXCR4, and the target cell lipid membrane (9, 61). In the currently accepted model, the binding of gp120 to CD4 triggers conformational changes that expose the highly conserved coreceptor binding site located on the bridging sheet, a domain that connects the inner and outer domains of the gp120 core and that overlaps with the 17b epitope (48, 58). Interaction with the coreceptor leads in turn to a major structural rearrangement of the gp41 subunits within the envelope glycoprotein trimer, resulting in a three-stranded coiled-coil fusion intermediate that exposes the hydrophobic fusion peptides. This intermediate then folds back on itself (to form a six-helix bundle), a transition that is thought to bring the viral and the cellular membranes in close proximity and allow them to fuse (12). Increased fusogenicity could result from a change in the efficiency of any of these steps. It was clear that $SHIV_{SF33A}$ envelope glycoproteins EnvA2 and EnvA5 did not fuse better because of a more efficient interaction with the CD4 receptor. On the contrary, there were indications that the

parental Env33 interacted more efficiently with CD4, even if we could not detect a major difference in CD4 binding affinity between the three envelope glycoproteins tested. In particular, the advantage in entry conferred by EnvA2 and EnvA5 was detected only in target cells that expressed high levels of CD4 but was abolished in conditions where CD4 was limiting. It is interesting that primary HIV-1 isolates show a stronger dependence on CD4 expression than tissue culture-adapted isolates (44). The pathogenic $SHIV_{SF33A}$, which evolved during an in vivo passage, may resemble primary HIV-1 isolate in terms of CD4 dependence.

Increased binding of the 17b antibody to the $SHIV_{SF33A}$ envelope glycoproteins indicates exposure of conserved structures that overlap with the coreceptor binding site (48, 58, 59). This conformation may facilitate the interaction with the coreceptor CXCR4 and may thus account for the increased fusion efficiency of $SHIV_{SF33A}$. The envelope glycoprotein of dualtropic clone SHIV-KB9 was shown to have a higher affinity for CCR5 than the parental SHIV-89.6 envelope glycoprotein, as determined by measuring the binding of recombinant gp120 to CCR5-expressing cells (30). The affinity for CCR5 correlated with the fusogenic capacity of SHIV-KB9-derived envelope glycoprotein mutants (16). Increased coreceptor binding may thus be a common feature of pathogenic SHIVs. It cannot be ruled out, however, that the conformation of the $SHIV_{SF33A}$ envelope glycoproteins facilitates a late step in fusion distinct from coreceptor binding. We did not observe that the advantage in entry conferred by EnvA2 and EnvA5 was proportionally higher at low CXCR4 expression levels, which would be expected if the affinity for CXCR4 was the limiting factor in entry. The affinity of the HIV-1 Env-CXCR4 interaction has proven difficult to measure and is thought to be low, with a dissociation constant in the 500 nM range (23). The Env-CD4 interaction is of higher affinity (in the 10 nM range) and is thought to be mainly responsible for the tethering of viral particles at the cell surface (9, 40). Thus, in the context of a CD4-dependent virus, the main function of the Env-CXCR4 interaction is likely to be the triggering of conformational changes that lead to fusion rather than attachment. An interesting hypothesis is that pathogenic SHIV envelope glycoproteins are in a pretriggered conformation that can more easily convert to the three-stranded coiled-coil fusion intermediate. A change in the kinetics of coiled-coil formation would have a major impact on the efficiency of entry, because fusion is a highly cooperative event and is thought to be rate limiting for entry (9). Mutant envelope glycoproteins with a lower energy requirement for fusion have been described in the well-characterized influenza virus model (52). Some of the associated mutations localize to surface envelope glycoprotein HA1 and appear to alter intersubunit contacts (8). Whether a parallel can be drawn with the envelope glycoproteins of pathogenic SHIVs requires further investigation.

Exposure of the 17b epitope in the absence of CD4 binding has been observed on several HIV-1 isolates and clones, most of them obtained after serial in vitro passages. For instance, the majority of CD4-independent HIV-1 isolates bind 17b more efficiently than their CD4-dependent counterparts (13, 24, 32). The envelope glycoprotein of the $HIV\text{-}1_{BORI\text{-}15}$ isolate, which was obtained by passage on microglial cells cultures that express little CD4, also displayed increased binding to 17b and increased fusogenicity (39). These in vitro-adapted isolates are particularly neutralization sensitive, one interpretation being that they adopt a more open conformation in which the V2 and V3 loops do not shield the coreceptor binding site (10, 31, 39). The characteristic of $SHIV_{SF33A}$ is to combine the properties of both CD4-independent isolates (i.e., 17b epitope exposure and increased fusogenicity) and of primary isolates (strong CD4 dependence and neutralization resistance). These unique characteristics likely result from the convergence of two selective forces, one favoring viruses that enter target cells more efficiently and the other favoring viruses that can escape the host immune response. The determinants that render SHIVs neutralization resistant have been partially elucidated. We have previously shown that changes in the distribution of N-linked glycans at the surfaces of the V1 and V3 loops contribute to $SHIV_{SF33A}$ neutralization resistance (4). In particular, the acquisition of a single N-linked glycan at the base of the V3 loop (amino acid 301 in SF33 gp120) was sufficient to confer resistance to autologous and heterologous HIV sera, as well as to MAbs directed against CD4 binding site and CD4i epitopes (38). The involvement of the variable loops in neutralization resistance is a theme common to pathogenic SHIVs and primary HIV-1 isolates (7, 17, 43, 51). Changes that contribute to neutralization resistance include the addition of N-linked carbohydrates that may shield underlying regions from antibodies but also structural rearrangements that relocate the V2 and V3 loops closer to the gp120 core (62). How the envelope glycoproteins can accommodate changes that confer both neutralization resistance and increased fusogenicity remains to be understood. It is important to determine whether the regions that mediate coreceptor binding in highly fusogenic SHIV differ from those in the parental viruses. In this respect, it is interesting that the 17b antibody can bind the surface of $SHIV_{SF33A}$ virions but that it only partially neutralizes the same virions. It is possible that 17b binding does not entirely block access to the coreceptor binding site on $SHIV_{SF33A}$ gp120 or that 17b binding can mimic the interaction with the coreceptor, which would allow the subsequent steps in fusion. The 17b antibody has been shown to enhance, rather than block, the entry of the HIV-1 YU2 isolate, which provides evidence that 17b-bound envelope glycoprotein oligomers can remain competent for entry (57).

In conclusion, this study demonstrates that changes in the V1-to-V5 region of the surface envelope glycoprotein are responsible for increases in fusogenicity, entry, cytopathicity, and neutralization resistance of $SHIV_{SF33A}$ molecular clones. These changes are associated with the spontaneous exposure of a CD4i epitope on the envelope glycoprotein oligomer, a conformation likely to facilitate fusion. Characterizing the structural determinants that allow SHIV envelope glycoproteins to become highly fusogenic while remaining neutralization resistant will be critical in understanding the basis of SHIV virulence.

ACKNOWLEDGMENTS

We thank Nathaniel Landau for the gift of numerous cell lines and plasmids, Mark Muesing for the R7/3 EGFP plasmid, James Robinson for the 17b antibody, and Dennis Burton for the IgG1b12 antibody. We are grateful to Leonidas Stamatatos for help with the virion binding experiments. We thank Ben Mulder for critical reading of the manuscript.

This work was supported by NIH grants AI41945 and CA72822 and by the Pasteur Institute (Paris, France).

REFERENCES

1. **Burton, D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, P. L. Nara, et al.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024–1027.
2. **Cayabyab, M., G. B. Karlsson, B. A. Etemad-Moghadam, W. Hofmann, T. Steenbeke, M. Halloran, J. W. Fanton, M. K. Axthelm, N. L. Letvin, and J. G. Sodroski.** 1999. Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXBc2). J. Virol. **73:** 976–984.
3. **Chen, Z., Y. Huang, X. Zhao, E. Skulsky, D. Lin, J. Ip, A. Gettie, and D. D. Ho.** 2000. Enhanced infectivity of an R5-tropic simian/human immunodeficiency virus carrying human immunodeficiency virus type 1 subtype C envelope after serial passages in pig-tailed macaques (*Macaca nemestrina*). J. Virol. **74:**6501–6510.
4. **Cheng-Mayer, C., A. Brown, J. Harouse, P. A. Luciw, and A. J. Mayer.** 1999. Selection for neutralization resistance of the simian/human immunodeficiency virus $SHIV_{SF33A}$ variant in vivo by virtue of sequence changes in the extracellular envelope glycoprotein that modify N-linked glycosylation. J. Virol. **73:**5294–5300.
5. **Cheng-Mayer, C., R. Liu, N. R. Landau, and L. Stamatatos.** 1997. Macrophage tropism of human immunodeficiency virus type 1 and utilization of the CC-CKR5 coreceptor. J. Virol. **71:**1657–1661.
6. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency type-1 in mononuclear phagocytes. Virology **206:**935–944.
7. **Crawford, J. M., P. L. Earl, B. Moss, K. A. Reimann, M. S. Wyand, K. H. Manson, M. Bilska, J. T. Zhou, C. D. Pauza, P. W. Parren, D. R. Burton, J. G. Sodroski, N. L. Letvin, and D. C. Montefiori.** 1999. Characterization of primary isolate-like variants of simian-human immunodeficiency virus. J. Virol. **73:**10199–10207.
8. **Daniels, R. S., J. C. Downie, A. J. Hay, M. Knossow, J. J. Skehel, M. L. Wang, and D. C. Wiley.** 1985. Fusion mutants of the influenza virus hemagglutinin glycoprotein. Cell **40:**431–439.
9. **Doms, R. W.** 2000. Beyond receptor expression: the influence of receptor conformation, density and affinity in HIV-1 infection. Virology **276:**229–237.
10. **Dumonceaux, J., C. Goujon, V. Joliot, P. Briand, and U. Hazan.** 2001. Determination of essential amino acids involved in the CD4-independent tropism of the X4 human immunodeficiency virus type 1 m7NDK isolate: role of potential N glycosylations in the C2 and V3 regions of gp120. J. Virol. **75:**5425–5428.
11. **Dunn, C. S., C. Beyer, M. P. Kieny, L. Gloeckler, D. Schmitt, J. P. Gut, A. Kirn, and A. M. Aubertin.** 1996. High viral load and CD4 lymphopenia in rhesus and cynomolgus macaques infected by a chimeric primate lentivirus constructed using the env, rev, tat, and vpu genes from HIV-1 Lai. Virology **223:**351–361.
12. **Eckert, D. M., and P. S. Kim.** 2001. Mechanisms of viral membrane fusion and its inhibition. Annu. Rev. Biochem. **70:**777–810.
13. **Edwards, T. G., T. L. Hoffman, F. Baribaud, S. Wyss, C. C. LaBranche, J. Romano, J. Adkinson, M. Sharron, J. A. Hoxie, and R. W. Doms.** 2001. Relationships between CD4 independence, neutralization sensitivity, and exposure of a CD4-induced epitope in a human immunodeficiency virus type 1 envelope protein. J. Virol. **75:**5230–5239.
14. **Endo, Y., T. Igarashi, Y. Nishimura, C. Buckler, A. Buckler-White, R. Plishka, D. S. Dimitrov, and M. A. Martin.** 2000. Short- and long-term clinical outcomes in rhesus monkeys inoculated with a highly pathogenic chimeric simian/human immunodeficiency virus. J. Virol. **74:**6935–6945.
15. **Etemad-Moghadam, B., D. Rhone, T. Steenbeke, Y. Sun, J. Manola, R. Gelman, J. W. Fanton, P. Racz, K. Tenner-Racz, M. K. Axthelm, N. L. Letvin, and J. Sodroski.** 2001. Membrane-fusing capacity of the human immunodeficiency virus envelope proteins determines the efficiency of $CD4^+$ T-cell depletion in macaques infected by a simian-human immunodeficiency virus. J. Virol. **75:**5646–5655.
16. **Etemad-Moghadam, B., Y. Sun, E. K. Nicholson, M. Fernandes, K. Liou, R. Gomila, J. Lee, and J. Sodroski.** 2000. Envelope glycoprotein determinants of increased fusogenicity in a pathogenic simian-human immunodeficiency virus (SHIV-KB9) passaged in vivo. J. Virol. **74:**4433–4440.
17. **Etemad-Moghadam, B., Y. Sun, E. K. Nicholson, G. B. Karlsson, D. Schenten, and J. Sodroski.** 1999. Determinants of neutralization resistance in the envelope glycoproteins of a simian-human immunodeficiency virus passaged in vivo. J. Virol. **73:**8873–8879.
18. **Harouse, J. M., A. Gettie, T. Eshetu, R. C. H. Tan, R. Bohm, J. Blanchard, G. Baskin, and C. Cheng-Mayer.** 2001. Mucosal transmission and induction of simian AIDS by CCR5-specific simian/human immunodeficiency virus $SHIV_{SF162P3}$. J. Virol. **75:**1990–1995.
19. **Harouse, J. M., A. Gettie, R. C. Tan, J. Blanchard, and C. Cheng-Mayer.** 1999. Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. Science **284:**816–819.
20. **Harouse, J. M., A. Gettie, R. C. H. Tan, T. Eshetu, M. Ratterree, J. Blanchard, and C. Cheng-Mayer.** 2001. Pathogenic determinants of the mucosally transmissible CXCR4-specific SHIVSF33A2 map to Env region. J. Acquir. Immune Defic. Syndr. **27:**222–228.
21. **Harouse, J. M., R. C. Tan, A. Gettie, P. Dailey, P. A. Marx, P. A. Luciw, and C. Cheng-Mayer.** 1998. Mucosal transmission of pathogenic CXCR4-utilizing SHIVSF33A variants in rhesus macaques. Virology **248:**95–107.
22. **Himathongkham, S., N. S. Halpin, J. Li, M. W. Stout, C. J. Miller, and P. A. Luciw.** 2000. Simian-human immunodeficiency virus containing a human immunodeficiency virus type 1 subtype-E envelope gene: persistent infection, $CD4^+$ T-cell depletion, and mucosal membrane transmission in macaques. J. Virol. **74:**7851–7860.
23. **Hoffman, T. L., G. Canziani, L. Jia, J. Rucker, and R. W. Doms.** 2000. A biosensor assay for studying ligand-membrane receptor interactions: binding of antibodies and HIV-1 Env to chemokine receptors. Proc. Natl. Acad. Sci. USA **97:**11215–11220.
24. **Hoffman, T. L., C. C. LaBranche, W. Zhang, G. Canziani, J. Robinson, I. Chaiken, J. A. Hoxie, and R. W. Doms.** 1999. Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein. Proc. Natl. Acad. Sci. USA **96:**6359–6364.
25. **Igarashi, T., C. R. Brown, Y. Endo, A. Buckler-White, R. Plishka, N. Bischofberger, V. Hirsch, and M. A. Martin.** 2001. Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): implications for HIV-1 infections of humans. Proc. Natl. Acad. Sci. USA **98:**658–663.
26. **Igarashi, T., Y. Endo, G. Englund, R. Sadjadpour, T. Matano, C. Buckler, A. Buckler-White, R. Plishka, T. Theodore, R. Shibata, and M. Martin.** 1999. Emergence of a highly pathogenic simian/human immunodeficiency virus in a rhesus macaque treated with anti-CD8 mAb during a primary infection with a nonpathogenic virus. Proc. Natl. Acad. Sci. USA **96:**14049–14054.
27. **Joag, S. V., I. Adany, Z. Li, L. Foresman, D. M. Pinson, C. Wang, E. B. Stephens, R. Raghavan, and O. Narayan.** 1997. Animal model of mucosally transmitted human immunodeficiency virus type 1 disease: intravaginal and oral deposition of simian/human immunodeficiency virus in macaques results in systemic infection, elimination of $CD4^+$ T cells, and AIDS. J. Virol. **71:**4016–4023.
28. **Joag, S. V., Z. Li, L. Foresman, E. B. Stephens, L. J. Zhao, I. Adany, D. M. Pinson, H. M. McClure, and O. Narayan.** 1996. Chimeric simian/human immunodeficiency virus that causes progressive loss of $CD4^+$ T cells and AIDS in pig-tailed macaques. J. Virol. **70:**3189–3197.
29. **Karlsson, G. B., M. Halloran, J. Li, I. W. Park, R. Gomila, K. A. Reimann, M. K. Axthelm, S. A. Iliff, N. L. Letvin, and J. Sodroski.** 1997. Characterization of molecularly cloned simian-human immunodeficiency viruses causing rapid $CD4^+$ lymphocyte depletion in rhesus monkeys. J. Virol. **71:**4218–4225.
30. **Karlsson, G. B., M. Halloran, D. Schenten, J. Lee, P. Racz, K. Tenner-Racz, J. Manola, R. Gelman, B. Etemad-Moghadam, E. Desjardins, R. Wyatt, N. P. Gerard, L. Marcon, D. Margolin, J. Fanton, M. K. Axthelm, N. L. Letvin, and J. Sodroski.** 1998. The envelope glycoprotein ectodomains determine the efficiency of CD4+ T lymphocyte depletion in simian-human immunodeficiency virus-infected macaques. J. Exp. Med. **188:**1159–1171.
31. **Kolchinsky, P., E. Kiprilov, P. Bartley, R. Rubinstein, and J. Sodroski.** 2001. Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. J. Virol. **75:**3435–3443.
32. **Kolchinsky, P., E. Kiprilov, and J. Sodroski.** 2001. Increased neutralization sensitivity of CD4-independent human immunodeficiency virus variants. J. Virol. **75:**2041–2050.
33. **LaBonte, J. A., T. Patel, W. Hofmann, and J. Sodroski.** 2000. Importance of membrane fusion mediated by human immunodeficiency virus envelope glycoproteins for lysis of primary CD4-positive T cells. J. Virol. **74:**10690–10698.
34. **LeGuern, M., T. Shioda, J. A. Levy, and C. Cheng-Mayer.** 1993. Single amino acid change in Tat determines the different rates of replication of two sequential HIV-1 isolates. Virology **195:**441–447.
35. **Li, J., C. I. Lord, W. Haseltine, N. L. Letvin, and J. Sodroski.** 1992. Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. J. Acquir. Immune Defic. Syndr. **5:**639–646.
36. **Luciw, P. A., C. P. Mandell, S. Himathongkham, J. Li, T. A. Low, K. A. Schmidt, K. E. Shaw, and C. Cheng-Mayer.** 1999. Fatal immunopathogenesis by SIV/HIV-1 (SHIV) containing a variant form of the HIV-1SF33 env gene in juvenile and newborn rhesus macaques. Virology **263:**112–127.
37. **Luciw, P. A., E. Pratt-Lowe, K. E. Shaw, J. A. Levy, and C. Cheng-Mayer.** 1995. Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV). Proc. Natl. Acad. Sci. USA **92:**7490–7494.
38. **Malenbaum, S. E., D. Yang, L. Cavacini, M. Posner, J. Robinson, and C. Cheng-Mayer.** 2000. The N-terminal V3 loop glycan modulates the interaction of clade A and B human immunodeficiency virus type 1 envelopes with CD4 and chemokine receptors. J. Virol. **74:**11008–11016.

39. **Martin, J., C. C. LaBranche, and F. Gonzalez-Scarano.** 2001. Differential CD4/CCR5 utilization, gp120 conformation, and neutralization sensitivity between envelopes from a microglia-adapted human immunodeficiency virus type 1 and its parental isolate. J. Virol. **75:**3568–3580.
40. **Moore, J. P., J. A. McKeating, Y. X. Huang, A. Ashkenazi, and D. D. Ho.** 1992. Virions of primary human immunodeficiency virus type 1 isolates resistant to soluble CD4 (sCD4) neutralization differ in sCD4 binding and glycoprotein gp120 retention from sCD4-sensitive isolates. J. Virol. **66:**235–243.
41. **Nathanson, N., V. M. Hirsch, and B. J. Mathieson.** 1999. The role of nonhuman primates in the development of an AIDS vaccine. AIDS **13:**S113–S120.
42. **Overbaugh, J., P. A. Luciw, and E. A. Hoover.** 1997. Models for AIDS pathogenesis: simian immunodeficiency virus, simian-human immunodeficiency virus and feline immunodeficiency virus infections. AIDS **11:**S47–S54.
43. **Parren, P. W., J. P. Moore, D. R. Burton, and Q. J. Sattentau.** 1999. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. AIDS **13:**S137–S162.
44. **Platt, E. J., S. L. Kozak, and D. Kabat.** 2000. Critical role of enhanced CD4 affinity in laboratory adaptation of human immunodeficiency virus type 1. AIDS Res. Hum. Retroviruses **16:**871–882.
45. **Poignard, P., T. Fouts, D. Naniche, J. P. Moore, and Q. J. Sattentau.** 1996. Neutralizing antibodies to human immunodeficiency virus type-1 gp120 induce envelope glycoprotein subunit dissociation. J. Exp. Med. **183:**473–484.
46. **Reimann, K. A., J. T. Li, R. Veazey, M. Halloran, I. W. Park, G. B. Karlsson, J. Sodroski, and N. L. Letvin.** 1996. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate Env causes an AIDS-like disease after in vivo passage in rhesus monkeys. J. Virol. **70:**6922–6928.
47. **Reimann, K. A., J. T. Li, G. Voss, C. Lekutis, K. Tenner-Racz, P. Racz, W. Lin, D. C. Montefiori, D. E. Lee-Parritz, Y. Lu, R. G. Collman, J. Sodroski, and N. L. Letvin.** 1996. An *env* gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. J. Virol. **70:**3198–3206.
48. **Rizzuto, C. D., R. Wyatt, N. Hernandez-Ramos, Y. Sun, P. D. Kwong, W. A. Hendrickson, and J. Sodroski.** 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science **280:**1949–1953.
49. **Shibata, R., M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, and A. Adachi.** 1991. Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J. Virol. **65:**3514–3520.
50. **Shibata, R., F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, T. Fredrickson, and M. A. Martin.** 1997. Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J. Infect. Dis. **176:**362–373.
51. **Si, Z., M. Cayabyab, and J. Sodroski.** 2001. Envelope glycoprotein determinants of neutralization resistance in a simian-human immunodeficiency virus (SHIV-HXBc2P 3.2) derived by passage in monkeys. J. Virol. **75:**4208–4218.
52. **Skehel, J. J., and D. C. Wiley.** 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. **69:**531–569.
53. **Stamatatos, L., and C. Cheng-Mayer.** 1995. Structural modulations of the envelope gp120 glycoprotein of human immunodeficiency virus type 1 upon oligomerization and differential V3 loop epitope exposure of isolates displaying distinct tropism upon virion-soluble receptor binding. J. Virol. **69:**6191–6198.
54. **Stamatatos, L., A. Werner, and C. Cheng-Mayer.** 1994. Differential regulation of cellular tropism and sensitivity to soluble CD4 neutralization by the envelope gp120 of human immunodeficiency virus type 1. J. Virol. **68:**4973–4979.
55. **Stamatatos, L., S. Zolla-Pazner, M. K. Gorny, and C. Cheng-Mayer.** 1997. Binding of antibodies to virion-associated gp120 molecules of primary-like human immunodeficiency virus type 1 (HIV-1) isolates: effect on HIV-1 infection of macrophages and peripheral blood mononuclear cells. Virology **229:**360–369.
56. **Stephens, E. B., S. Mukherjee, M. Sahni, W. Zhuge, R. Raghavan, D. K. Singh, K. Leung, B. Atkinson, Z. Li, S. V. Joag, Z. Q. Liu, and O. Narayan.** 1997. A cell-free stock of simian-human immunodeficiency virus that causes AIDS in pig-tailed macaques has a limited number of amino acid substitutions in both SIVmac and HIV-1 regions of the genome and has altered cytotropism. Virology **231:**313–321.
57. **Sullivan, N., Y. Sun, J. Binley, J. Lee, C. F. Barbas III, P. W. Parren, D. R. Burton, and J. Sodroski.** 1998. Determinants of human immunodeficiency virus type 1 envelope glycoprotein activation by soluble CD4 and monoclonal antibodies. J. Virol. **72:**6332–6338.
58. **Sullivan, N., Y. Sun, Q. Sattentau, M. Thali, D. Wu, G. Denisova, J. Gershoni, J. Robinson, J. Moore, and J. Sodroski.** 1998. CD4-induced conformational changes in the human immunodeficiency virus type 1 gp120 glycoprotein: consequences for virus entry and neutralization. J. Virol. **72:**4694–4703.
59. **Thali, M., J. P. Moore, C. Furman, M. Charles, D. D. Ho, J. Robinson, and J. Sodroski.** 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J. Virol. **67:**3978–3988.
60. **Wyatt, R., P. D. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski.** 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature **393:**705–711.
61. **Wyatt, R., and J. Sodroski.** 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science **280:**1884–1888.
62. **Ye, Y., Z. H. Si, J. P. Moore, and J. Sodroski.** 2000. Association of structural changes in the V2 and V3 loops of the gp120 envelope glycoprotein with acquisition of neutralization resistance in a simian-human immunodeficiency virus passaged in vivo. J. Virol. **74:**11955–11962.