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ABSTRACT

Binding of HIV-1 and SIV gp120 exterior envelope glycoprotein to CD4 triggers conformational changes in gp120 that promote its interaction with one of the chemokine receptors, usually CCR5, ultimately leading to gp41-mediated virus-cell membrane fusion and entry. We previously described that topological Layers (Layer 1, Layer 2 and Layer 3) in the gp120 inner domain contribute to gp120-trimer association in the unliganded state but also help secure CD4 binding. Relative to Layer 1 of HIV-1 gp120, the SIVmac239 gp120 Layer 1 plays a more prominent role in maintaining gp120-trimer association but is minimally involved in promoting CD4 binding, which could be explained by the existence of a well-conserved Tryptophan 375 (Trp 375) in HIV-2/SIVsmm. Here we investigated the role of SIV Layer 3 on viral entry, cell-to-cell fusion and CD4 binding. We observed that a network of interactions involving some residues of the β8-α5 region in SIVmac239 Layer 3 may contribute to CD4 binding by helping shape the nearby Phe 43 cavity which directly contacts CD4. In summary, our results suggest that SIV Layer 3 has a greater impact on CD4 binding than in HIV-1. This work defines lineage-specific differences in Layer 3 from HIV-1 and SIV.

IMPORTANCE

CD4-induced conformational changes in the gp120 inner domain involve rearrangements between three topological layers. While the role of Layers 1-3 for HIV-1 and 1-2 for SIV on gp120 transition to the CD4-bound conformation has been reported, the role of SIV Layer 3 remains unknown. Here we report that SIV Layer 3 has a greater

impact on CD4 binding than in HIV-1 gp120. This work defines lineage specific differences in Layer 3 from HIV-1 and SIV.

INTRODUCTION

Binding of HIV-1 and SIV gp120 exterior envelope glycoprotein to the initial receptor, CD4 (1, 2), triggers conformational changes in gp120 that promote its interaction with one of the chemokine receptors, usually CCR5 (3-10), ultimately leading to gp41-mediated virus-cell membrane fusion and entry (11-14). We recently described that topological layers in the gp120 inner domain contribute to gp120-trimer association in the unliganded state and to secure CD4 binding (15). Indeed, the transition of gp120 from the unbound to the CD4-bound conformation is modulated by the inner domain Layers 1-3. While HIV-1 Layers 1 and 2 are required to keep CD4 in place by decreasing the off-rate of the gp120-CD4 interaction (15), Layer 3 is implicated in the initial contact and modulates the on-rate of this association (16). Interestingly, lineage-specific differences on the role of Layer 1 and 2 regarding their contribution to gp120-trimer association and CD4 binding were recently described (17). Relative to Layer 1 of HIV-1 gp120, the SIVmac239 gp120 Layer 1 plays a more prominent role in maintaining gp120-trimer association and is minimally involved in promoting CD4 binding (17). HIV-2/SIVsmm gp120 glycoproteins, like those of most monkey SIVs, typically have a tryptophan residue at position 375 (18). In HIV-1, substitution of tryptophan for serine 375 (S375W), which is well-conserved in the major group of HIV-1, results in the spontaneous sampling of a conformation closer to CD4-bound state (19). Residue 375 is located in what is known as the Phe 43 cavity, where Phe 43 of CD4 makes numerous contacts with conserved gp120 residues critical for CD4 binding (20). Some gp120 residues that border this cavity contribute to an aromatic array that helps stabilize the CD4-bound conformation (15, 20, 21). Therefore it is possible that relative to HIV-1

gp120, the HIV-2/SIVsmm gp120 glycoproteins, by virtue of the Phe 43 cavity-filling Trp 375, might naturally exhibit a greater propensity to sample the CD4-bound conformation explaining a decreased requirement for Layers 1 and 2 in CD4 binding. However, how the presence of a tryptophan at position 375 affects the functional role of SIVmac239 gp120 Layer 3, previously shown to play a predominant role in securing CD4 binding in HIV-1 gp120, is unknown. Here, we characterize the importance of this element to SIVmac239 Env integrity and to the process of viral entry, and investigate its role in the transition to the CD4-bound conformation.

MATERIALS AND METHODS

Modeling full-length SIV gp120.

Modeling of the full-length SIVmac239 gp120 was done by using the X-ray crystal structure (PDB identifier 3JWD) of the ternary complex consisting of an HIV-1HXBc2 gp120 core with intact N and C termini, two-domain CD4, and the antigen-binding fragment of the human antibody 48d (21) as a template. Modeling was performed on the Discovery Studio software platform (Accelrys Software, Inc.). SIVmac239 gp120 glycoprotein modeling was based on protein sequence alignment with full-length HIV-1HXBc2 gp120, using the Modeler (version 9) program in the Accelrys software package. The gp120 V4 variable loop, consisting of the sequence WSTEGSNNT, was disordered in the X-ray crystal structure (21) and was added by modeling.

Cell lines.

293T human embryonic kidney, Cf2Th canine thymocytes (American Type Culture Collection) and TZM-bl cell lines (NIH AIDS Research and Reference Reagent Program) were grown at 37°C and 5% CO2 in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Sigma) and 100 µg/ml of penicillin-streptomycin (Mediatech). Cf2Th cells stably expressing human CD4 and CCR5 (Cf2Th-CD4-CCR5) (22) were grown in medium supplemented with 0.4 mg/ml of G418 (Invitrogen) and 0.15 mg/ml of hygromycin B (Roche Diagnostics). Cf2Th-CCR5 cells were grown in medium supplemented with 0.4 mg/ml of G418 (Invitrogen). The TZM-bl cell line is a HeLa cell line stably expressing high levels of CD4 and CCR5 and

possessing an integrated copy of the luciferase gene under the control of the HIV-1 long terminal repeat (23).

Site-directed mutagenesis.

Mutations were introduced individually into the previously described vector expressing the SIVmac239 envelope glycoproteins (pSIVmac239) (24, 25). Site-directed mutagenesis was performed using the QuikChange II XL site-directed mutagenesis protocol (Stratagene). The presence of the desired mutations was determined by automated DNA sequencing. The numbering of the SIVmac239 and HIV-1 envelope glycoprotein amino acid residues is based on that of the prototypic HXBc2 strain of HIV-1, where 1 is the initial methionine.

Immunoprecipitation and CCR5 binding of envelope glycoproteins.

For pulse-labeling experiments, 3×10^5 293T cells were cotransfected by the calcium phosphate method with pLTR-Tat and the pSIVmac239 vector expressing the SIVmac239 envelope glycoproteins. One day after transfection, cells were metabolically labeled for 16 h with 100 μCi/ml [^{35}S]methionine-cysteine ([^{35}S] protein labeling mix; Perkin-Elmer) in Dulbecco's modified Eagle's medium lacking methionine and cysteine and supplemented with 5% dialyzed fetal bovine serum. Cells were subsequently lysed in RIPA buffer (140 mM NaCl, 8 mM Na2HPO4, 2 mM NaH2PO4, 1% NP-40, and 0.05% sodium dodecyl sulfate [SDS]). Precipitation of radiolabeled SIVmac239 envelope glycoproteins from cell lysates or medium was performed with a mixture of sera from SIV-infected macaques. The association index is a measure of the ability of the mutant gp120 molecule to remain associated with the Env trimer complex on the expressing cell, relative to that of the wild-type Env trimer. The association

index is calculated as follows: association index = ([mutant gp120]$_{cell}$ × [wild-type gp120]$_{supernatant}$)/ ([mutant gp120]$_{supernatant}$ × [wild-type gp120]$_{cell}$). The processing index is a measure of the conversion of the mutant gp160 Env precursor to mature gp120, relative to that of the wild-type Env trimer. The processing index was calculated by the formula: processing index = ([total gp120]$_{mutant}$ × [gp160]$_{wild-type}$)/ ([gp160]$_{mutant}$ × [total gp120]$_{wild-type}$).

Alternatively, medium containing radiolabeled envelope proteins was immunoprecipitated with CD4-Ig for 1 h at 37°C in the presence of 50 µl of 10% protein A-Sepharose (Amersham Biosciences). Of note, while some differences between human and rhesus CD4/CCR5 have been reported (24, 26, 27) we decided to use both human CD4-Ig and cells expressing human CCR5 to be consistent with previous reports characterizing the HIV and SIV gp120 inner domain layers (15, 16). For CCR5 binding, normalized amounts of radiolabeled SIVmac239 gp120 envelope glycoproteins were incubated in the presence or absence of 200 nm of sCD4 for 1 h at 37°C. Subsequently, 2 × 10^6 Cf2Th-CCR5 cells were added for an additional 1 h at 37°C and washed twice with phosphate-buffered saline (PBS) prior to cell lysis in RIPA buffer. Cell lysates were immunoprecipitated with a mixture of sera from SIV-infected macaques. All samples were loaded on polyacrylamide gels and analyzed by autoradiography and by using a PhosphorImager (Molecular Dynamics).

Recombinant luciferase viruses.

Recombinant viruses containing the firefly luciferase gene were produced by calcium phosphate transfection of 293T cells with the HIV-1 proviral vector pNL4.3 Env- Luc and the plasmid expressing the wild-type or mutant SIVmac239 envelope glycoproteins at a ratio of 2:1. Two days after transfection, the cell supernatants were harvested; the

158 reverse transcriptase activities of all viruses were measured as described previously
159 (28). The virus-containing supernatants were stored in aliquots at −80°C.
160
161 **Infection by single-round luciferase viruses.**
162 Cf2Th-CD4-CCR5 target cells were seeded at a density of 5×10^3 cells/well in 96-well
163 luminometer- compatible tissue culture plates (Dynex) 24 h before infection.
164 Recombinant viruses (10,000 reverse transcriptase units) in a final volume of 100 µl
165 were then added to the target cells, followed by incubation for 48 h at 37°C; the medium
166 was then removed from each well, and the cells were lysed by the addition of 30 µl of
167 passive lysis buffer (Promega) and three freeze-thaw cycles. A microplate luminometer
168 was used to measure the luciferase activity of each well after the addition of 100 µl of
169 luciferin buffer (15 mM MgSO4, 15 mMKPO4 [pH 7.8], 1 mM ATP, and
170 1mMdithiothreitol) and 50 µl of 1mMD-luciferin potassium salt (BD Pharmingen).
171
172 **Cell-cell fusion.**
173 To assess cell-to-cell fusion, 3×10^5 293T cells were cotransfected by the calcium
174 phosphate method with an HIV-1 Tat-expressing plasmid, pLTR-Tat, and the
175 pSIVmac239 vector expressing the SIVmac239 envelope glycoproteins. Two days after
176 transfection, 3×10^4 293T cells were added to TZM-bl target cells that were seeded at a
177 density of 3×10^4 cells/well in 96-well luminometer-compatible tissue culture plates 24 h
178 before the assay. Cells were co-incubated for 6 h at 37°C, after which they were lysed
179 by the addition of 30 µl of passive lysisbuffer (Promega) and three freeze-thaw cycles.
180 Luciferase activity in each well was measured as described above.

Purification of recombinant SIVmac239 gp120 glycoproteins

FreeStyle 293F cells (Invitrogen) were grown in FreeStyle 293F medium (Invitrogen) to a density of 1 x 10^6 cells/ml at 37°C with 8% CO_2 with regular agitation (125 rpm). Cells were transfected with a codon-optimized plasmid expressing His_6-tagged wild-type or mutant SIV_{mac239} gp120 using the 293Fectin reagent, as directed by the manufacturer (Invitrogen). One week later, the cells were pelleted and discarded. The supernatants were filtered (0.22-μ filter) (Corning) and the gp120 glycoproteins were purified by nickel affinity columns, as directed by the manufacturer (Invitrogen) followed by FPLC purification of monomeric gp120, as described (29). The gp120 preparations were dialyzed against PBS and stored in aliquots at -80°C. To assess purity, recombinant proteins were loaded on SDS-PAGE polyacrylamide gels and stained with Coomassie Blue.

SPR biosensor analysis.

Surface plasmon resonance (SPR) biosensor data were collected on a Biacore 3000 optical biosensor (General Electric). CD4-Ig was immobilized onto separate flow cells within the same sensor chip (CM5; GE) to a surface density of around 500 response units (RU) using standard amine coupling chemistry (30). The binding capacities of CD4 surfaces were kept low to avoid mass transport effects and steric hindrance. Flow cell 1 or 3 was left blank as a control for nonspecific binding and refractive index changes. With the instrument operating in a parallel sensing mode, soluble gp120 was injected over flow cells 1 and 2 or 3 and 4 at different concentrations ranging from 100 to 750

nM at a flow rate of 30 µl/min for 3 min. This was followed by a 10-min dissociation phase to allow an estimation of off-rates and binding affinities. Sensor data were prepared for kinetic analysis by subtracting binding responses collected from the blank reference surface. The association and dissociation phase data were fitted simultaneously with BIAevalution, version 3.2, RC1 software using a 1:1 Langmuir model of binding.

212 **RESULTS**

213 **The SIVmac239 gp120 layer 3 inner domain mutants**

214 The nine intra-chain disulfide bonds in HIV-1 gp120 glycoproteins are conserved in all
215 primate immunodeficiency virus gp120 glycoproteins, assisting the alignment of the
216 HIV-1 and SIVmac239 gp120 sequences (18, 31). To evaluate the functional role of
217 SIV gp120 Layer 3 and how it compares with HIV-1, the SIVmac239 gp120 Layer 3 and
218 Phe 43 cavity primary amino acid sequence was aligned with that of HIV-1 group M and
219 HIV-2 gp120 (Figure 1A). Five Layer 3 residues (247, 248, 480, 482 and 483) were
220 identical and only the nature of two others, 251 and 479, was preserved (Figure 1A).
221 Comparison of the Phe 43 cavity sequences indicates that HIV-2 and SIVmac have a
222 large residue (Trp) at position 375 that likely fills the Phe 43 cavity (18) (Figure 1A)
223 whereas HIV-1 has an “empty” Phe 43 cavity by virtue of its smaller residue (a serine) at
224 this position. Altogether, these results suggest that substantial differences exist
225 between the inner domain Layer 3 of HIV-1 and SIVmac239/HIV-2 gp120. We
226 introduced point mutations in residues 248 to 254 and 476 to 483 of SIVmac239 and
227 evaluated the ability of the variants to interact with the receptor CD4 and coreceptor
228 CCR5, we also evaluated their ability to mediate viral infectivity, cell-to-cell fusion and
229 their contribution to trimer stability (association index) and processing of the gp160
230 precursor (processing index) (Table 1).

231

232 **Proteolytic processing and subunit association of SIVmac239 variants**

233 Proteolytic processing of the gp160 precursor and association of the gp120 and gp41
234 subunits of each SIVmac239 Env variant were evaluated by transfecting 293T followed

by radiolabelling and immunoprecipitation of cell lysates and supernatant with polyclonal sera from SIV-infected macaques. All of the mutants were expressed efficiently and only mutants T248A, R249A, L481A and L483A exhibited a marked decrease in proteolytic processing of the gp160 Env precursor (Table 1) (Figure 2A). A similar phenotype was previously observed for T248A in HIV-1 gp120, perhaps due to its proximity to cysteine 247 which might be important for Env folding. While in HIV-1 gp120 only changes in residue W479 significantly disrupted the non-covalent association of gp120 with the Env trimer (16), in SIVmac239 changes in residues 478-481 affected trimer stability below half of their wild-type counterpart (Table 1, Figure 2B and Figure 5A). Similar to the unique position of W479 in HIV-1 gp120, residues 478-481 of SIVmac239 are located at the center of a hydrophobic interface between Layers 2 and 3 (Figure 1 D and E) and their mutation might affect trimer stability through effects on Layer 2-Layer 3 association, which could indirectly affect gp120-trimer association by altering either the gp120 trimer association domain or the gp120-gp41 interface.

Function of the mutant SIV envelope glycoproteins

We next evaluated the effect of alteration of Layer 3 residues on the ability of SIVmac239 Env to mediate viral infectivity (Figure 3A) and cell-to-cell fusion (Figure 3B). In striking contrast with what we reported for HIV-1 gp120 Layer 3 mutants where only W479 presented a significant decrease in viral infectivity (16), we observed that the majority of SIVmac239 gp120 Layer 3 changes (T248A, R249A, E477A, L478A, Y479A, R480A, L481A, E482A, L483A) impacted viral infectivity below 20% of the wild-type. However, all the variants mediated cell-to-cell fusion much more efficiently than cell-free

258 virus infection. This phenotype has previously been observed for alterations in the inner
259 domain Layers 1 and 2 and the β-sandwich that effect gp120-trimer association (15,
260 32). While a defect in processing (T248A, R249A, R480A, L481A, L483A) and trimer
261 stability (L478A, Y479A, R480A and L481A) could explain part of the major impairment
262 to mediate viral infectivity, these parameters did not account for the marked loss of
263 infectivity of E477A and E482A, suggesting that additional mechanisms could account
264 for their impaired ability to mediate viral infection.
265
266 **Interaction of the mutant SIVmac239 envelope glycoproteins with CD4 and CCR5**
267 Alteration of the interface between Layers 1 and 2 of the HIV-1 gp120 inner domain has
268 been shown to decrease CD4 binding, revealing an important mechanism whereby HIV-
269 1 gp120 regulates CD4-induced conformational rearrangement and achieves a
270 reduction in off-rate (15, 21, 33). To investigate the contribution of Layer 3 of SIV gp120
271 to CD4 binding, the CD4-binding ability of the panel of SIVmac239 Layer 3 gp120
272 mutants was examined. Wild-type and mutant SIVmac239 envelope glycoproteins were
273 transiently expressed in 293T cells, which were radiolabeled for 16 h. The amount of
274 radiolabeled gp120 glycoproteins shed into the cell medium was normalized by
275 immunoprecipitation with polyclonal serum from SIV-infected macaques before
276 assessing the ability to bind CD4-Ig. As shown in Table 1 and Figure 4A, a major defect
277 on CD4 binding was observed for the majority of SIV239 Layer 3 variants, in agreement
278 with a critical role of Layer 3 in CD4 interaction. Indeed, with the exception of Q254A
279 and A476G, the rest of the mutants (248-253 and 477-483) bound CD4-Ig below 40% of
280 their wild-type counterpart (Figure 4A and Figure 5B). As previously shown for HIV-1

gp120 Layer 3 variants, the observed decreases in CD4 binding were mainly due to decreased on-rates (A476G, E477A, Y479A and E482A) compared with that of WT gp120 except for T248A (Table 1). Thus, gp120 mutants with alterations in Layer 3 fail to engage CD4 efficiently. Therefore, multiple residues in Layer 3 of the gp120 inner domain that, based on our modeling, do not directly contact CD4 nonetheless contribute to the affinity of the gp120-CD4 interaction. A network of interactions involving some residues of the β8-α5 region in Layer 3 may contribute to CD4 binding by helping shape the nearby Phe 43 cavity that directly contacts CD4 (20, 34, 35).

The functional consequence of CD4 binding for HIV-1 and SIV envelope glycoproteins is the interaction with co-receptor CCR5 or CXCR4 (3-10). We analyzed whether introducing changes in Layer 3 of the SIVmac239 gp120 inner domain affected CCR5 recognition. Radiolabeled wild-type and mutant SIV gp120 glycoproteins were incubated in the presence or absence of sCD4 for 1 h at 37 ºC prior to incubation with cells expressing the CCR5 coreceptor for 1 h at 37ºC. After washing and lysis of the cells, bound gp120 was detected by immunoprecipitation. As shown in Table 1 and Figure 4B, L478A was the only variant presenting reduced CCR5 binding to less than 40%. This defect was more pronounced upon sCD4 addition, suggesting that this mutant has some difficulties in assuming the CD4-bound conformation required to engage its co-receptor. The defect in CCR5 binding could explain the lower cell-to-cell fusion efficiency brought by L478A (Figure 3B and Table 1). However, its decreased subunit association, gp160 processing and CD4 binding could also account for this phenotype (Table 1).

DISCUSSION

In the unliganded trimer, the gp120 must maintain its non-covalent association with gp41. Layers 1 and 2 of the gp120 inner domain were recently shown to be important in maintaining the association of gp120 with the Env trimer in HIV but also SIV lineages (15, 17). In HIV-1, Layer 3 was also shown to contribute to the association of gp120 with the unliganded Env trimer, likely through an indirect mechanism (16). Here we extend these results to the SIV lineage since mutation of residues L478, Y479 and L481 to alanine resulted in a significant decrease in trimer stability. These residues are located at the center of a hydrophobic interface between Layers 2 and 3 and in an analogue way to what was reported for W479 in HIV-1, where when mutated to an alanine (W479A) it results in a major shedding phenotype in R5-tropic Envs but to a lesser extent in R5X4 or X4 Envs, (16), alteration of these residues might alter Layer 2-Layer 3 interactions required for proper gp120-trimer association. Shifts between the inner domain and outer domain that occur as a result of these changes in the Layer 2-Layer 3 interface might affect the orientation of the variable regions which are known to affect trimer stability (36, 37).

In the transition from the unliganded state to the CD4-bound state, the inner domain of gp120 experiences major conformational rearrangements (15, 38-42). Located at the interface between the inner domain and outer domain, Layer 3 has been shown to play a pivot-like role in the layered allosteric changes of the HIV-1 gp120 inner domain by decreasing the spontaneous sampling of the CD4-bound conformation, mainly through a decrease on-rate (16). Analogous to the role played in HIV-1 we

328 found that SIVmac239 Layer 3 also played a critical role in securing CD4 binding mainly
329 through an on-rate effect (Table 1). Indeed, an on-rate decrease was observed for
330 A476G, E477A, Y479A and E482A variants. However, for other variants such as
331 T248A, the decrease in CD4 binding was mediated by an accelerated off-rate (Table 1).
332 Moreover, we found that in contrast to HIV-1 Layer 3, where only four residues
333 contributed to CD4 binding (T248, H249, N478 and W479), eleven out of the thirteen
334 residues mutated (with the exception of Q254A and A476G) dramatically affected CD4
335 binding. Decreased CD4 binding combined with defects in trimer stability likely explain
336 the marked inability to mediate viral entry for the majority of SIVmac239 Layer 3
337 variants.
338
339 Altogether, our results show that subtle differences exist in the organization of
340 the inner domain network of residues governing SIV and HIV-1 gp120 Layers
341 interaction. As summarized in Figure 6, the presence of a tryptophan at position 375,
342 which fills the Phe 43 cavity, forces the gp120 to spontaneously sample the CD4-bound
343 conformation in SIV lineages. This differential arrangement of the inner domain Layers
344 and the Phe43 cavity between HIV-1 and SIV gp120 glycoproteins might help explain
345 why SIV envelope glycoproteins are generally less dependent on CD4 for CCR5
346 interaction and able to infect cells that express low levels of CD4, and can maintain a
347 long-lived sCD4-activated state (27, 43-48). Since sampling the CD4-bound
348 conformation might have unintended negative effects for the virus (decrease trimer
349 stability, exposure of neutralizing epitopes (15-17, 19, 49)) this propensity is
350 compensated by the strong contribution of Layer 1 to trimer stability. Therefore, in SIV

gp120 tryptophan 375 and Layer 3 play a predominant role on CD4 interaction. In HIV-1 gp120, which lacks a tryptophan at position 375, the propensity to assume the CD4-bound conformation is lower and therefore, Layers 1 and 2 help secure CD4 binding by decreasing the off-rate of gp120-CD4 interaction, as previously described (15).

The major involvement of SIV gp120 Layer 3 in CD4 binding suggests that Layer 3 residues are important for optimal exposure of the CD4-binding site. This is particularly true in SIV which has a large-filling residue at position 375 and where Layer 3 appears to maintain a critical relationship between the gp120 inner and outer domains required for the intermolecular signaling required for gp41 HR1 exposure upon CD4 interaction. Our work completes a detailed analysis of HIV and SIV inner domain layers and shows how subtle lineage-specific differences in the gp120 inner domain affect Env function.

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567 **FIGURE LEGENDS**

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569 **Figure 1. Alignment and Structure of the inner domain of HIV-1 gp120 and**
570 **modeled SIV gp120 in the CD4-bound conformation.** (**A**) The primary amino acid
571 sequences of the gp120 inner domain Layer 3 and Phe 43 cavity of HIV-1 (accession
572 number K03455), HIV-2 (accession number AAC95347.1) and SIVmac239 (accession
573 number M33262) are aligned. Sequence identity is indicated by a solid vertical line.
574 Gaps in the sequence are indicated by dashes. Residue numbering is based on that of
575 the HXBc2 strain of HIV-1 (31). (**B**) View of the conformation adopted by the inner
576 domain Layers 1, 2 and 3 in the structure of the HIV-1_{HXBc2} gp120 core with N/C termini
577 in the CD4-bound state (21) is shown. The outer domain (OD) of gp120 is colored
578 yellow. The N and C termini are colored cyan. The components of the gp120 inner
579 domain (ID) are the β-sandwich (red) and three loop-like extensions: Layer 1
580 (magenta), Layer 2 (green) and Layer 3 (orange). (**C**) Same perspective than in B but
581 using a modeled SIVmac239 gp120 glycoprotein, as described in Materials and
582 Methods. (D) A close-up view of the interactions between HIV-1 Layer 2 and Layer 3.
583 Critical Layer 3 residues that are between Layer 2 and Layer 3 are indicated. (E) A
584 close-up view of the interactions between SIVmac239 Layer 2 and Layer 3. Critical
585 residues of Layer 3 that are between Layer 2 and Layer 3 are indicated.

586

587 **Figure 2. Precursor processing and gp120-trimer association of SIVmac239**
588 **envelope glycoprotein mutants.** Cell lysates and supernatants (SN) of ^{35}S-labeled
589 cells transiently expressing the SIVmac239 WT and mutant envelope glycoproteins

were precipitated with serum from an SIV-infected macaque. The precipitates were washed, run on SDS-poly-acrylamide gels, and analyzed by densitometry. (**A**) The processing index is a measure of the conversion of the mutant gp160 envelope glycoprotein precursor to mature gp120 relative to that of the wild-type envelope glycoproteins. (**B**) The association index is a measure of the ability of the mutant gp120 molecule to remain associated with the envelope glycoprotein complex on the expressing cell relative to that of the wild-type envelope glycoproteins. The processing index and association index were calculated as described in Materials and Methods. Data shown represent the average +/- standard deviation of at least four independent experiments.

Figure 3. Functionality of SIV gp120 envelope glycoprotein variants. (A) Relative infectivity was assessed on Cf2Th-CD4/CCR5 cells using RT-normalized amounts of pseudoviruses bearing SIV239 WT or Layer 3 variants. Data shown here is the ratio of mutant/wild-type virus infectivity. (**B**) Cell-to-cell fusion activity was assessed by co-incubation between 293T cells expressing envelope glycoprotein variants and TZM-bl cells for 6 hours at 37℃. Luciferase activity in the mixture of cell lysate was measured and normalized to that mediated by WT envelope glycoprotein. Data shown represent the average +/- standard deviation of at least three independent experiments.

Figure 4. Binding of soluble SIV gp120 glycoproteins to CD4-Ig and CCR5. Normalized amounts of radiolabeled wild-type and mutant gp120 glycoproteins were incubated with 2μg of CD4-Ig for 1 hour at 37°C. The precipitates were washed, run on

SDS-polyacrylamide gels, and analyzed by densitometry (**A**). Representative results from at least three independent experiments are shown. (**B**). Normalized amounts of radiolabeled gp120 glycoproteins were incubated in the absence (white bars) or presence (grey bars) of 200 nM sCD4 prior to addition to cells expressing CCR5. After one hour at 37°C, the amount of bound mutant gp120 was determined and normalized to the observed amount of bound wild-type (wt) gp120. Incubation with sCD4 increased the binding of wt SIV gp120 to CCR5 by 7-fold. The data shown represent the means +/- SEM of three independent experiments.

Figure 5. Residues important for gp120-trimer association and CD4 binding. Layer 3 of HIV-1 gp120 (21) and the modeled SIVmac239 gp120 are shown from the same perspective as those in Figure 1, B and C. In **A**, the ribbon and side chain residues that were altered in this and a previous study (16) are colored according to the gp120-trimer association index (Red: association index < 0.5 and Green: association index $\geq$ 0.7). In **B**, the ribbon and side chain residues are colored according to CD4-Ig binding ability (Red: relative CD4-Ig binding $\leq$ 0.5 and Green: relative CD4-Ig binding > 0.5).

Figure 6. Summary of functional differences between HIV-1 and SIVmac239 gp120 glycoproteins. One of the three gp120 subunits of the HIV-1 and SIVmac239 envelope glycoprotein trimer is depicted. In the orientation shown, the trimer axis runs vertically on the left of each figure and the viral membrane is at the top of the figure. The gp120 N and C termini are colored cyan, the β-sandwich red, Layer 1 purple, Layer 2 green,

636 Layer 3 yellow, and the outer domain (OD) ochre. The Trp 375 side chain that fills the
637 Phe 43 cavity of SIV gp120 is depicted. The major contributions of the gp120 N and C
638 termini, the inner domain (the β-sandwich, Layer 1, Layer 2 and Layer 3) and the Phe
639 43 cavity to gp120-trimer association and/or CD4 binding are shown. Note that the
640 contribution of the filled SIVmac239 gp120 Phe 43 cavity to CD4 binding is lacking in
641 HIV-1 gp120; this lack is compensated by a contribution of the HIV-1 gp120 inner
642 domain Layer 1 to CD4 binding, as reported (17). While Layer 3 is important for
643 securing CD4 binding both in HIV-1 (16) and SIV gp120, the contribution appears to be
644 more important in SIVmac239 gp120 due to the contribution of Layer 3 to maintain the
645 integrity of the Phe 43 cavity.