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Lineage-specific differences in the gp120 Inner Domain Layer 3 of Human and Simian Immunodeficiency Viruses

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1 **Lineage-specific differences in the gp120 Inner Domain Layer 3 of Human and Simian**
2 **Immunodeficiency Viruses**

3
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29 **ABSTRACT**

30 Binding of HIV-1 and SIV gp120 exterior envelope glycoprotein to CD4 triggers
31 conformational changes in gp120 that promote its interaction with one of the chemokine
32 receptors, usually CCR5, ultimately leading to gp41-mediated virus-cell membrane
33 fusion and entry. We previously described that topological Layers (Layer 1, Layer 2 and
34 Layer 3) in the gp120 inner domain contribute to gp120-trimer association in the
35 unliganded state but also help secure CD4 binding. Relative to Layer 1 of HIV-1 gp120,
36 the SIVmac239 gp120 Layer 1 plays a more prominent role in maintaining gp120-trimer
37 association but is minimally involved in promoting CD4 binding, which could be
38 explained by the existence of a well-conserved Tryptophan 375 (Trp 375) in HIV-
39 2/SIVsmm. Here we investigated the role of SIV Layer 3 on viral entry, cell-to-cell fusion
40 and CD4 binding. We observed that a network of interactions involving some residues
41 of the $\beta 8$ - $\alpha 5$ region in SIVmac239 Layer 3 may contribute to CD4 binding by helping
42 shape the nearby Phe 43 cavity which directly contacts CD4. In summary, our results
43 suggest that SIV Layer 3 has a greater impact on CD4 binding than in HIV-1. This work
44 defines lineage-specific differences in Layer 3 from HIV-1 and SIV.

45

46 **IMPORTANCE**

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

51 impact on CD4 binding than in HIV-1 gp120. This work defines lineage specific
52 differences in Layer 3 from HIV-1 and SIV.
53

54 **INTRODUCTION**

55 Binding of HIV-1 and SIV gp120 exterior envelope glycoprotein to the initial receptor,
56 CD4 (1, 2), triggers conformational changes in gp120 that promote its interaction with
57 one of the chemokine receptors, usually CCR5 (3-10), ultimately leading to gp41-
58 mediated virus-cell membrane fusion and entry (11-14). We recently described that
59 topological layers in the gp120 inner domain contribute to gp120-trimer association in
60 the unliganded state and to secure CD4 binding (15). Indeed, the transition of gp120
61 from the unbound to the CD4-bound conformation is modulated by the inner domain
62 Layers 1-3. While HIV-1 Layers 1 and 2 are required to keep CD4 in place by
63 decreasing the off-rate of the gp120-CD4 interaction (15), Layer 3 is implicated in the
64 initial contact and modulates the on-rate of this association (16). Interestingly, lineage-
65 specific differences on the role of Layer 1 and 2 regarding their contribution to gp120-
66 trimer association and CD4 binding were recently described (17). Relative to Layer 1 of
67 HIV-1 gp120, the SIVmac239 gp120 Layer 1 plays a more prominent role in maintaining
68 gp120-trimer association and is minimally involved in promoting CD4 binding (17). HIV-
69 2/SIVsmm gp120 glycoproteins, like those of most monkey SIVs, typically have a
70 tryptophan residue at position 375 (18). In HIV-1, substitution of tryptophan for serine
71 375 (S375W), which is well-conserved in the major group of HIV-1, results in the
72 spontaneous sampling of a conformation closer to CD4-bound state (19). Residue 375
73 is located in what is known as the Phe 43 cavity, where Phe 43 of CD4 makes
74 numerous contacts with conserved gp120 residues critical for CD4 binding (20). Some
75 gp120 residues that border this cavity contribute to an aromatic array that helps stabilize
76 the CD4-bound conformation (15, 20, 21). Therefore it is possible that relative to HIV-1

77 gp120, the HIV-2/SIVsmm gp120 glycoproteins, by virtue of the Phe 43 cavity-filling Trp
78 375, might naturally exhibit a greater propensity to sample the CD4-bound conformation
79 explaining a decreased requirement for Layers 1 and 2 in CD4 binding. However, how
80 the presence of a tryptophan at position 375 affects the functional role of SIVmac239
81 gp120 Layer 3, previously shown to play a predominant role in securing CD4 binding in
82 HIV-1 gp120, is unknown. Here, we characterize the importance of this element to
83 SIVmac239 Env integrity and to the process of viral entry, and investigate its role in the
84 transition to the CD4-bound conformation.

85

86

87

88 **MATERIALS AND METHODS**

89 **Modeling full-length SIV gp120.**

90 Modeling of the full-length SIVmac239 gp120 was done by using the X-ray crystal
91 structure (PDB identifier 3JWD) of the ternary complex consisting of an HIV-1HXBc2
92 gp120 core with intact N and C termini, two-domain CD4, and the antigen-binding
93 fragment of the human antibody 48d (21) as a template. Modeling was performed on the
94 Discovery Studio software platform (Accelrys Software, Inc.). SIVmac239 gp120
95 glycoprotein modeling was based on protein sequence alignment with full-length HIV-
96 1HXBc2 gp120, using the Modeler (version 9) program in the Accelrys software
97 package. The gp120 V4 variable loop, consisting of the sequence WSTEGSNNT, was
98 disordered in the X-ray crystal structure (21) and was added by modeling.

99

100 **Cell lines.**

101 293T human embryonic kidney, Cf2Th canine thymocytes (American Type Culture
102 Collection) and TZM-bl cell lines (NIH AIDS Research and Reference Reagent
103 Program) were grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium
104 (Invitrogen) containing 10% fetal bovine serum (Sigma) and 100 µg/ml of penicillin-
105 streptomycin (Mediatech). Cf2Th cells stably expressing human CD4 and CCR5
106 (Cf2Th-CD4-CCR5) (22) were grown in medium supplemented with 0.4 mg/ml of G418
107 (Invitrogen) and 0.15 mg/ml of hygromycin B (Roche Diagnostics). Cf2Th-CCR5 cells
108 were grown in medium supplemented with 0.4 mg/ml of G418 (Invitrogen). The TZM-bl
109 cell line is a HeLa cell line stably expressing high levels of CD4 and CCR5 and

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

112

113 **Site-directed mutagenesis.**

114 Mutations were introduced individually into the previously described vector expressing
115 the SIVmac239 envelope glycoproteins (pSIVmac239) (24, 25). Site-directed
116 mutagenesis was performed using the QuikChange II XL site-directed mutagenesis
117 protocol (Stratagene). The presence of the desired mutations was determined by
118 automated DNA sequencing. The numbering of the SIVmac239 and HIV-1 envelope
119 glycoprotein amino acid residues is based on that of the prototypic HXBc2 strain of HIV-
120 1, where 1 is the initial methionine.

121

122 **Immunoprecipitation and CCR5 binding of envelope glycoproteins.**

123 For pulse-labeling experiments, 3×10^5 293T cells were cotransfected by the calcium phosphate
124 method with pLTR-Tat and the pSIVmac239 vector expressing the SIVmac239 envelope
125 glycoproteins. One day after transfection, cells were metabolically labeled for 16 h with 100
126 $\mu\text{Ci/ml}$ [^{35}S]methionine-cysteine ([^{35}S] protein labeling mix; Perkin-Elmer) in Dulbecco's
127 modified Eagle's medium lacking methionine and cysteine and supplemented with 5% dialyzed
128 fetal bovine serum. Cells were subsequently lysed in RIPA buffer (140 mM NaCl, 8 mM
129 Na_2HPO_4 , 2 mM NaH_2PO_4 , 1% NP-40, and 0.05% sodium dodecyl sulfate [SDS]).
130 Precipitation of radiolabeled SIVmac239 envelope glycoproteins from cell lysates or medium
131 was performed with a mixture of sera from SIV-infected macaques. The association index is a
132 measure of the ability of the mutant gp120 molecule to remain associated with the Env trimer
133 complex on the expressing cell, relative to that of the wild-type Env trimer. The association

134 index is calculated as follows: $\text{association index} = \frac{([\text{mutant gp120}]_{\text{cell}} \times [\text{wild-type}$
135 $\text{gp120}]_{\text{supernatant}})}{([\text{mutant gp120}]_{\text{supernatant}} \times [\text{wild-type gp120}]_{\text{cell}})}$. The processing index is a
136 measure of the conversion of the mutant gp160 Env precursor to mature gp120, relative to that
137 of the wild-type Env trimer. The processing index was calculated by the formula: processing
138 $\text{index} = \frac{([\text{total gp120}]_{\text{mutant}} \times [\text{gp160}]_{\text{wild-type}})}{([\text{gp160}]_{\text{mutant}} \times [\text{total gp120}]_{\text{wild-type}})}$.

139 Alternatively, medium containing radiolabeled envelope proteins was
140 immunoprecipitated with CD4-Ig for 1 h at 37°C in the presence of 50 µl of 10% protein
141 A-Sepharose (Amersham Biosciences). Of note, while some differences between
142 human and rhesus CD4/CCR5 have been reported (24, 26, 27) we decided to use both
143 human CD4-Ig and cells expressing human CCR5 to be consistent with previous reports
144 characterizing the HIV and SIV gp120 inner domain layers (15, 16). For CCR5 binding,
145 normalized amounts of radiolabeled SIVmac239 gp120 envelope glycoproteins were
146 incubated in the presence or absence of 200 nm of sCD4 for 1 h at 37°C. Subsequently,
147 2×10^6 Cf2Th-CCR5 cells were added for an additional 1 h at 37°C and washed twice
148 with phosphate-buffered saline (PBS) prior to cell lysis in RIPA buffer. Cell lysates were
149 immunoprecipitated with a mixture of sera from SIV-infected macaques. All samples
150 were loaded on polyacrylamide gels and analyzed by autoradiography and by using a
151 PhosphorImager (Molecular Dynamics).

152

153 **Recombinant luciferase viruses.**

154 Recombinant viruses containing the firefly luciferase gene were produced by calcium
155 phosphate transfection of 293T cells with the HIV-1 proviral vector pNL4.3 Env- Luc and
156 the plasmid expressing the wild-type or mutant SIVmac239 envelope glycoproteins at a
157 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 MgSO_4 , 15 mM MKPO_4 [pH 7.8], 1 mM ATP, and
170 1mM dithiothreitol) and 50 μl of 1mM D-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 lysis buffer (Promega) and three freeze-thaw cycles.
180 Luciferase activity in each well was measured as described above.

181

182 **Purification of recombinant SIV_{mac239} gp120 glycoproteins**

183 FreeStyle 293F cells (Invitrogen) were grown in FreeStyle 293F medium (Invitrogen) to
184 a density of 1×10^6 cells/ml at 37°C with 8% CO₂ with regular agitation (125 rpm). Cells
185 were transfected with a codon-optimized plasmid expressing His₆-tagged wild-type or
186 mutant SIV_{mac239} gp120 using the 293Fectin reagent, as directed by the manufacturer
187 (Invitrogen). One week later, the cells were pelleted and discarded. The supernatants
188 were filtered (0.22- μ filter) (Corning) and the gp120 glycoproteins were purified by nickel
189 affinity columns, as directed by the manufacturer (Invitrogen) followed by FPLC
190 purification of monomeric gp120, as described (29). The gp120 preparations were
191 dialyzed against PBS and stored in aliquots at -80°C. To assess purity, recombinant
192 proteins were loaded on SDS-PAGE polyacrylamide gels and stained with Coomassie
193 Blue.

194

195 **SPR biosensor analysis.**

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

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

210

211

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

235 by radiolabelling and immunoprecipitation of cell lysates and supernatant with polyclonal
236 sera from SIV-infected macaques. All of the mutants were expressed efficiently and only
237 mutants T248A, R249A, L481A and L483A exhibited a marked decrease in proteolytic
238 processing of the gp160 Env precursor (Table 1) (Figure 2A). A similar phenotype was
239 previously observed for T248A in HIV-1 gp120, perhaps due to its proximity to cysteine
240 247 which might be important for Env folding. While in HIV-1 gp120 only changes in
241 residue W479 significantly disrupted the non-covalent association of gp120 with the Env
242 trimer (16), in SIVmac239 changes in residues 478-481 affected trimer stability below
243 half of their wild-type counterpart (Table 1, Figure 2B and Figure 5A). Similar to the
244 unique position of W479 in HIV-1 gp120, residues 478-481 of SIVmac239 are located at
245 the center of a hydrophobic interface between Layers 2 and 3 (Figure 1 D and E) and
246 their mutation might affect trimer stability through effects on Layer 2-Layer 3
247 association, which could indirectly affect gp120-trimer association by altering either the
248 gp120 trimer association domain or the gp120-gp41 interface.

249

250 **Function of the mutant SIV envelope glycoproteins**

251 We next evaluated the effect of alteration of Layer 3 residues on the ability of
252 SIVmac239 Env to mediate viral infectivity (Figure 3A) and cell-to-cell fusion (Figure
253 3B). In striking contrast with what we reported for HIV-1 gp120 Layer 3 mutants where
254 only W479 presented a significant decrease in viral infectivity (16), we observed that the
255 majority of SIVmac239 gp120 Layer 3 changes (T248A, R249A, E477A, L478A, Y479A,
256 R480A, L481A, E482A, L483A) impacted viral infectivity below 20% of the wild-type.
257 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

281 gp120 Layer 3 variants, the observed decreases in CD4 binding were mainly due to
282 decreased on-rates (A476G, E477A, Y479A and E482A) compared with that of WT
283 gp120 except for T248A (Table 1). Thus, gp120 mutants with alterations in Layer 3 fail
284 to engage CD4 efficiently. Therefore, multiple residues in Layer 3 of the gp120 inner
285 domain that, based on our modeling, do not directly contact CD4 nonetheless contribute
286 to the affinity of the gp120-CD4 interaction. A network of interactions involving some
287 residues of the β 8- α 5 region in Layer 3 may contribute to CD4 binding by helping shape
288 the nearby Phe 43 cavity that directly contacts CD4 (20, 34, 35).

289

290 The functional consequence of CD4 binding for HIV-1 and SIV envelope
291 glycoproteins is the interaction with co-receptor CCR5 or CXCR4 (3-10). We analyzed
292 whether introducing changes in Layer 3 of the SIVmac239 gp120 inner domain affected
293 CCR5 recognition. Radiolabeled wild-type and mutant SIV gp120 glycoproteins were
294 incubated in the presence or absence of sCD4 for 1 h at 37 °C prior to incubation with
295 cells expressing the CCR5 coreceptor for 1 h at 37°C. After washing and lysis of the
296 cells, bound gp120 was detected by immunoprecipitation. As shown in Table 1 and
297 Figure 4B, L478A was the only variant presenting reduced CCR5 binding to less than
298 40%. This defect was more pronounced upon sCD4 addition, suggesting that this
299 mutant has some difficulties in assuming the CD4-bound conformation required to
300 engage its co-receptor. The defect in CCR5 binding could explain the lower cell-to-cell
301 fusion efficiency brought by L478A (Figure 3B and Table 1). However, its decreased
302 subunit association, gp160 processing and CD4 binding could also account for this phenotype
303 (Table 1).

304

305 **DISCUSSION**

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

321

322 In the transition from the unliganded state to the CD4-bound state, the inner
323 domain of gp120 experiences major conformational rearrangements (15, 38-42).
324 Located at the interface between the inner domain and outer domain, Layer 3 has been
325 shown to play a pivot-like role in the layered allosteric changes of the HIV-1 gp120 inner
326 domain by decreasing the spontaneous sampling of the CD4-bound conformation,
327 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

351 gp120 tryptophan 375 and Layer 3 play a predominant role on CD4 interaction. In HIV-
352 1 gp120, which lacks a tryptophan at position 375, the propensity to assume the CD4-
353 bound conformation is lower and therefore, Layers 1 and 2 help secure CD4 binding by
354 decreasing the off-rate of gp120-CD4 interaction, as previously described (15).

355

356 The major involvement of SIV gp120 Layer 3 in CD4 binding suggests that Layer
357 3 residues are important for optimal exposure of the CD4-binding site. This is
358 particularly true in SIV which has a large-filling residue at position 375 and where Layer
359 3 appears to maintain a critical relationship between the gp120 inner and outer domains
360 required for the intermolecular signaling required for gp41 HR1 exposure upon CD4
361 interaction. Our work completes a detailed analysis of HIV and SIV inner domain layers
362 and shows how subtle lineage-specific differences in the gp120 inner domain affect Env
363 function.

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372

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567 **FIGURE LEGENDS**

568

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 ³⁵S-labeled

589 cells transiently expressing the SIVmac239 WT and mutant envelope glycoproteins

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

600

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

609

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

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

621

622 **Figure 5. Residues important for gp120-trimer association and CD4 binding.**

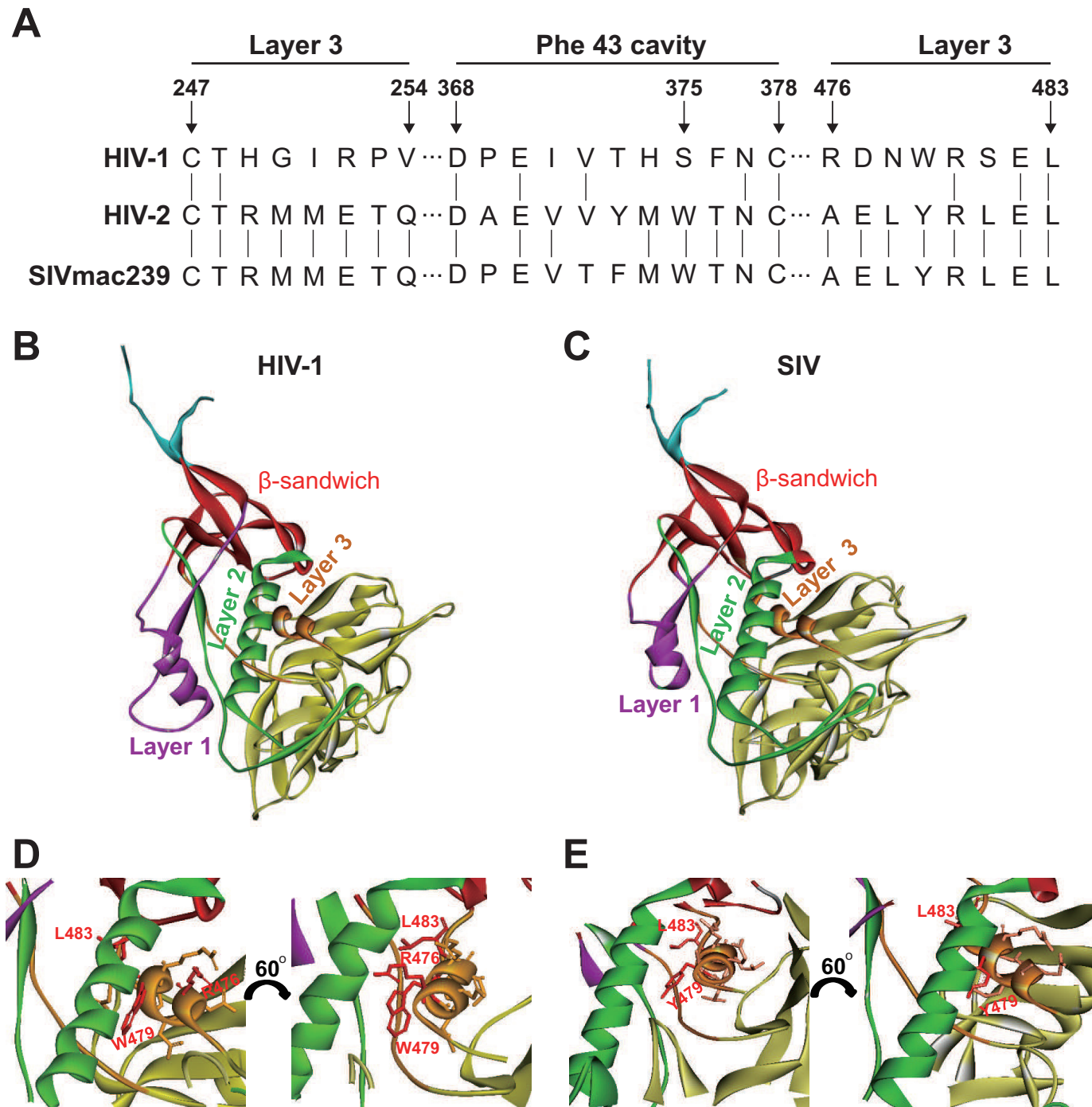
623 Layer 3 of HIV-1 gp120 (21) and the modeled SIVmac239 gp120 are shown from the
624 same perspective as those in Figure 1, B and C. In **A**, the ribbon and side chain
625 residues that were altered in this and a previous study (16) are colored according to the
626 gp120-trimer association index (Red: association index < 0.5 and Green: association
627 index \geq 0.7). In **B**, the ribbon and side chain residues are colored according to CD4-Ig
628 binding ability (Red: relative CD4-Ig binding \leq 0.5 and Green: relative CD4-Ig binding >
629 0.5).

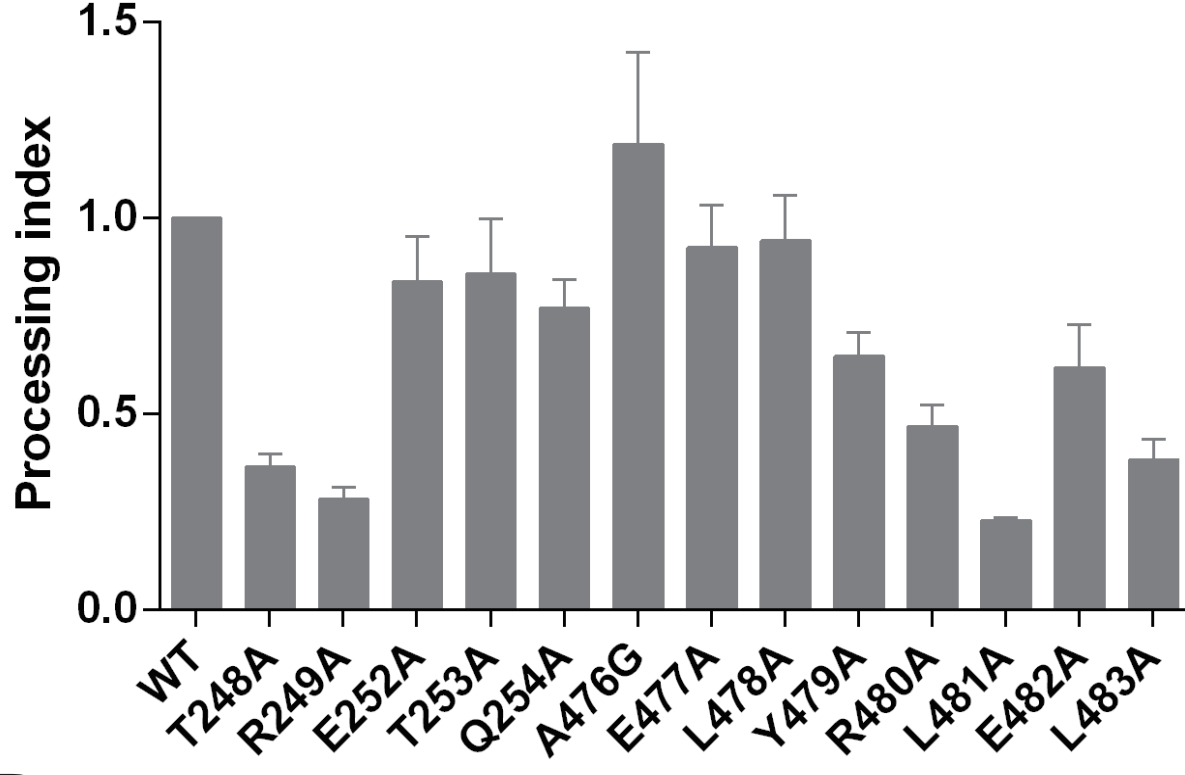
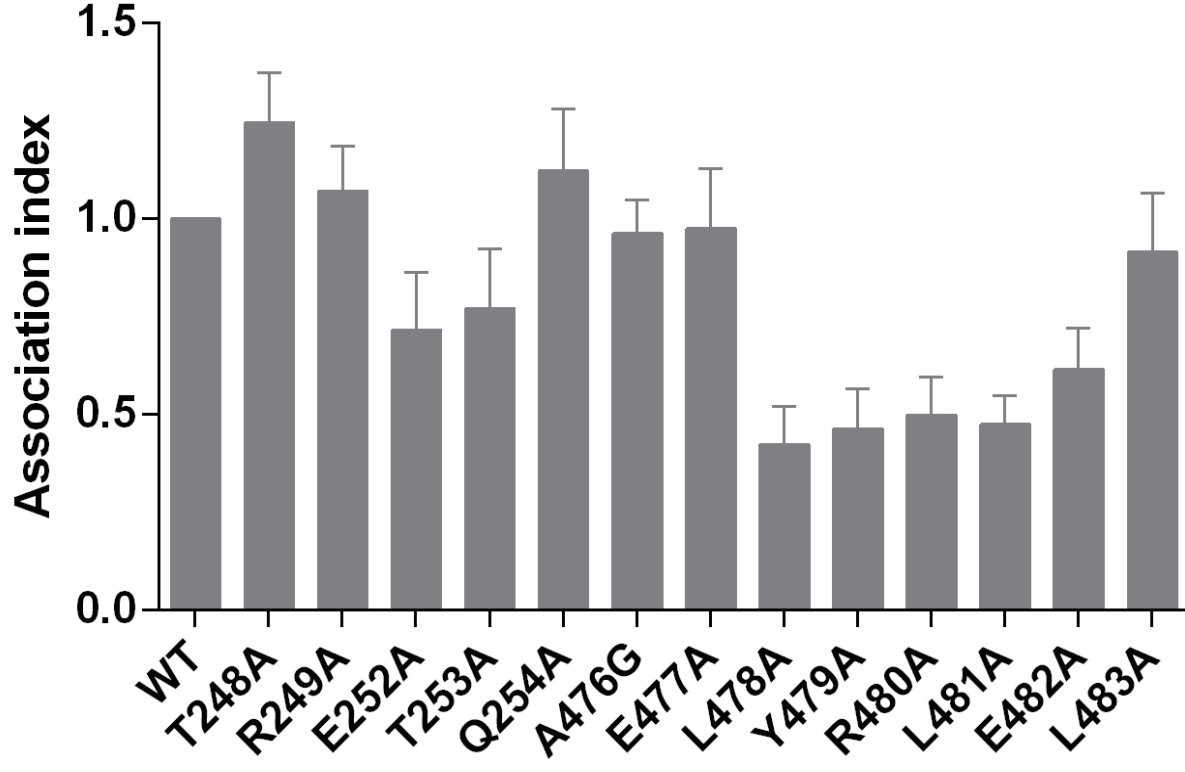
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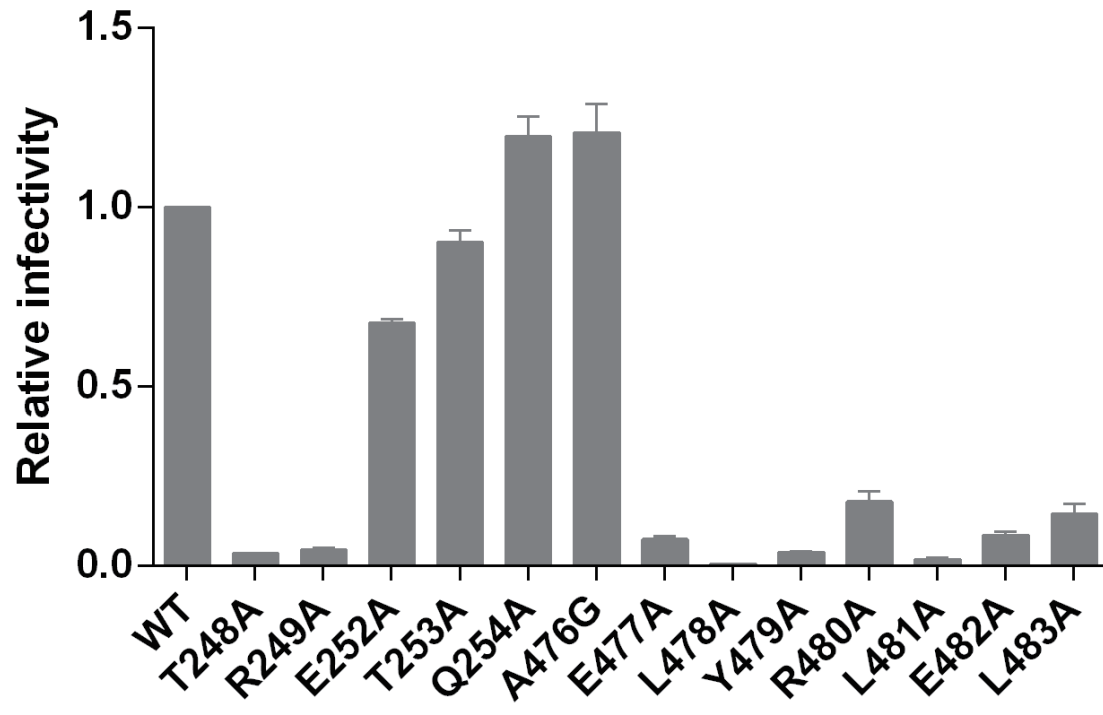
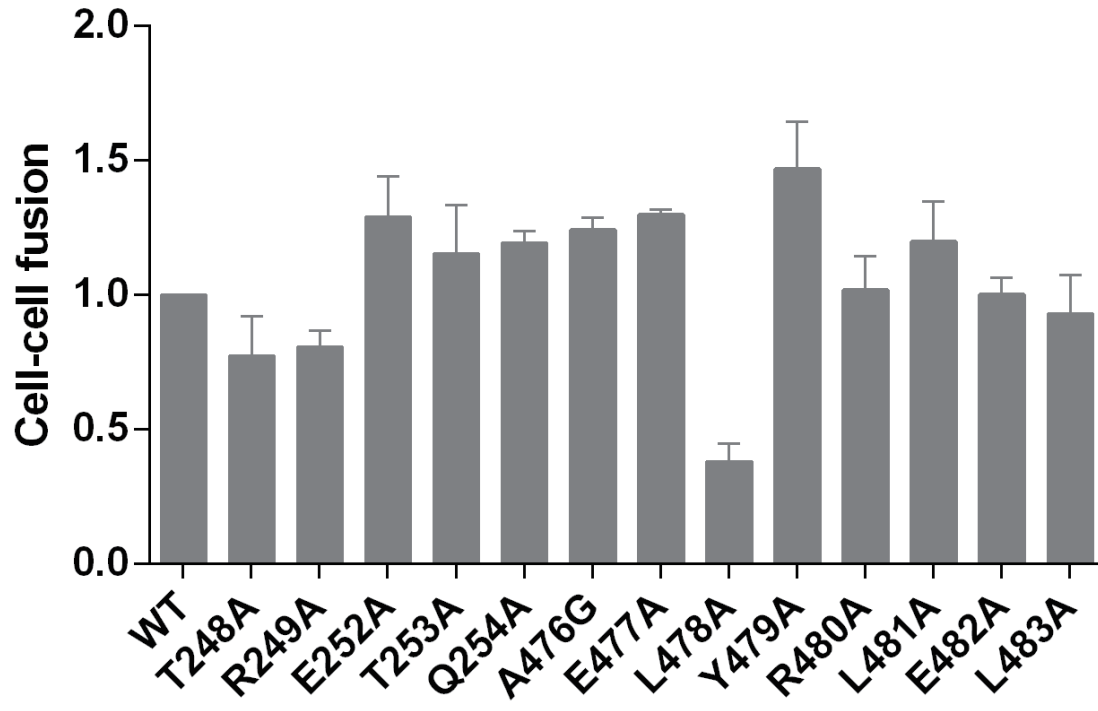
631 **Figure 6. Summary of functional differences between HIV-1 and SIVmac239 gp120**

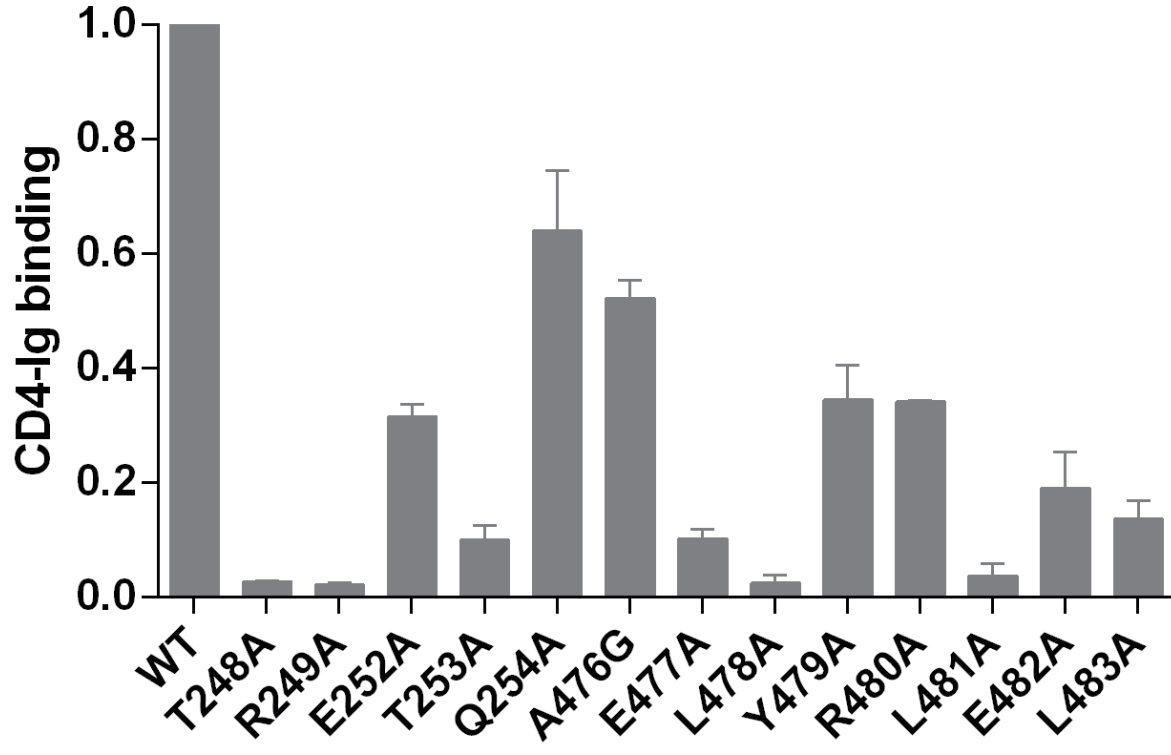
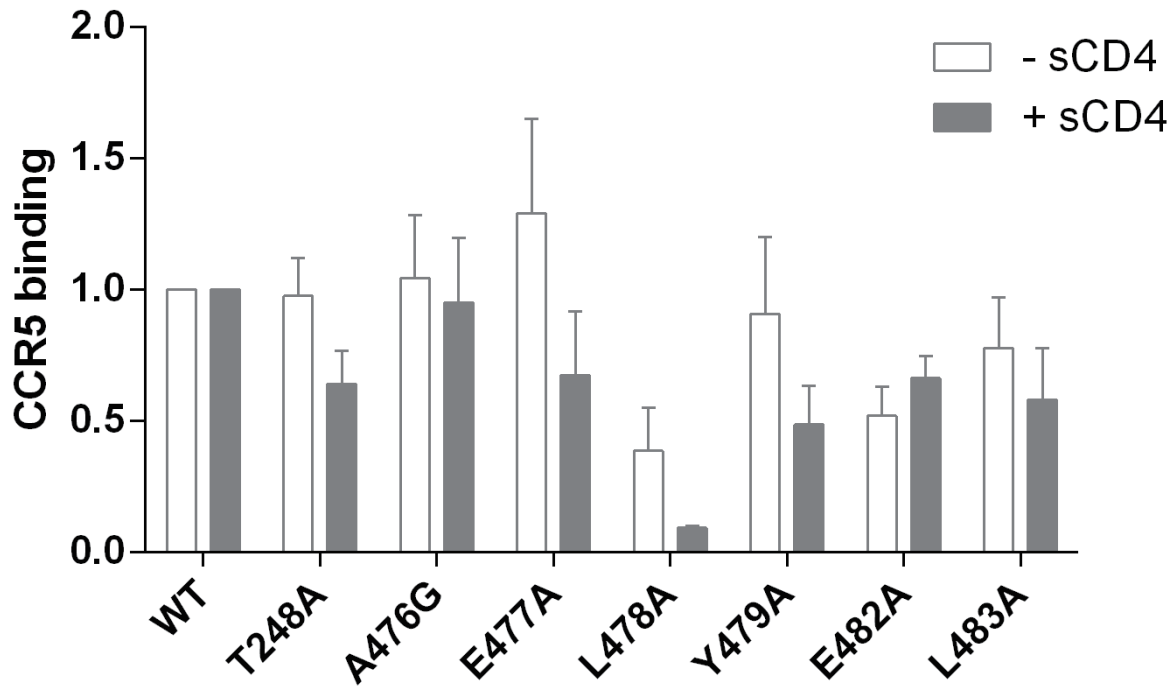
632 **glycoproteins.** One of the three gp120 subunits of the HIV-1 and SIVmac239 envelope
633 glycoprotein trimer is depicted. In the orientation shown, the trimer axis runs vertically
634 on the left of each figure and the viral membrane is at the top of the figure. The gp120 N
635 and C termini are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

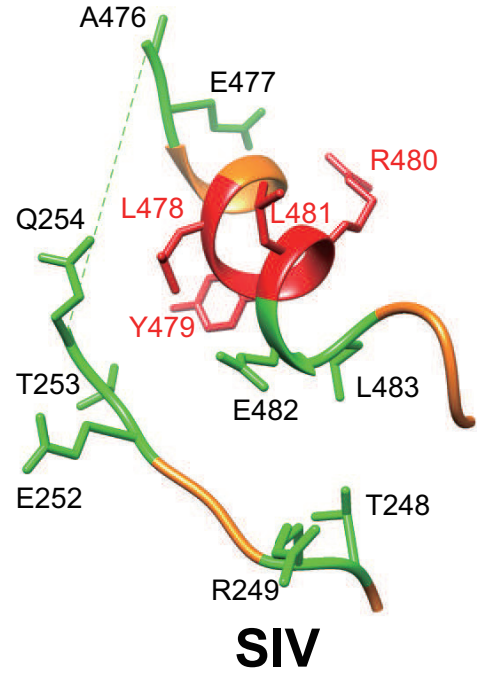
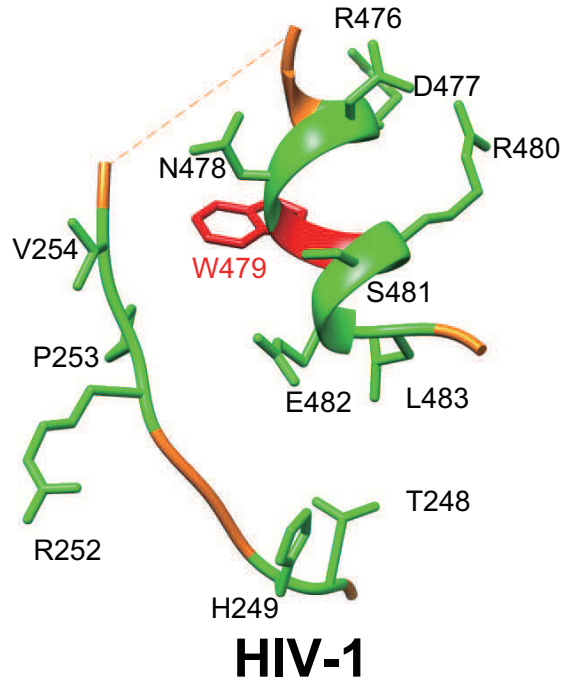
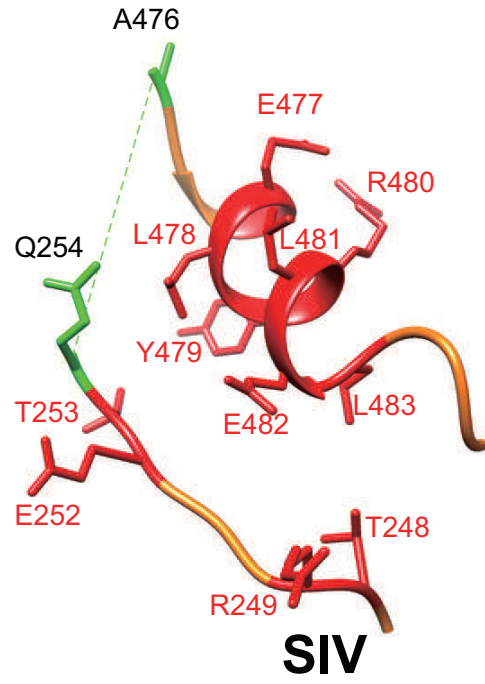
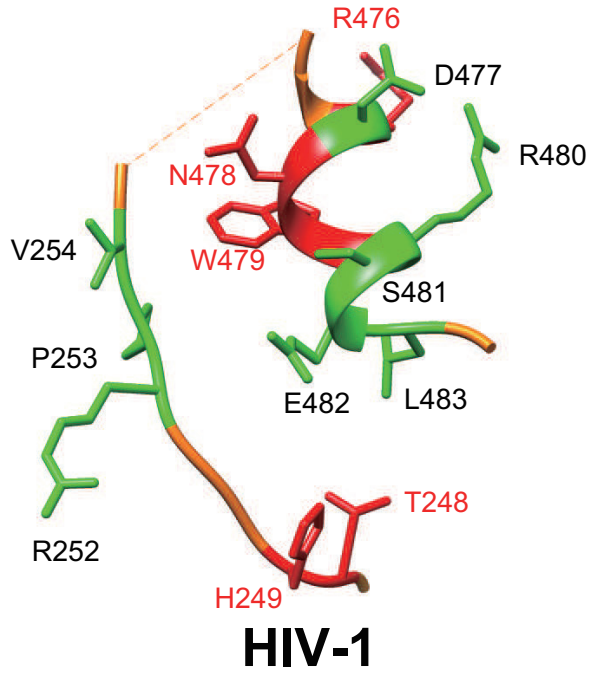
636 Layer 3 yellow, and the outer domain (OD) ochre. The Trp 375 side chain that fills the
637 Phe 43 cavity of SIV gp120 is depicted. The major contributions of the gp120 N and C
638 termini, the inner domain (the β -sandwich, Layer 1, Layer 2 and Layer 3) and the Phe
639 43 cavity to gp120-trimer association 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.



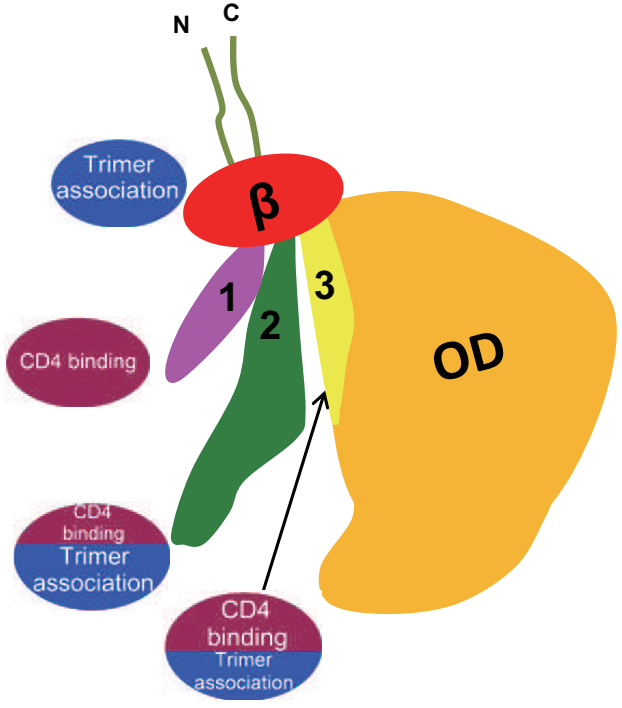
A**B**

A**B**

A**B**

A
gp120 - trimer association**B**
gp120 - CD4 binding

HIV-1 gp120



SIV gp120

