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1 **Title:** HIV-1 BF intersubtype recombinant Vpu second alpha helix plays an important role in
2 the viral release and BST-2 degradation
3
4 **Running Title:** HIV-1 BF intersubtype recombinant Vpu
5
6 **Authors:** Cristian De Candia, Constanza Espada, Gabriel Duette, Horacio Salomón, and
7 Mauricio Carobene§
8 **Affiliation**: Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS),
9 Universidad de Buenos Aires, Paraguay 2155 Piso11, C1121AGB, Buenos Aires, Argentina
10
11 § Corresponding author: Mauricio Carobene, PhD
12 E-mail: mcarobe@fmed.uba.ar
13 Phone: 54 11 4508-3689 / 3671
14 Fax: 54 11 4508-3705
15
16
17 **Word count text: 6554**
18 **Word count abstract: 472**
19 **Figure count: 7**

Abstract

We previously reported a naturally occurring BF intersubtype recombinant Vpu variant with augmented capacity to enhance viral replication. Structural analysis of this variant revealed that its transmembrane domain (TMD) and α-helix I in the cytoplasmic domain (CTD) corresponded to subtype B, whereas CTD α-helix II corresponded to subtype F1. J gtg'y g'""' aimed at evaluating the role of Vpu CTD α-helix II domain on viral release enhancement and down-modulation of BST-2 and CD4 from cell surface. In addition, as serine residues in either Vpu amino acid positions 61 or 64 have been shown to regulate Vpu intracellular half-life, which in turn could influence the magnitude of viral release, we also studied the impact of these residues in the VpuBF functions, since S61 and S64 are infrequently found among BF recombinant Vpu variants. Our results showed that interchange of Vpu α-helix II between subtypes (B→F) directly correlated with enhancement of viral release and, to a lesser extent, with changes in the capacity to down-modulate BST-2 and CD4 of the resulting chimera. No differences on viral release and BST-2 down-modulation were observed between VpuBF and VpuBF-E61S. On the other hand, VpuBF-A64S showed a slightly reduced capacity to enhance viral production but was modestly more efficient than VpuBF in down-modulating BST-2. In summary, our observations clearly evidence that α-helix II is actively involved in Vpu viral release-promoting activity, and that intersubtype recombination between subtypes B and F1 originated a protein variant with higher potential to boost the spread of the recombinant strain that harbors it.

46 **Introduction**

47 The viral protein U (Vpu), encoded in the HIV-1 genome (Cohen *et al.*, 1988; Strebel *et al.*,
48 1988), is a type I transmembrane protein consisting of a short N-terminal domain, a single
49 transmembrane α-helix domain, two cytosolic α-helices separated by a flexible connector
50 loop, and a short C-terminal tail. This protein is known for performing two important
51 functions during HIV-1 infection of target cells: a) it mediates the proteasomal degradation of
52 newly synthesized CD4 receptor in the endoplasmic reticulum (Magadan *et al.*, 2010;
53 Margottin *et al.*, 1998), and b) it enhances the release of virions from infected cells by
54 antagonizing the restriction imposed by BST-2 (CD317/HM1.24/tetherin), an INFα-inducible
55 host restriction factor (Neil *et al.*, 2008; Van Damme *et al.*, 2008). In addition, it has been
56 recently demonstrated that SLAMF6/NTB-A, a transmembrane protein that induces natural
57 killer cell-mediated killing of infected cells, is also down-regulated by Vpu from the plasma
58 membrane (Shah *et al.*, 2010).

59 Early reports suggested that different Vpu domains were directing separate functions,
60 identifying its cytoplasmic domain (CTD) as critical for CD4 degradation, and the
61 transmembrane domain (TMD) as involved in promotion of viral release (Schubert *et al.*,
62 1996a; Schubert *et al.*, 1996b; Schubert *et al.*, 1994); however, recent studies demonstrated
63 that discrete regions in both CTD and TMD are required to carry out its main functions
64 (Magadan *et al.*, 2010; Mangeat *et al.*, 2009; Nomaguchi *et al.*, 2010; Petit *et al.2011*).

65 We recently reported a naturally occurring BF intersubtype recombinant Vpu with improved
66 *in vitro* capacity to enhance viral replication when compared to a subtype B variant. The
67 analysis of numerous BF intersubtype recombinant Vpu sequences revealed a highly
68 conserved recombination pattern, similar to that observed in the prototypic BF recombinant
69 strain CRF12_BF, in which the membrane-spanning and α-helix I domains correspond to
70 subtype B, and the α-helix II to subtype F1 (De Candia *et al.*, 2010). This pattern displays a
71 breakpoint in a flexible and highly conserved amino acid sequence located between α-helix I

72 and II. This region contains two critical serine residues (S52 and S56) which phosphorylation
73 by casein kinase II (CK-II) is crucial for proteasome-mediated CD4 degradation (Magadan *et*
74 *al.*, 2010; Margottin *et al.*, 1998) and for the control of BST-2 intracellular trafficking and/or
75 degradation (Douglas *et al.*, 2009; Goffinet *et al.*, 2009; Goffinet *et al.*, 2010; Mangeat *et al.*,
76 2009; Mitchell *et al.*, 2009; Miyagi *et al.*, 2009; Schmidt *et al.*, 2011; Tervo *et al.*, 2011).
77 On the other hand, according to an early report by Estrabaud et al, Vpu degradation is a
78 proteasome-mediated process that involves phosphorylation of a serine residue, either at
79 positions 61 or 64 (S61 or S64), that triggers Vpu degradation by a not well elucidated β-
80 TrCP-independent pathway (Estrabaud *et al.*, 2007). Replacement of S61 with alanine
81 correlated with a marked increase in the protein half-life and, consequently, with the
82 enhancement of viral release. This mutation did not affect CD4 degradation (Estrabaud *et al.*,
83 2007; Hill *et al.*, 2010). We found that S61 is absent in the CRF12_BF Vpu sequence and in
84 most of the related BF recombinant sequences, and that only 6.6% of them have a serine
85 residue at position 64 (De Candia *et al.*, 2010).
86 Taking all this evidence into account, the present work was aimed at evaluating the
87 contribution of α-helix-II in Vpu CTD and the impact of serine residues at positions 61 or 64,
88 on viral release promotion and BST-2 and CD4 down-modulation capacity, in order to clarify
89 the relationship between the structural and functional variations observed in the BF
90 recombinant Vpu.
91

92 **Results**

93

94 **BF Vpu's α-helix II determines an increase in viral release in Vpu-dependent cell line**

95 **models**

96 To evaluate the role of the α-helix II in viral release and to assess the potential consequences
97 of recombination involving this Vpu domain on it functions, two different recombinant Vpu
98 variants were generated: i) **VpuBF/B**, where α-helix-II from a CRF12_BF-like variant was
99 replaced with the α-helix-II from the NL4-3 reference strain, and ii) **VpuB/F1**, where α-helix-
100 II from NL4-3 was replaced with the α-helix-II from the CRF12_BF-like variant (Fig. 1a,b).
101 To evaluate the protein expression levels, the cloning strategy used included the incorporation
102 of a 6xHis-tag at the C-terminus of each recombinant protein variant. SDS-PAGE and
103 Western blot analysis, as described in the Methods section, showed that all Vpu variants were
104 expressed at similar levels (Fig. 2). These proteins were fully functional regarding the Vpu
105 canonical activities.
106 HeLa cells were used as a Vpu-dependent model to evaluate viral production. As described in
107 the Methods section, pNL4-3 U35 and each of the Vpu expression vectors pCG-VpuB, pCG-
108 VpuF1, pCG-VpuBF, pCG-Vpu B/F1 and pCG-Vpu BF/B, were used to separately co-
109 transfect HeLa cell cultures. Co-transfection with the empty pCG vector was used as a
110 negative control. Forty eight hours post-transfection, viral production was monitored by
111 quantifying p24 antigen in cell culture supernatants. Similar transfection efficiency was
112 achieved in all cases, as determined by the percentage of GFP-positive cells evaluated by
113 FACS (data not shown).
114 As depicted in Fig. 3 (a), transfection with VpuBF expression vector resulted in an
115 approximately 2-fold increase in p24 production when compared to VpuB, which is in line
116 with our previous observations (De Candia *et al.*, 2010). Interestingly, VpuB/F1 was more
117 efficient in promoting viral release than both VpuB and VpuF1 ($p>0.01$). Of note, viral

118 production measured in VpuBF/B was lower than that associated to the naturally occurring
119 VpuBF (p>0.01), resembling the VpuB biological behaviour. In this regard, no statistical
120 differences were observed in p24 production among Vpu BF/B vs. VpuB, or VpuBF vs. Vpu
121 B/F1. VpuF1 p24 production was slightly but significantly higher than VpuB (p<0.01) and
122 significantly lower than VpuBF (p<0.01).
123 These results clearly point out that, in our intersubtype recombination study model, Vpu α-
124 helix II is involved in the viral release promotion function of Vpu.
125
126 **A64S mutation in BF intersubtype recombinant Vpu affects viral release**
127 Phosphorylation of serine residues by a protein kinase is a process known to induce functional
128 changes in a target protein or to regulate protein's fate (Ciesla *et al.*, 2011; Johnson &
129 Barford, 1993; Tarrant & Cole, 2009). As stated above, phosphorylation of serine residues at
130 positions 52 and 56 (S52, S56) in HIV-1 Vpu protein regulates its BST-2 and CD4 down-
131 modulation capacity, while phosphorylation of serine residues either at positions 61 or 64
132 (S61 or S64) has been found to influence the protein half-life (Estrabaud *et al.*, 2007). As
133 shown in Fig. 1 (a), reference strain NL4-3 Vpu has a serine residue at position 61 (S61) and a
134 valine residue at position 64 (A64), while BF intersubtype recombinant Vpu has a glutamic
135 residue at position 61 (E61) and an alanine residue at position 64 (A64).
136 To test the hypothesis that absence of S61 and/or S64 in the BF recombinant Vpu could
137 contribute to increase its capacity to promote viral production, mutant Vpu variants, in which
138 amino acids at positions 61 or 64 were replaced with serine residues, (pCG-VpuBF E61S and
139 pCG-VpuBF A64S) (Fig. 1a) were generated. VpuB expression vectors in which S61A and
140 V64S mutations were introduced (pCG-VpuB S61A, pCG-VpuB V64S) were included as
141 controls in the study.
142 The impact of these changes in p24 production was evaluated by performing Hela cell co-
143 transfections and p24 quantitation as described above. We evaluated the protein expression

144 level of each construct by Western blot (Fig. 2). No significant differences in p24 production
145 were observed among VpuBF or VpuBF E61S. However, VpuBF A64S viral production was
146 slightly but significantly lower than viral production for VpuBF ($p<0.01$)(Fig. 3b).
147 Different results were obtained for VpuB; in line with those reported by Estrabaud et al
148 (Estrabaud *et al.*, 2007), replacement of S61 with alanine (VpuB S61A) was associated to a
149 significant increase in p24 production when compared to the wild type protein (VpuB),
150 confirming the contribution of S61 in the viral enhancement function of this particular (NL4-
151 3) viral strain. When S61 was absent and the valine residue at position 64 was replaced with a
152 serine residue (VpuB V64S) no significant difference in p24 production was observed.
153
154 **Pure subtype, chimeric and serine-mutant Vpu variants have different capacities to**
155 **down-modulate BST-2**
156 To determine the relative capacity of pure subtype, chimeric and serine-mutant Vpu variants
157 to down-modulate BST-2 from cell surface, HeLa cell cultures were transfected with each
158 Vpu-GFP expression vector as described above. Transfection with pCG-GFP empty vector
159 was used as negative control. Transfection efficiency, assessed as percentage of GFP-positive
160 cells, was similar in all cases (data not shown).
161 Forty eight hours post-transfection, BST-2 cell surface expression was assessed by FACS and
162 expressed as mean fluorescence intensity (MFI) (Fig. 4).
163 All the variants studied retained their capacity to down-modulate BST-2 from cell surface. As
164 shown in Fig. 4 (a), VpuBF was more efficient in modulating BST-2 when compared with
165 VpuB and VpuF ($p<0.01$). Among the variants studied, VpuF was clearly the least efficient in
166 down-modulating BST-2. No significant differences were observed when comparing VpuBF
167 and VpuB/F1 or VpuB and VpuBF/B. Remarkably, Vpu BF and Vpu B/F1 variants were
168 more efficient in modulating BST-2 than Vpu BF/B and VpuB respectively, indicating that α-
169 helix II is also implicated in Vpu-dependent BST-2 cell surface down-modulation.

170 Nevertheless, viral release and BTS-2 cell surface expression observed for the BF
171 recombinant variants did not show any direct correlation.
172 As for the role of serine residues on BST-2 expression (Fig. 4b), no statistically significant
173 differences were observed between VpuB and VpuB S61A or VpuBF and VpuBF E61S.
174 Interestingly, VpuBF A64S showed a slightly lower capacity to down-modulate BST-2 from
175 cell surface than VpuBF ($p < 0.01$).
176
177 **Pure subtype and chimeric Vpu have different capacities to down-modulate CD4**
178 **expression**
179 We subsequently examined and compared the ability of pure subtype, chimeric and serine-
180 mutant Vpu variants to induce the down-modulation of CD4 from the cell surface. HeLa T4
181 cells were transfected separately with each Vpu-GFP expression vector. Transfection with
182 pCG-GFP empty vector was used as negative control.
183 Forty eight hour post-transfection, CD4 cell surface expression in Vpu-expressing cells was
184 evaluated by FACS, and expressed as MFI. Similar transfection efficiency, determined as
185 percentage of GFP-positive cells by FACS, was obtained in all cases (data not shown).
186 All Vpu variants efficiently reduce CD4 cell surface expression when compared to empty
187 vector (Fig. 5). As shown in Fig. 5 (a), VpuF1, VpuBF and Vpu B/F1 were more efficient in
188 down-modulating CD4 from the cell surface than VpuB ($p < 0.01$). No statistically significant
189 differences were observed between VpuBF and VpuB/F1, or between VpuB and VpuBF/B.
190 Regarding the role of the serine residue at positions 61 and 64 on CD4 cell surface expression,
191 no statistically significant differences were observed between the serine-mutants Vpu studied
192 (Fig. 5b).
193

194 **Discussion**

195 Despite the extensive knowledge about HIV molecular epidemiology, studies on HIV
196 replication and pathogenesis among non-B isolates are still scarce. However, some clear
197 subtype-specific features have been reported (Abraha *et al.*, 2009; Arien *et al.*, 2005; Geuenich
198 *et al.*, 2009; Holguin *et al.*, 2006; Ramirez de Arellano *et al.*, 2006).

199 It has been proposed that the fact that only HIV-1 strains from the M group express a fully
200 functional Vpu protein may explain, at least in part, the global HIV/AIDS pandemic for the
201 HIV-1 M group (Petit *et al.*, 2011; Sauter *et al.*, 2011; Sauter *et al.*, 2009). Notably, among the
202 51 CRFs described until now, just 4 of them exhibit a recombinant structure involving the Vpu
203 coding sequence; of them, CRF02_AG and CRF12_BF are the most represented recombinant
204 forms in the infected population (Hemelaar *et al.*, 2011). This might reflect some not yet clear
205 recombination restrictions at this region and the importance of the conservation/preservation of
206 its function.

207 In a previous study we found that a naturally occurring BF intersubtype recombinant Vpu has
208 an improved *in vitro* capacity to enhance viral replication. In addition, it was observed that the
209 recombination pattern of other numerous BF recombinant sequences is very similar to that
210 observed in the CRF12_BF, first described in Argentina. In a comparative analysis between
211 subtype B and BF recombinant Vpu amino acid sequences, major differences were found in the
212 α-helix II and C-terminal domains. This protein region has been strongly associated to Vpu
213 functions, therefore partial and full deletions of the cytoplasmic domain, affected negatively its
214 capacity to down-modulate CD4 (Pacyniak *et al.*, 2005) or to promote viral release (Dube *et*
215 *al.*, 2009).

216 Taking this evidence into account, the aim of this work was to determine the relevance of α-
217 helix II on the functional changes observed for the BF recombinant Vpu. To achieve this, two
218 chimeric proteins where the α-helix II were exchanged between NL4-3, and BF recombinant
219 variants were generated and used to perform *in vitro* studies.

220 Our results indicate that the replacement of α-helix II (B→F), resembling the structure of the
221 naturally occurring recombinant variant, directly correlated with the enhancement of viral
222 release, and with a moderate increase in BST-2 and CD4 down-modulation capacity. This
223 observation evidences that the α-helix II participates actively in the Vpu viral release-
224 promoting activity.
225 We also found that subtype F1 Vpu was relatively less efficient in reducing BST-2 cell surface
226 expression levels than the subtype B and BF recombinant Vpu studied. Nevertheless, its
227 capacity to promote viral release was slightly higher than that observed in the subtype B
228 variant.
229 Of note, the relative augmentation of viral production among the variants studied did not
230 directly correlate with their capacity to down-modulate BST-2 from the cell surface. This
231 discrepancy has been previously observed (Dube *et al.*, 2010; Goffinet *et al.*, 2010; Kuhl *et al.*,
232 2011; Mitchell *et al.*, 2009; Miyagi *et al.*, 2009; Nomaguchi *et al.*, 2010) allowing to
233 hypothesize that both activities may be relatively independent. In this regard, Schmidt et al
234 (Schmidt *et al.*, 2011) found that Vpu may interfere with both the BST-2 recycling from the
235 cell surface and the anterograde transport of newly synthesized BST-2, without affecting its
236 internalization rate, which leads to a reduction in its virion-tethering activity at cell surface.
237 Thus, BST-2 intracellular subpopulations with different trafficking characteristics and/or
238 susceptibility to Vpu may exist, and have a higher antiviral activity potential than molecules at
239 cell surface, explaining the lack of a precise correlation among viral production and BST-2 cell
240 surface expression.
241 On the other hand, the motif (E/D)XXXL(V/M/I) within α-helix II, which is highly conserved
242 among Vpu proteins from HIV-1 M group subtypes A, B, D, G and H, has been recently
243 identified as required for efficient BST-2 down-modulation and enhancement of viral release
244 (Kueck & Neil, 2012). Vpu variants used in this work display differences in the location and
245 amino acid composition of this motif, i.e. $E_{59}XXXL_{63}V$ in subtype B and $(D/E)_{62}XXXL_{66}(A)$ in

246 subtype F1 and BF recombinant, and as suggested by Kuek and Neil (Kueck & Neil, 2012),
247 such motifs may not be functionally equivalent.
248 Proteasomal degradation of Vpu, which occurs through a β-TRCP complex independent
249 pathway, has been shown to involve phosphorylation of a serine residue at position 61
250 (Estrabaud *et al.*, 2007). Mutation of S61 led to accumulation of Vpu within cells and
251 augmentation of viral release. S61 is not present in CRF12_BF or related BF recombinant Vpu
252 sequences, and only 6.6% of BF sequences had S64 (De Candia *et al.*, 2010). These evidences
253 prompted us to examine the effect of S61 and S64 on the BF recombinant Vpu function. Our
254 results showed that both VpuBF E61S and the wild-type VpuBF E61 variants behaved
255 similarly in terms of promoting viral release in a tetherin-expressing cell model. Nevertheless,
256 we found a modest reduction in viral production when alanine residue at position 64 was
257 replaced by serine. This change also correlated to a slight increase in the capacity of the
258 VpuS64 variant to down-modulate BST-2. No changes in CD4 degradation were observed in
259 VpuBF E61S or VpuBF A64S. Although the biological impact of these small differences
260 remains to be established, these results suggest that regulation of Vpu degradation, and the
261 relationship with the protein functions, is a complex process for which other steps may be
262 required., regardless of the simple phosphorylation event,
263 In summary, data presented here clearly show that structural modifications introduced by the
264 intersubtype recombination in BF Vpu at the level of its α-helix II correlates with the
265 previously observed augmentation in viral production, and with changes in its capacity to
266 modulate the steady-state BST-2 and/or CD4 cell surface levels. Regarding the role of residues
267 S61 and S64 in the BF Vpu function, we found that S64 but not S61 did affect the capacity of
268 Vpu to down-modulate BST-2 and to promote viral release of the variant studied. To the best of
269 our knowledge this is the first report obtained from other than a B subtype Vpu variant.
270 Altogether, these findings shed more light on the molecular basis of the Vpu function,
271 underscoring the power of recombination as a variability source during HIV-1 evolution.

272 In an elegant report by Sato et al, it was shown that Vpu enhances the kinetics of cell-free virus
273 propagation, especially during the initial phase of infection, which leads to a rapid systemic
274 HIV-1 dissemination *in vivo* (Sato *et al.*, 2012). Thus, if intersubtype recombination has the
275 potential to generate structurally re-organized proteins with improved functions, such as the BF
276 intersubtype recombinant Vpu, then viruses harbouring them may have biological advantages
277 that play an important role in the successful spread of such HIV-1 recombinant variants.

278
279

280 **Methods**

281

282 **Plasmids**

283 To perform the functional analysis, we generated vectors co-expressing Vpu and GFP from a
284 single bicistronic RNA via an internal ribosome entry site (IRES). Briefly, *vpu* genes from
285 HIV-1 subtype B molecular clone pNL4-3 (Adachi *et al.*, 1986), subtype F1 molecular clone
286 p93BR020.1 (Gao *et al.*, 1998) and pVpuBF vector previously described (De Candia *et al.*,
287 2010) were used as templates for a PCR designed to introduce the XbaI restriction site and a
288 C-terminus 6xHis tag/MluI restriction site at 5' and 3' ends of the vpu coding sequence,
289 respectively. Primers used for this reaction were:

290 VpuB-XbaI (5´-CCTCTAGATAATGCAACCTATAATAG-3´),

291 VpuB-6xHis-MluI (5´-CACGCGT**TTA**GTGATGGTGATGATGATGGCCGGATCCCAGATCATCAATATCC-3´),

292 VpuF1-XbaI (5´- CCTCTAGATAATGTCAAATTTGTTAGC-3´),

293 VpuF1-6xHis-MluI (5´- CACGCGT**TTA**GTGATGGTGATGATGATGGCCGGATCCCAGATTATTAATGTCC-3´),

294 VpuBF XbaI (5´-CCTCTAGATAATGCAATCTTTAG-3´) and

295 VpuBF 6xHis-MluI (5´-CACGCGT**TTA**GTGATGGTGATGATGATGGCCGGATCCCAGAATATCAATATTC-3´)

296 Nucleotide changes corresponding to restriction sites and sequence coding for the 6xHis Tag
297 are underlined. Original Vpu stop codon was eliminated with the addition of the
298 GSGHHHHHH amino acid sequence, and a new one was generated at the end of the tag
299 (highlighted in bold).

300 The amplicons obtained were first cloned into a commercial vector (pGEM T-easy, Promega)
301 and then subcloned into the XbaI/MluI harboring bicistronic cytomegalovirus-based pCG
302 expression vector co-expressing GFP (Lock *et al.*, 1999; Mariani & Skowronski, 1993),
303 generating expression vectors pCG-VpuB, pCG-VpuF1 and pCG-VpuBF. All mutagenesis
304 reactions were performed using a highly processive DNA polymerase with proofreading 3'-5'
305 exonuclease activity (Platinum®PfxDNA Polymerase, Invitrogen). The Vpu expression
306 vectors were then sequenced to assess *vpu* sequences integrity (Big Dye terminator kit and an

307 ABI 3100 automated sequencer, Applied Biosystems Inc, Foster City CA). Nucleotide
308 sequences from both strands were analyzed and manually adjusted using Sequencher 4.0.5
309 software (Gene Codes Co, USA).
310 For the construction of chimeric Vpu proteins, plasmids pCG-VpuB and pCG-VpuBF were
311 digested with XbaI and BpiI, both, digestion products (vector and inserts containing coding
312 sequences for the α-helix II and C-terminal domains), were gel purified and appropriate
313 ligations were carried out using T4 DNA ligase (Promega). The resulting plasmids were
314 named as pCG-VpuBF/B, where α-helix-II from CRF12_BF-like variant was replaced with
315 that from NL4-3, and pCG-VpuB/F1, where α-helix-II from NL4-3 was replaced with that
316 from the CRF12_BF-like variant (Fig. 1).
317 Mutation of serine at position 61 or 64 in pCG-VpuBF, serine at position 64 in pCG-VpuB
318 and alanine at position 61 in pCG-VpuB were introduced by site-directed mutagenesis.
319 Briefly, PCR reaction was carried out by using the following set of forward primers, where
320 codon substitutions are underlined:
321 VpuB S61A (5`-GGAGAAGTAGCAGCACTTGTGG-3`)
322 VpuB V64S (5´-GAAGTAGCAGCACTTTCGGAGATG-3´)
323 VpuBF E61S (5`-GGATGCATCAGAATTGGCAGC-3`)
324 VpuBF A64S (5´-GGATGCAGAAGAATTGTCAGC-3´)
325 The amplicons were subcloned into a commercial vector (pGEM-T® easy vector, Promega),
326 and then subcloned into pCG expression vector. The resulting vectors were sequenced to
327 ensure that mutation was introduced and to assess any possible unwanted changes introduced
328 during mutagenesis. The resulting plasmids were named as pCGVpuBF E61S, pCG-VpuBF
329 A64S, pCG-VpuB S61A and pCG-VpuB V64S (Fig. 1).
330 pNL4-3 U35 (Strebel *et al.*, 1988), a derivate of pNL4-3 containing an insertion within the
331 *vpu* ORF that causes a translational frame shift which results in a 35-amino acid truncated
332 form of the Vpu protein, was used as negative control.
333

334 **Cells**

335 HeLa and HeLa CD4+ cells were used in this study. HeLa cells were cultured in DMEM
336 medium (Gibco®) supplemented with 10% fetal bovine serum (FBS, Gibco®), 2 mM L-
337 glutamine (Gibco®) and penicillin/streptomycin (100 IU/ml and 100 µg/ml, respectively,
338 Gibco®). HeLa CD4+ cells were cultured in the same medium plus 500 µg/ml G418. Cell lines
339 were obtained through the AIDS Research and Reference Reagent Program.

340

341 **Viral release assay**

342 HeLa cells were co-transfected with 2 µg of HIV-1 proviral DNA (pNL4-3 U35) and 0.5 µg
343 of each of the Vpu expression vectors. Co-transfection with 500 ng of pCG-GFP empty vector
344 was set as negative control. Transient transfections were carried out using Lipofectamine2000
345 (Invitrogen) following manufacturer's instructions. Cell culture supernatants were collected
346 24 and 48 hours post-transfection. HIV-1 p24 antigen levels were measured using a
347 commercial ELISA kit (Murex, UK).

348

349 **Down-modulation of BST-2 and CD4 cell surface expression assays**

350 HeLa and HeLa CD4+ cells were transfected with 0.8 µg of the Vpu expression vectors
351 separately using Lipofectamine2000 (Invitrogen) following manufacturer's instructions. An
352 empty vector was used as negative control. At 48 h post-transfection, Hela cells were
353 harvested and incubated with the α-BST-2 primary antibody (obtained from the AIDS
354 Research and Reference Reagent Program), washed twice with ice-cold 1% BSA-PBS and
355 finally incubated with PE-conjugated anti-rabbit IgG secondary antibody (BD Bioscience) in
356 1% BSA-PBS. Cells were washed twice with ice-cold 1% BSA-PBS and acquired in a BD
357 FACSCanto flow cytometer. Data acquisition and analysis were performed using the BD
358 FACSDiva software, determining mean fluorescence intensity (MFI) of PE for transfected
359 cells (GFP positive) within the same well.

360 HeLa $CD4^+$ cell were stained with PE conjugated anti-CD4 monoclonal antibody (BD
361 Bioscience) in 1% BSA-PBS. Cells were washed twice with ice-cold 1% BSA-PBS and
362 analyzed as described above.
363
364 **Western blotting**
365 Vpu expression was assessed by transfecting HeLa cells with 4 µg of each pCG-Vpu
366 expression vector using Lipofectamine2000 (Invitrogen) following manufacturer's
367 instructions. At 48 hour post-transfection, cells were harvested and lysed in RIPA buffer (1%
368 NP-40, 0.5 % Na-DOC, 0.1 % SDS, 0.15 M NaCl; 50 mM Tris-HCl pH 7.4; 5 mM EDTA,
369 protease inhibitor cocktail). Lysates were boiled at 95°C for 5 min, separated on 12.5% SDS-
370 PAGE gels under reducing conditions. After gel electrophoresis, proteins were transferred
371 onto PVDF membranes. The expression of each Vpu variant in whole cellular lysates was
372 analyzed by immunoblot using 1:2000 diluted mouse anti-6xHis (BD Pharmingen™) and
373 secondary peroxidase labelled sheep anti-mouse antibody (Amersham Biosciences) at 1:2000
374 for chemiluminescent detection (ECL, GE Healthcare). β-actin was used as loading control by
375 immunoblot using 1:3000 diluted rabbit anti- β-actin (Abcam) and secondary peroxidase
376 labelled goat anti-rabbit antibody (Abcam) at 1:2000 for chemiluminescent detection (ECL,
377 GE Healthcare). Band intensity was quantified using the ImageJ software.
378
379 **Statistical analysis**
380 ANOVA with Tukey´s post-test (one-way ANOVA for comparison between groups) was
381 used. $P < 0.01$ was considered statistically significant.
382
383 **Competing interests**
384 The authors declare that they have no competing interests.
385

386 **Acknowledgements**

387 We thank Sergio Mazzini for his excellent work in manuscript editing and language revision.
388 CDC, CE, HS and MC were supported by the Argentinean National Research Council
389 (CONICET). This research was partially funded by the Argentinean Agency for the Promotion
390 of Science and Technology (ANPCYT, Grant N° 0714) and by the Argentinean National
391 Research Council (PIP Grant Nº112-200801-01773)

392

393

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548
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551

552 **Figure legends**

553

554 **Figure 1**

555 **Schematic representation of the HIV-1 Vpu expression vectors. a)** Amino acid alignment

556 of Vpu proteins from HIV-1 subtype B, F, BF intersubtype recombinant and representations

557 of the structural domains as well as the phosphorylation sites (asterisks) of Vpu. The

558 substituted amino acids within α-helix II are represented in bold. **b)** Schematic representation

559 of the Vpu chimeras.

560

561 **Figure 2**

562 **Expression of B, F1, BF, chimeric and mutant Vpu proteins.** Western blot of 6xHis-tagged

563 Vpu in the pCG-Vpu-IRES-GFP vector showing expression of the various Vpu constructs in

564 lysates of transfected HeLa cells. Empty vector was used as negative control, while β-actin

565 was used as loading control.

566

567 **Figure 3**

568 **Ability of *vpu* mutants to enhance viral release. a) Viral release induced by Vpu**

569 **chimeras. b) Viral release induced by Vpu mutants.** HeLa cells were co-transfected with

570 pNL4-3 U35 and Vpu-GFP expressing vectors. Co-transfection with pCG-GFP empty vector

571 was set as negative control. Viral release was evaluated by p24 antigen content in cell culture

572 supernatants (ng/ml) 48 h post-transfection. Error bars indicate standard errors. Results are

573 representative of two independent experiments. Statistical significance is represented as

574 *$p<0.01$.

575

576 **Figure 4**

577 **Vpu chimeras and mutants affect cell surface BST-2 down- modulation. a) BST-2 down-**
578 **modulation by Vpu chimeras. b) BST-2 down-modulation by Vpu mutants.** HeLa cell
579 cultures were transfected separately with pCG-Vpu-IRES-GFP expressing vectors. Empty
580 vector was used as negative control. BST-2 surface expression represented as Mean
581 Fluorescence Intensity (MFI) of GFP-positive cells. Bar graphs represent the means ± SD
582 from three independent experiments. Histograms represent relative cell number vs. relative
583 levels of BST-2 fluorescence intensity for GFP-positive cells. Black lines indicate the results
584 of Vpu-GFP expression vector, and gray dotted line indicates the result of GFP empty control
585 vector. Statistical significance is represented as $^{*}p<0.01$.
586
587 **Figure 5**
588 **Down- modulation of CD4 cell surface expression by Vpu chimeras and mutants**. **a) CD4**
589 **down-modulation by Vpu chimeras. b) CD4 down-modulation by Vpu mutants.** HeLa
590 $CD4^{+}$ cell cultures were transfected separately with pCG-Vpu-IRES-GFP expressing vectors.
591 Empty vector was used as negative control. CD4 surface expression represented as Mean
592 Fluorescence Intensity (MFI) of GFP-positive cells. Bar graphs represent the means ± SD
593 from three independent experiments. Histograms represent relative cell number vs. relative
594 levels of CD4 fluorescence intensity for GFP-positive cells. Black lines indicate the results of
595 Vpu-GFP expression vector, and gray dotted line indicates the result of GFP empty control
596 vector. Statistical significance is represented as $^{*}p<0.01$.

A

```
                                   Transmembrane              α-helix I      CK-II      α-helix II
                                                                             sites
                                                                            *    *
                            1        10        20        30        40        50        60        70        80
VpuB (NL4-3)                MQP-IIVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVSALVEMGVEMGHHAPWDIDDL
VpuB S61A                   ...-.........................................................A....................
VpuB V64S                    ...-.........................................................A..S.................
VpuBF (CRF12_BF-like)       ..SLV.L........A.L....T..L...............L..IR.........D.DAEE.AAL.-D..PLI.GN..I.
VpuBF E61S                  ..SLV.L........A.L....T..L...............L..IR.........D.DASE.AAL.-D..PLI.GN..I.
VpuBF A64S                  ..SLV.L........A.L....T..L...............L..IR.........D.DAEE.SAL.-D..PLI.GN..I.
VpuF1 (93BR020.1)           .SNLLAIG.A..I..L..T....T.AY...K.LV.....N..YK.IS...........DAEE.AAL.-.V.PFI.G..NN.
Vpu B/F1                    ...-...................................................D.DAEE.AAL.-D..PLI.GN..I.
Vpu BF/B                    ..SLV.L........A.L....T..L...............L..IR..................................
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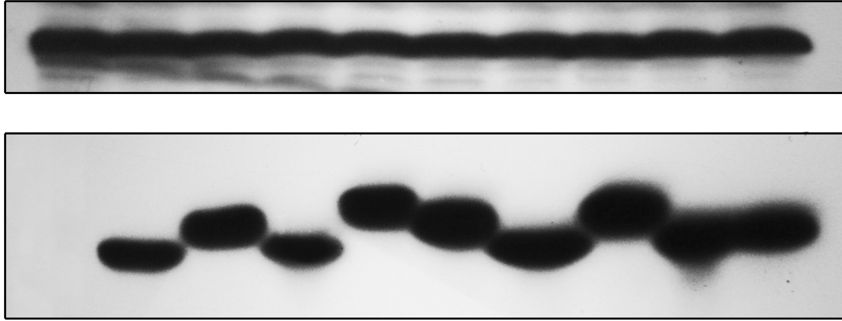
B

VpuB (NL4-3)

VpuBF (CRF12_BF-like)

Vpu B/F1

Vpu BF/B


Empty
VpuB
VpuB S61A
VpuB V64S
Vpu BF/B
VpuBF
VpuBF E61S
VpuBF A64S
Vpu B/F1
VpuF1
β–actin
Vpu

a

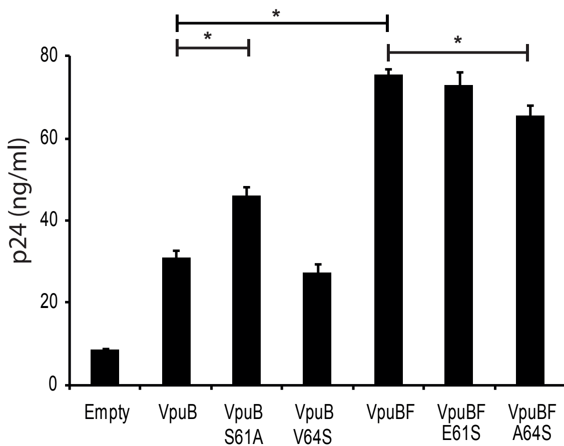
* * * * * *

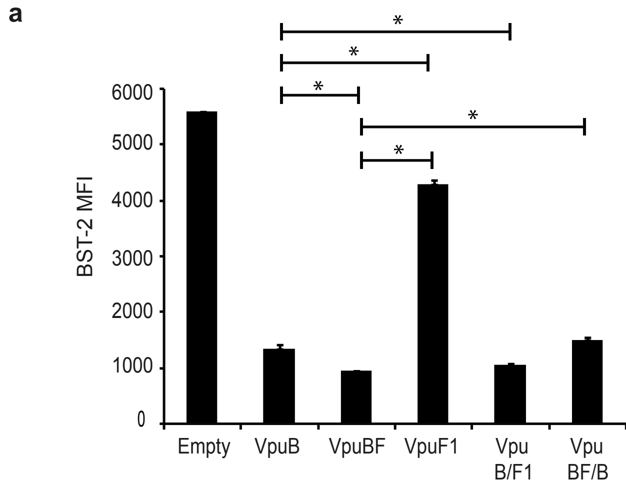
p24 (ng/ml)

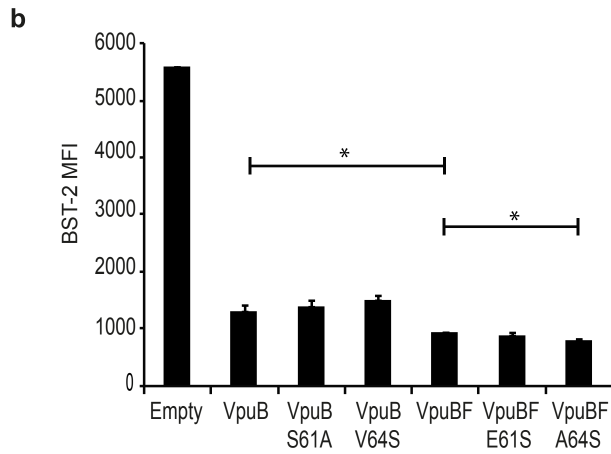
80
60
40
20
0

Empty VpuB VpuBF VpuF1 Vpu B/F1 Vpu BF/B

b




a
BST-2 MFI
6000
5000
4000
3000
2000
1000
0
*
*
*
*
*
Empty
VpuB
VpuBF
VpuF1
Vpu B/F1
Vpu BF/B


b
BST-2 MFI
6000
5000
4000
3000
2000
1000
0
*
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Empty
VpuB
VpuB S61A
VpuB V64S
VpuBF
VpuBF E61S
VpuBF A64S
Cell counts
BST-2 expression
VpuB
VpuBF
VpuF1
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Vpu BF/B
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VpuBF
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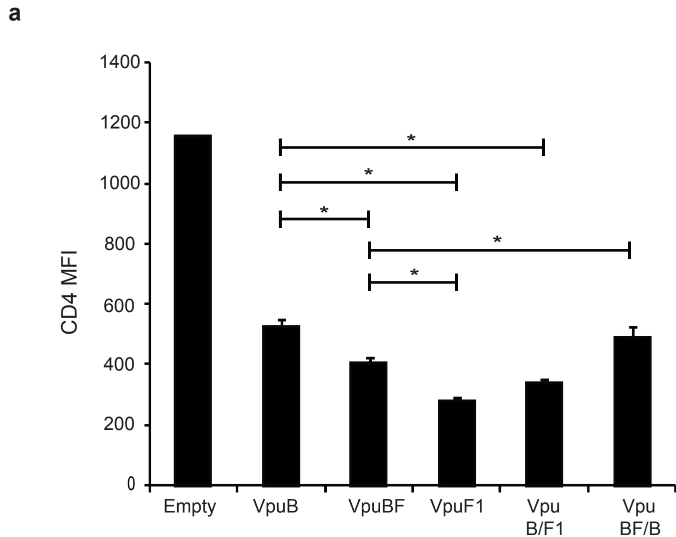
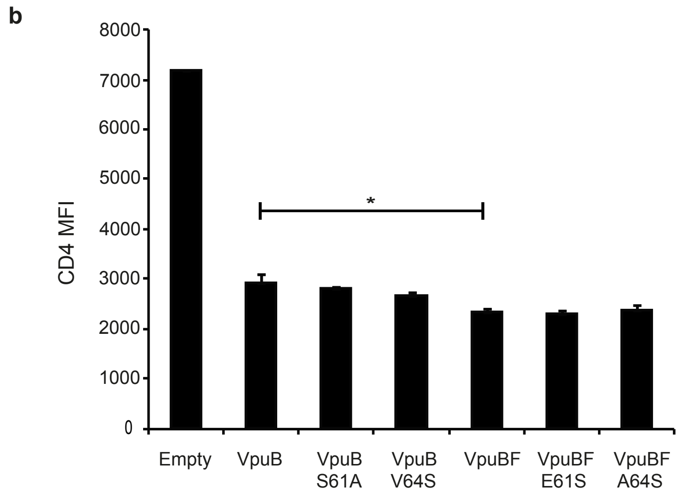
VpuBF A64S





Cell counts

VpuB | VpuBF | VpuF1

Vpu B/F1 | Vpu BF/B

VpuB | VpuB S61A | VpuB V64S

VpuBF | VpuBF E61S | VpuBF A64S

CD4 expression