Antagonism of BST-2/tetherin is a conserved function of the Env glycoprotein of primary HIV-2 isolates

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Abstract

Although HIV-2 does not encode a *vpu* gene, the ability to antagonize BST-2 is conserved in some HIV-2 isolates where it is controlled by the Env glycoprotein. We previously reported that a single amino acid difference between the lab-adapted ROD10 and ROD14 Envs controlled the enhancement of virus release (referred to here as Vpu-like) activity. Here we investigated how conserved the Vpu-like activity is in primary HIV-2 isolates. We found that almost half of the 35 tested primary HIV-2 Env isolates obtained from 7 different patients enhanced virus release. Interestingly, most HIV-2 patients harbored a mixed population of viruses containing or lacking Vpu-like activity. Vpu-like activity and Envelope functionality varied significantly among Env isolates; however, there was no direct correlation between these two functions suggesting they evolved independently. In comparing the Env sequences from one HIV-2 patient, we found that similar to the ROD10/ROD14 Envs, a single amino acid change (T568I) in the ectodomain of the TM subunit was sufficient to confer Vpu-like activity to an inactive Env variant. Surprisingly, however, absence of Vpu-like activity was not correlated with absence of BST-2 interaction. Taken together, our data suggest that maintaining the ability to antagonize BST-2 is of functional relevance not only to HIV-1 but to HIV-2 as well. Our data show that as with Vpu, binding of HIV-2 Env to BST-2 is important but not sufficient for antagonism. Finally, as observed previously, the Vpu-like activity in HIV-2 Env can be controlled by single residue changes in the TM subunit.

Importance

Lentiviruses such as HIV-1 and HIV-2 encode accessory proteins whose function is to overcome host restriction mechanisms. Vpu is a well-studied HIV-1 accessory protein that enhances virus release by antagonizing the host restriction factor BST-2. HIV-2 does not encode a *vpu* gene. Instead, the HIV-2 Env glycoprotein was found to antagonize BST-2 in some isolates. Here, we cloned multiple Env sequences from 7 HIV-2-infected patients and found that about half were able to antagonize BST-2. Importantly, most HIV-2 patients harbored a mixed population of viruses containing or lacking the ability to antagonize BST-2. In fact, in comparing Env sequences from one patient combined with site-directed mutagenesis, we were able to restore BST-2 antagonism to an inactive Env protein by a single amino acid change. Our data suggest that targeting BST-2 by HIV-2 Env is a dynamic process that can be regulated by simple changes in the Env sequence.

Introduction

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) infections are well defined as viral zoonoses. Phylogenetic analysis shows that HIV-1 is closely related to simian immunodeficiency virus (SIV) from chimpanzees (SIVcpz), and HIV-2 is closely related to SIV from sooty mangabeys (SIVsm) (1). At least nine lineages of HIV-2 have been identified, referred to as HIV-2 groups A through I. However, only groups A and B are known to cause human epidemics. In fact, group A viruses account for the vast majority of HIV-2 infections worldwide, which are concentrated mainly in West Africa, Europe, and some Asian countries (1-3). Like all primate retroviruses, HIV-2 encodes three structural proteins (Gag, Pol, and Env) and a set of accessory proteins (Vif, Vpx, Vpr, and Nef). Most if not all of the accessory proteins serve to antagonize host restriction factors, which are part of the host's innate immune system and are considered a first line of defense against viruses. Overall, the genomes of HIV-1 and HIV-2 are very similar. Two notable differences are (i) the presence of a *vpu* gene in HIV-1 which is absent in HIV-2 and (ii) the absence of a *vpx* gene in HIV-1 which is present in HIV-2. Vpu targets bone marrow stromal antigen 2 (BST-2) and induces degradation of CD4 while Vpx induces degradation of sterile alpha motif and HD domain-containing protein 1 (SAMHD1) (for review see (4)). There is no known functional homolog to Vpx in HIV-1 to target SAMHD1 and while Nef is well-known to down-regulate CD4 from the cell surface (5), the ability to induce proteasomal degradation of CD4 is limited to viruses expressing Vpu (6, 7). Thus, the Vpu and Vpx proteins are not functional homologs. On the other hand, the ability to enhance virus release by antagonizing BST-2 is not limited to Vpu encoding viruses. In fact, in HIV-2 antagonizing BST-2 is a functional property of the Env glycoprotein (8, 9), while in SIV this function is

executed by the Nef protein (10-13). For the remainder of this manuscript we refer to the ability of HIV-2 to enhance virus release as Vpu-like activity.

BST-2, also known as tetherin or CD317, is a 30- to 36-kD type II transmembrane protein that inhibits the release of retrovirus particles by physically tethering virions to the cell surface (14, 15). The exact mechanism of how Vpu antagonizes BST-2 is still unclear. However, it is thought to involve a process that interferes with the resupply of newly synthesized BST-2 from the ER to the cell surface (reviewed in (4)). Similar to HIV-1 Vpu, the ability of HIV-2 Env to overcome the restrictive phenotype of Vpu-deficient HIV-1 was known long before the cellular target was identified (8, 9, 16, 17). Direct evidence that HIV-2 Env, like Vpu, antagonizes human BST-2 was provided for two HIV-2 laboratory isolates (ROD10 and RODA (16, 18, 19)) and for one SIVtan isolate, which was adapted for replication in a human CD4+ T cell line (20). It is also interesting to note that serial passaging of a *nef*-deleted SIV in rhesus macaques resulted in the acquisition of mutations in the cytoplasmic domain of gp41 that conferred resistance to rhesus BST-2 (21). In contrast, the Env proteins of HIV-2 and SIVtan were found to target BST-2 through ectodomain interactions (20, 22) leading to the recruitment of a clathrin adaptor AP2 complex via a membrane-proximal GYXXϕ motif in the cytoplasmic domain of gp41 and resulting in the sequestration of BST-2 in the trans-Golgi network (TGN) (23).

We had previously found that a single amino acid change in the ectodomain of the HIV-2 Env TM subunit can regulate the ability of HIV-2 Env to enhance virus release (24). However, these studies were done with highly lab-adapted virus isolates and it was not clear how relevant the Vpu-like activity was *in vivo*. To address this question we cloned primary HIV-2 *env* sequences from viruses that had been isolated by coculture of patient peripheral blood

mononuclear cells (PBMCs) with PBMCs from uninfected individuals (25). In total, we isolated 35 full-length HIV-2 Env sequences from 8 patients. All 35 Env isolates were analyzed for Vpu-like activity in a virus release assay and their envelope function was tested by pseudotyping Env-defective HIV-2. We found that all Env proteins were functional in the pseudotyping assay although there was significant variability in the relative pseudotyping efficiency. Interestingly, almost half of the primary HIV-2 isolates also exhibited Vpu-like activity and viruses with Env proteins capable or incapable of antagonizing BST-2 were found to coexist in the same patient. Finally, mutational analysis of an Env isolate lacking Vpu-like activity revealed that a single amino acid change could lead to gain of Vpu-like function. Interestingly, gain of Vpu-like activity was not caused by a gain of interaction with BST-2 since both inactive and active Envs interacted with BST-2 with similar efficiency. Taken together, our data reveal that the ability to target BST-2 is conserved not only in HIV-1 but in HIV-2 as well. Our data also show that the ability of HIV-2 to target BST-2 is a dynamic process that can be regulated by very subtle changes in the Env amino acid sequence. These changes can occur in the same patient *in vivo* without correlating to the functionality of the Env proteins with respect to producing infectious virus. Finally, consistent with our observations on Vpu, the ability of HIV-2 Env to interact with BST-2 is presumably necessary but not sufficient for antagonism.

Materials and Methods

Cell culture and transfections. HeLa, HeLa-TZM-bl, and 293T cells were propagated in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS). For transfection, cells were grown in 25 cm^2 flasks to about 80% confluency. Cells were transfected using LipofectAMINE PLUS™ (Invitrogen Corp, Carlsbad CA) following the manufacturer's recommendations. A total of 6 µg of plasmid DNA per 25 cm^2 flask was used. Total amounts of transfected DNA was kept constant in all samples of any given experiment by adding empty vector DNA as appropriate. Cells were harvested 24 h post transfection.

Viral RNA extraction, HIV-2 envelope cloning, and sequence analysis. Virus culture samples from 8 patients infected with HIV-2 were obtained from the Research Institute for Medicines (iMed.ULisboa), University of Lisbon, Portugal (25, 26). Patient data are summarized in table 1. For each sample, 140 µl of culture supernatant were used to extract viral RNA using a QIAamp Viral RNA mini kit (Qiagen). RNA was eluted in 60 µl of elution buffer and immediately subjected to first strand cDNA synthesis using the SuperScript III Reverse Transcriptase kit according to manufacturer's instructions (Invitrogen Life Technologies). The resulting cDNA was subjected to 1st round PCR using primers to conserved regions upstream or downstream of *env* (table 2: 5'-primers F3 or A1m2F; 3'-primers R1 or NT5mR). PCR products were cloned into the pCR4-TOPO vector (Invitrogen) and sequenced. Specific primers were designed for sub-cloning of individual *env* isolates into a mammalian expression vector (table 2). Note that the 3'-primers were designed to add an HA tag to the C-terminus of Env. Also, the 5' primer (HIV-2 Rev-Xba-F) was designed to include the first exon of Rev. Using these primers, a 2600~2700 bp fragment encompassing the entire *env* gene and the *rev* gene was amplified from individual

145 TOPO clones by 2nd round PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen)
146 and cloned into the Env-expression vector pCM10 (24). This vector allows for the expression of
147 Env proteins in a Tat- and Rev-independent manner. As a control we also created C-terminally
148 HA-tagged variants of the HIV-2 ROD10 and ROD14 Env using primers listed in table 2. For
149 consistency, these vectors also included the upstream first exon of Rev. All PCR fragments were
150 cloned via the primer-encoded *Xba*I and *Xho*I restriction sites into the corresponding sites in
151 pCM10 (24).
152
153 **Phylogenetic analysis.** Clonal envelope sequences from each patient were codon aligned with a
154 set of reference sequences representative of HIV-2 groups A and B obtained from the Los
155 Alamos HIV Sequence Database (http://www.hiv.lanl.gov/) using MUSCLE (27) and the
156 alignment was manually edited with GeneDoc
157 (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html). Maximum likelihood
158 (ML) phylogenetic analysis was performed using the best-fit model of molecular evolution
159 estimated by Modeltest v3.7 using the Bayesian Information Criterion. ML tree was inferred
160 with program MEGA6 (28). To find the ML tree the nearest neighbor interchange (NNI) iterative
161 heuristic method was used. The reliability of the obtained topology was estimated by bootstrap
162 (1000 replicates). The nucleotide sequence data was deposited in GenBank under the following
163 accession numbers: KX791206-KX791239.
164
165 **Site-directed mutagenesis.** HIV-2 envelope point mutants were created using QuikChange™
166 site-directed mutagenesis (Stratagene, La Jolla CA) and primer pairs m1-m7 (table 2). Mutations
167 were verified by sequencing.

168

169 **Antibodies.** HIV-1 Gag proteins were identified using human HIV-1 IG (NIH Research and
170 Reference reagent program (Cat #3957). HIV-2 Gag proteins were identified using HIV-2 patient
171 serum (NIH Research and Reference reagent program (Cat #1495; discontinued)). Mouse anti-
172 tubulin and mouse anti-HA mABs were from Sigma (Sigma-Aldrich, St. Louis MO; cat# T-9026
173 & H9658, respectively).

174

175 **Western blotting.** Cells were washed with ice-cold PBS twice and lysed with 1× SDS protein
176 loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and
177 0.01% bromophenol blue). Samples were then heated at 95°C for 10 min with occasional
178 vortexing of the samples. The lysates were resolved by SDS-PAGE and transferred to
179 polyvinylidene fluoride membranes (EMD Millipore, Billerica MA). The membrane was blocked
180 with dry milk (5% solution in 1x TNT buffer [10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.3%
181 Tween-20]) and probed with the primary antibodies in TNT buffer followed by incubation with
182 alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis MO). Finally,
183 signals were detected using chemiluminescence following the manufacturer's recommendations
184 (Applied Biosystems, Foster City CA). α-tubulin was used as a loading control.

185

186 **Assessment of viral particle release.** Pulse-chase analysis was performed as described
187 previously with some modifications (9). Briefly, HeLa cells were co-transfected with 4 μg of
188 Vpu-defective pNL4-3/Udel-1 (29) and 2 μg of one of the HA-tagged Env expression vectors
189 using LipofectAMINE PLUS™. Cells were pulse-labeled 24 h later with [^{35}S]-EXPRE^{35}S^{35}S-
190 label (2 mCi/ml; Perkin Elmer, Waltham MA) for 30 min at 37°C and chased in 1 ml of

prewarmed complete DMEM-FBS for 0, 2.5, or 5 h. At each time point, cells were collected and lysed in 400 µl of lysis buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1% Triton-X100). Cell lysates were precleared by incubation at 4°C for 1 h with protein A-Sepharose beads (Sigma). The cell-free culture supernatants were mixed with 200 µl of lysis buffer. Cell lysates and detergent-treated supernatants were immunoprecipitated with HIV-IgG (NIH Research and Reference reagent program (cat#3957)). Immunoprecipitates were solubilized by heating in sample buffer and separated by SDS-PAGE using 12% polyacrylamide gels. Gels were treated for 20 min with 1M Na-salicylic acid and dried. Radioactive bands were visualized by fluorography using Bio-Max MR film (Eastman Kodak, Rochester NY). Quantitation of the relevant bands was performed with a Fujix BAS 2000 Bio-Image Analyzer. The efficiency of particle release at each time point was calculated by dividing the amount of Gag proteins present in the virus fraction by the total of cell- and virus-associated Gag proteins. The ratio of virion-associated versus total Gag protein was then plotted as a function of time.

Virus preparation. Virus stocks were prepared by transfection of 293T cells with appropriate plasmid DNAs. Virus-containing supernatants were harvested 24 h after transfection. Cellular debris was removed by centrifugation (5 min; 1,500 rpm) and the clarified supernatants were filtered (0.45 µm) to remove residual cellular contaminants. Supernatants were quantified by reverse transcriptase assay (30) and used for infection of TZM-bl indicator cells.

Viral infectivity assay. A 200 µl aliquot of viral stock was used to infect TZM-bl cells (CD4+, CCR5+, CXCR4+) in a 24-well plate (5×10^4 cells were seeded 1 day prior to infection) in a total volume of 1 ml. Typically, infections were performed in duplicate. Infection was allowed to

proceed for 48 h at 37°C. Medium was removed, and cells were lysed in 200 µl of Promega 1× reporter lysis buffer (Promega Corp., Madison WI) and frozen at -80°C for a minimum of 30 min. To determine the luciferase activity in the lysates, 10 µl of each lysate was combined with 50 µl of luciferase substrate (Steady-Glo; Promega Corp., Madison WI), and light emission was measured using a Modulus II microplate reader (Turner Biosystems Inc., Sunnyvale CA). Values were corrected for differences in input virus (based on RT assay).

Co-immunoprecipitation analyses. 293T cells were transfected with expression vectors for HIV-2 Env and BST-2 as indicated in the text. Cells were harvested 24 h posttransfection, washed twice with cold PBS, lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40; supplemented with Complete™ protease inhibitor cocktail [Roche Life Science, Indianapolis IN]) at 4°C for 20 min, and then clarified by centrifugation at 15,000 × g for 10 min. Ten percent of the lysate was used as input control and the remaining lysate was used for immunoprecipitation of HA-tagged antigens. Precleared cell lysates were mixed with anti-HA antibody-conjugated agarose beads (Sigma-Aldrich, Inc., St. Louis MO) and incubated at 4°C for 4 h. Samples were then washed three times with RIPA buffer. Proteins were eluted by boiling beads in sample buffer and subjected to immunoblot analysis with antibodies to HA and BST-2.

Results

Phylogenetic analysis of primary HIV-2 isolates.

We obtained virus culture samples from eight HIV-2-infected individuals (P1 - P8; table 1). *Env* sequences were amplified by RT-PCR. Since we expected significant sequence variation in the *env* gene, we first amplified *env* sequences using PCR primers mapping to more conserved regions in the upstream *vpr* and downstream *nef* genes (table 2). Resulting cDNAs were cloned into pCR4-TOPO and individual clones from each sample were sequenced. *Env* sequences isolated from a given patient were labeled according to the patient code followed by the clone number. For instance, sample P3-11 represents clone 11 from patient 3. Of the clones analyzed, 35 expressed detectable protein levels. Clones that did not express detectable protein because of deletions or truncations were excluded from further analysis. Also, despite several attempts we were unable to obtain more than a single clone from patient 5. This clone was severely truncated and non-functional and we therefore decided to exclude it from our study as well.

Phylogenetic analysis was performed based on 9 group A, 4 group B, and 1 AB reference sequences published in the NCBI database (http://www.ncbi.nlm.nih.gov) together with the 34 full-length HIV-2 *env* sequences identified in the present study (Fig. 1). We found that the *env* sequences from all seven HIV-2 patients clustered significantly with HIV-2 group A reference sequences. Sequences of Env variants isolated from the same patient were fairly conserved (96-99% at the amino acid level [data not shown]). Variation across the entire *env* gene sequence when samples from all patients were analyzed was as high as 20% at the nucleotide level and up to 25% at the amino acid level (data not shown).

Antagonism of BST-2 by HIV-2 envelope glycoproteins.

HIV-2 does not encode a *vpu* gene. Nevertheless, we and others previously reported that certain HIV-2 isolates, such as HIV-2 ROD10, encode a Vpu-like activity that results in enhanced virus release and maps to the HIV-2 Env protein (8, 16). Interestingly, the closely related ROD14 Env lacks a Vpu-like activity due to a single amino acid change in Env (24). Indeed, after the identification of BST-2 as the cellular target of Vpu (15, 31), it was confirmed that HIV-2 Env, like Vpu, antagonizes BST-2 to counteract BST-2-mediated tethering of virus particles to the host cell membrane (18, 19).

To assess the ability of our Env isolates to antagonize BST-2, we sub-cloned the full-length *env* genes into the Env expression vector pCM10 (24). To be able to track expression and virus incorporation of the Env products, all constructs, including ROD10 and ROD14, were modified to add a C-terminal HA tag. Rev independence was achieved by including the first exon of Rev upstream of the Env coding sequence. Vpu-like activity was determined by comparing the effects of ROD10 Env (positive control) and ROD14 Env (negative control) to the various primary HIV-2 envelope isolates on the release of Vpu-defective HIV-1 NL4-3 using a pulse/chase metabolic labeling assay described previously (8, 9). Vpu-deficient HIV-1 was chosen as a model system since we had previously demonstrated the Vpu-like activity of HIV-2 Env in this system (9). Also, antibodies for immunoprecipitation of HIV-1 Gag proteins are more readily available than antibodies to HIV-2 Gag. Experiments were performed in transiently transfected HeLa cells, which express high levels of endogenous BST-2 (32). Representative experimental data are shown for 6 Env variants isolated from patient 4 (Figs. 2A & 2B). In all experiments cells were pulse-labeled for 30 min and chased for up to 5 h as described in Materials and Methods. At each time point, equal aliquots of cells were harvested and virions released into the supernatant were collected. Each fraction was lysed in lysis buffer, and viral

proteins were subjected to immunoprecipitation with an HIV-1 patient serum. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography (Fig. 2A). Expression of comparable levels of HIV-2 Env was confirmed by immunoblotting (Fig. 2B). Quantitation of results from two independent experiments is presented in figure 2C. All other Env isolates were analyzed in a similar fashion and quantitation of the data is summarized in figure 3 and figure 4A.

As expected, virus release in the presence of the ROD14 Env was poor and similar to that observed in the absence of Env (Fig. 2C, compare ROD14 vs Env(-)). In contrast, co-expression of the ROD10 Env significantly enhanced the release of viral Gag proteins. Of the 6 tested Env isolates from patient P4, three (P4-1, P4-7, P4-8) behaved like the ROD14 Env and exhibited a Vpu(-) phenotype. Two of the Env isolates (P4-6 & P4-11) significantly enhanced virus release when compared to ROD14 Env although they were not quite as effective as the lab-adapted ROD10 Env (Fig. 2C). Finally, the Env protein from isolate P4-3 exhibited an intermediate phenotype. Thus, three of the six Env isolates derived from patient 4 exhibited some degree of Vpu-like activity. Overall, half (17/34 = 50%) of the Env isolates tested in this study were able to enhance the release of virus particles to varying degrees and thus revealed Vpu-like activity (Fig. 3 and Fig. 4A). To ascertain that the observed effects of HIV-2 Env on virus release are dependent on BST-2, we assessed virus release from BST-2 negative 293T cells. 293T cells were transfected with the *env*-defective pROD10.env1 (4 µg) (8) either in the absence of Env (no Env) or together with 2 µg of individual Env variants. Virus release was quantified 24 later by determining the virus-associated reverse transcriptase activity in the culture supernatants (Fig. 4B). As expected, the effects of HIV-2 Env proteins on virus release in the absence of BST-2 were small when compared to their effects on virus release from BST-2 expressing cells

(compare figure 4, panels A & B). Some Env variants had a slight enhancing effect (e.g. ROD10 and ROD14) while other Env proteins had a modest inhibitory effect (e.g. P2-1, P6-1, or P6-3). We conclude that the ability to antagonize BST-2 is conserved in about half of the HIV-2 Env variants. The ability to antagonize BST-2 was not specific to Env variants from specific patients. Indeed, most patients harbored viruses with Env proteins that contained or lacked BST-2 antagonizing activity.

HIV-2 Envs differ in their ability to produce infectious viruses.

We next tested the ability of our Env isolates to support the production of infectious viruses by coexpression with the *env*-deficient pROD10.env1 and tested the infectivity of the resulting virus preparations in a single-round infectivity assay. To avoid interference of virus production by BST-2 we used BST-2-negative 293T cells for this experiment. Cells were transfected with pROD10.env1 (4 µg) either in the absence of Env (2 µg empty vector [Ctrl]) or together with 2 µg of individual Env variants. Virus-containing supernatants were used for the infection of TZM-bl cells and virus-induced luciferase activity was determined 48 hr later. We found that four of the six P4 Env variants (Fig. 2D; P4-1, P4-3, P4-6, and P4-11) produced particles with significantly higher infectivity than viruses containing the lab-adapted ROD10 Env. Interestingly, the two Env isolates from patient P4 with the highest Vpu-like activity (P4-6 & P4-11) also scored highest in Env function. Analysis of all Env isolates for their ability to produce infectious virus is summarized in figure 4C. We observed significant variation among different Env variants. Overall, however, there was no direct correlation between envelope function and the ability to antagonize BST-2 (Fig. 4, compare panels A & C) suggesting that these functions of the HIV-2 Env protein evolved independently.

A naturally occurring substitution in HIV-2 Env regulates its Vpu-like virus release activity.

In a previous study we observed that a single amino acid change in ROD14 Env to the corresponding residue in ROD10 (T598A) was sufficient to restore Vpu-like activity (24). Sequence analysis of patient 4 isolates using P4-7 Env, which exhibits a Vpu(-) phenotype, as reference sequence revealed a number of amino acid differences among the individual isolates that were spread out across the entire Env sequence (Fig. 5). However, there were no common amino acid differences between variants with and without Vpu-like activity. Of note, the two Env isolates with the strongest Vpu-like phenotype (P4-6 and P4-11; see Fig. 2C) differed from the P4-7 reference sequence in 2 identical small deletions and only 9 amino acid positions, 8 of which were common to P4-6 and P4-11 (Fig. 5, pink background). Most of the sequence differences indicated by the pink background, together with additional changes, were also found in the other patient 4 Env sequences.

To test which of these sequence differences or deletions accounted for the Vpu-like phenotype of the P4-6/P4-11 Envs, we introduced amino acid changes/deletions into the P4-7 backbone (Fig. 5, m1-m7) either individually or in combination and assessed the resulting constructs in a gain-of-function analysis for their ability to enhance virus release using pulse-chase metabolic labeling as described for figure 2A (Fig. 6A). Analysis of Env expression by immunoblotting showed only minor variations in Env protein levels (Fig. 6B). Quantitation of the pulse/chase analysis data revealed that most of the Env mutants including the deletions, retained the Vpu(-) phenotype associated with the parental P4-7 isolate (Fig. 6C). Interestingly, however, mutation of T568 in P4-7 Env to isoleucine (Fig. 5, m5) conferred Vpu-like activity to

348 the P4-7 Env variant (Fig. 6C, P4-7m5). Of note, residue 568 is isoleucine in all P4 Env isolates
349 except P4-7, even those without Vpu-like activity (Fig. 5). indicating that isoleucine at this
350 position is important but not sufficient to confer Vpu-like activity to all Env variants.
351
352 **Co-immunoprecipitation of HIV-2 Env with BST-2.**
353 The inability of ROD14 Env to antagonize BST-2 was recently associated with a lack of
354 physical interaction of the two proteins (33). To confirm this observation we performed co-
355 immunoprecipitation studies in 293T cells by co-expressing HA-tagged ROD10 or ROD14 Env
356 with BST-2 (Fig. 7A). As a control, BST-2 was expressed in the absence of Env protein (Fig. 7A,
357 Ctrl). Transfected cells were harvested 24 h post transfection, lysed, and envelope proteins were
358 immunoprecipitated with an anti-HA monoclonal antibody. Total input samples and
359 immunoprecipitates were separated by SDS-PAGE and subjected to immunoblot analysis with
360 antibodies to HA or BST-2 (Fig. 7A). We found that BST-2 efficiently interacted with the
361 ROD10 Env protein. Consistent with the earlier report (33), interaction of BST-2 with ROD14
362 Env was significantly reduced although not entirely eliminated.
363 The interaction of P4-11, P4-7, and the P4-7m5 Env variants with BST-2 was determined
364 in a similar manner (Fig. 7B). Empty vector (Ctrl) and ROD10 Env-expressing vector (ROD10)
365 were included as controls. Interestingly, BST-2 interacted efficiently with the HIV-2 Env
366 variants P4-7, P4-11, as well as the gain-of-function mutant P4-7m5, irrespective of their Vpu
367 phenotype (Fig. 7B). Taken together, our data suggest that binding of Env to BST-2 is not
368 sufficient to antagonize BST-2 activity.
369

370 **Discussion**

371 The functional significance of BST-2/tetherin down-modulation by primate lentiviruses
372 viruses is still unclear. It has been suggested that BST-2 down-modulation serves to protect
373 infected cells from antibody-dependent cellular cytotoxicity (ADCC) by minimizing cell-surface
374 exposure of viral antigen (34-36). It is also possible that down-modulation of BST-2 benefits the
375 virus by increasing virus spread through cell-free transmission (reviewed in (37)). There is,
376 however, no doubt that controlling BST-2 is critical for primate lentiviruses since HIV-1, HIV-2,
377 SIV, FIV, and EIAV all have evolved mechanisms to antagonize BST-2. What is particularly
378 striking is the fact that these viruses use distinct strategies to target and neutralize BST-2. In the
379 case of HIV-1, Vpu has evolved as the BST-2 antagonist (14, 15). For most SIVs, Nef has
380 acquired the ability to target BST-2 (10-13). The latter include SIVcpz, the presumed ancestor of
381 HIV-1, which encodes a *vpu* gene, yet uses Nef to control BST-2 (12, 38) suggesting that the
382 original function of Vpu was not the targeting of BST-2. Like most SIV strains, HIV-2 lacks a
383 *vpu* gene. While HIV-2 does encode a *nef* gene, it does not use Nef to antagonize BST-2 but has
384 found yet another way by using its Env protein (8, 16, 18). Finally, FIV and EIAV acquired
385 similar Env-dependent strategies as HIV-2 (39, 40). Thus, there are at least three lentiviral
386 proteins with the demonstrated capacity to target and antagonize BST-2.

387 The reasons why BST-2 is not targeted by a common lentiviral protein are unclear.
388 However, it could be that in evolutionary terms, BST-2 represents a more recent challenge that
389 lentiviruses have had to cope with in different ways. Since BST-2 does not impose an absolute
390 restriction on virus replication, viruses may have had the luxury to gradually develop BST-2
391 resistance by expanding the functional breadth of available viral proteins. An interesting example
392 is the acquisition of a Vpu-like activity by the Env protein of a nef-deleted SIV following serial

393 passaging in rhesus macaques (21). Nevertheless, antagonism of BST-2 by any of the three viral
394 factors follows more or less the same pathway and is initiated by the physical interaction with
395 BST-2. For Vpu, this interaction clearly involves the TM domain (41-47) although the
396 involvement of the Vpu cytoplasmic domain has also been reported (48-52). For Nef, the
397 interaction with BST-2 is limited to the BST-2 cytoplasmic domain for the simple reason that
398 Nef does not have a TM- or ecto-domain but is attached to membrane through a myristic acid
399 moiety (10-12, 38). For HIV-2 Env, interactions with BST-2 have been reported to involve the
400 membrane-proximal ectodomain (17, 18, 22). However, as with Vpu, the cytoplasmic domain
401 may have a role in the antagonism of BST-2 as well (33). Exactly where in the cell the
402 interaction of BST-2 with Vpu, Nef, or Env is initiated is currently unclear. The co-expression of
403 BST-2 with Vpu, Env, or Nef can result in the surface down-modulation of BST-2 (reviewed in
404 (53)). However, whether surface down-modulation of BST-2 is an actual prerequisite or a
405 downstream consequence of BST-2 antagonism is still unclear. We previously found that in the
406 context of an acute spreading infection of T-cells, Vpu-dependent enhancement of virus release
407 does not coincide with BST-2 surface down-modulation (32). We also reported that antibody-
408 based interference with BST-2 must occur prior to BST-2 reaching the cell surface (54)
409 suggesting that the interaction of BST-2 with virus assembly complexes that ultimately results in
410 the membrane tethering is initiated inside the cells. This is true for HIV-1 as well as HIV-2 (54).

411 Our hypothesis that the ability to antagonize BST-2 is a more recent functional
412 acquisition of HIV-2 is supported by the fact that only about half of the functional Env isolates
413 characterized in our study have Vpu-like activity. Furthermore, the fact that there is significant
414 variation in the extent to which individual Env proteins can antagonize BST-2 supports the
415 model that antagonizing BST-2 may be an ongoing evolutionary process. This is supported by

416 the observation that we were able to isolate Env variants that contained or lacked Vpu-like
417 activity from most patient samples (see Figs. 1 & 4). More importantly, the ability or inability to
418 antagonize BST-2 is not a stable functional property but was sensitive to single amino acid
419 changes. Examples are the previously reported naturally occurring T598A mutation (24) as well
420 as the naturally occurring T568I mutation described in the current study (Fig. 8). It is interesting
421 that in both cases the presence of a threonine residue with its polar side chain was replaced by an
422 amino acid with a hydrophobic side chain suggesting structural changes are involved in the
423 acquisition of Vpu-like activity. It was previously reported that mutations resulting in a loss of
424 Vpu-like activity in HIV-2 Env were associated with a loss or at least a reduction in BST-2-Env
425 binding (33). Our own results are in partial agreement with those data in the sense that the
426 T598A mutation in ROD10/14 appeared to reduce - although not completely abolish - the
427 binding affinity to BST-2 (Fig. 7A). Interestingly, however, we did not observe a difference in
428 the interaction of BST-2 with Env variants P4-7 (Vpu(-) phenotype) and P4-11 (Vpu(+)
429 phenotype) or with the P4-7m5 back-mutation (T568I) that restored the Vpu-like activity in P4-7
430 Env (Fig. 7B). These results are in line with results from experiments involving a gpi-anchored
431 version of HIV-2 Env, which was able to interact with BST-2 but did not antagonize BST-2
432 function (33), and strongly suggest that binding of Env to BST-2 in itself is not sufficient to
433 antagonize BST-2 function.
434

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615 **Figure Legends**

616 **Figure 1: Molecular phylogenetic analysis of envelope gene sequences.**

617 The evolutionary history was inferred by using the Maximum Likelihood method based on the
618 General Time Reversible model (GTR+G+I) (28). The tree with the highest log likelihood (-
619 25625.8806) is shown. The percentage of trees in which the associated taxa clustered together is
620 shown next to the branches. Only values ≥ 70% are displayed. Initial tree(s) for the heuristic
621 search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix
622 of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and
623 then selecting the topology with superior log likelihood value. A discrete Gamma distribution
624 was used to model evolutionary rate differences among sites (5 categories (+G, parameter =
625 0.6138)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I],
626 25.7204% sites). The tree is drawn to scale, with branch lengths measured in the number of
627 substitutions per site. All positions containing gaps and missing data were eliminated. Each
628 reference HIV-2 strain is represented by its genetic group and name at the right. HIV-2 isolates
629 in green exhibit Vpu-like virus release activity (see Figs. 3 & 4A); HIV-2 isolates in red do not
630 exhibit Vpu-like activity.

631

632 **Figure 2: The HIV-2 envelope glycoprotein enhances HIV-1 particle release. (A)** Kinetic
633 analysis of viral particle release by Vpu-deficient HIV-1 in the presence of the different HIV-2
634 Env isolates. HeLa cells were transfected with pNL4-3/Udel-1 together with HA-tagged HIV-2
635 Env vectors pROD14-Env, pROD10-Env, and pHA vector (Env(-)) as controls, as well as
636 vectors for the expression of HA-tagged Envs from HIV-2 patient 4 isolates P4-1, P4-3, P4-6,
637 P4-7, P4-8, and P4-11. Samples were subjected to pulse-chase analysis and viral proteins

638 recovered by immunoprecipitation were separated by 12% SDS–PAGE. The major HIV-1 Gag
639 proteins p55gag and p24CA are identified on the right. A representative experiment is shown.
640 **(B)** Relative expression of Env in the transfected cells was verified by western blot analysis
641 using an HA-specific mAB. Expression of cellular α-tubulin served as a loading control (tub).
642 **(C)** Efficiency of virus release was determined by quantifying bands in panels A corresponding
643 to the precursor and mature Gag proteins at each time point. Results were plotted as a function of
644 time. Maximal virus release by ROD10 at the 5 hr time point was defined as 100% and the
645 remaining data points were normalized accordingly. Data are presented as mean +/- S.E.M. from
646 two independent experiments. **(D)** To assess the ability of HIV-2 Env variants to produce
647 infectious virus, 293T cells were transfected with 4 µg of envelope-deficient pROD10.env1
648 DNA in the presence of empty pHA vector (Env(-)), or HA-tagged pROD14-Env, pROD10-Env,
649 or HIV-2 patient 4 isolates P4-1, P4-3, p4-6, P4-7, P4-8, and P4-11 as indicated. Virus-
650 containing supernatants were harvested 24 h later and a portion of the filtered culture supernatant
651 was used for the infection of TZM-bl cells. Luciferase activity was measured 48 h after infection
652 and normalized for input virus. The result shown is representative of two independent
653 experiments. Infectivity of viruses pseudotyped with the ROD10 Env was defined as 1.
654 Differences in viral infectivity of the other samples are expressed as fold change relative to
655 ROD10 Env. Graphs represent the mean +/- S.E.M. of duplicate infections.
656
657 **Figure 3: Antagonism of BST-2 by HIV-2 Env variants.** Pulse/chase analyses were performed
658 for all Env variants as described for figure 2A. Quantitation was done as described for figure 2C.
659 Data were grouped by patient and are presented as mean +/- S.E.M. from two independent
660 experiments.

Fig. 4: Summary of the functional data for all HIV-2 Env variants. (A) Effect of HIV-2 Env on the release of HIV-1 from BST-2-expressing HeLa cells. Release of Vpu-deficient HIV-1 in the presence of the different HIV-2 Env variants was determined by pulse/chase analysis as described for figure 2A. Virus release observed after 5 hr of chase was quantified as described for figure 2C. Virus release in the absence of Env was defined as 1 and is marked by a horizontal line. Virus release in the presence of individual Env variants was calculated as fold-change relative to the Env-negative sample. Data are presented as mean +/- S.E.M. from at least two independent experiments. A 1.5-fold increase is marked by a second horizontal line and represents an empirical cut-off to define Vpu-like activity. **(B)** Effect of HIV-2 Env on the release of HIV-2 in the absence of BST-2. BST-2-negative 293T cells were transfected with 4 µg of envelope-deficient pROD10.env1 DNA in the presence of empty pHA vector (Env(-)), or HA-tagged pROD14-Env, pROD10-Env, or HIV-2 patient isolates. Virus-containing supernatants were harvested 24 h later and virus production was quantified by measuring the virus-associated reverse transcriptase activity. Virus production in the absence of Env was defined as 1 (marked by a horizontal line). Effects of individual Env proteins on virus release were calculated as fold-difference relative to the Env-negative sample. Graphs represent the mean +/- S.E.M. from two independent experiments. Colors indicate individual patients. **(C)** Effect of HIV-2 Env on viral infectivity. Virus samples from panel B were used for the infection of TZM-bl cells. Infections were done in duplicates. Luciferase activity was measured 48 h after infection and normalized for input virus. Infectivity of viruses pseudotyped with the ROD10 Env was defined as 1 and is marked by a horizontal line. Differences in viral infectivity of the other samples are expressed as fold change relative to ROD10 Env. Graphs represent the mean +/- S.E.M. of at least two independent experiments performed in duplicate infections.

684

685 **Figure 5: Sequence comparison of Env variants from patient P4.** Amino acid sequences from
686 all six patient 4-derived Env variants were aligned. Identical sequences appear as dots. The
687 transmembrane domain (TM domain) is marked by a gray background. Regions tested in figure 6
688 for their ability to convey Vpu-like activity are marked by a pink background. Alanine 598
689 (A598), which is critical for the ability of ROD10 Env to antagonize BST-2 (24) is highlighted
690 by a green background.

691

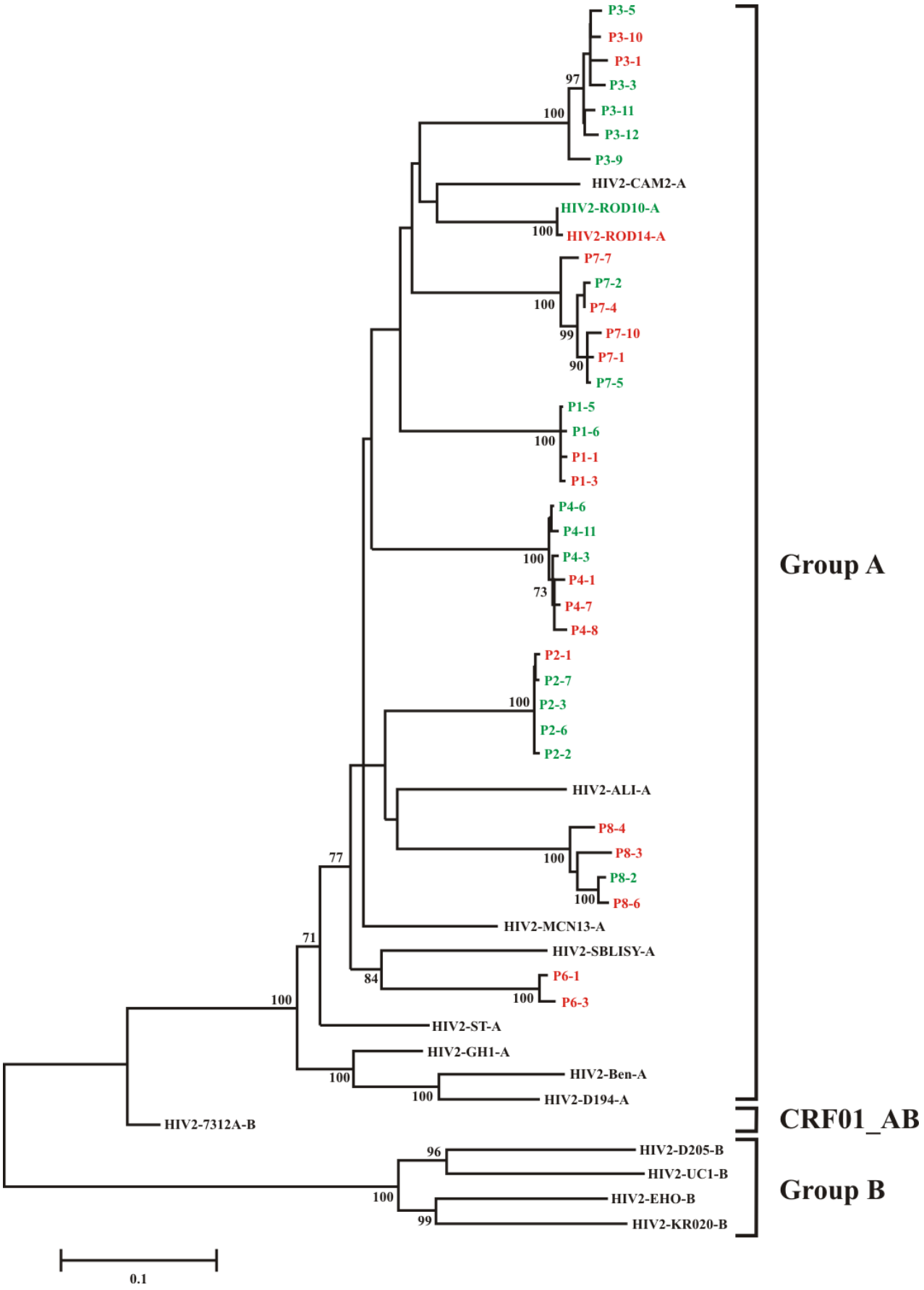
692 **Figure 6. The ectodomain of the TM subunit of HIV-2 Env is critical for enhancing virus**
693 **release. (A)** Amino acid differences in P4-11 Env highlighted in figure 4 were transferred
694 individually or in combination as indicated into the backbone of P4-7 Env. The ability of the
695 resulting mutants to antagonize BST-2 was tested in HeLa cells by pulse/chase analysis as
696 described for figure 2A. **(B)** Expression of Env mutants was verified by western blot analysis
697 using cellular α-tubulin as a loading control (tub). **(C)** Kinetic data from panel A were quantified
698 as described for figure 2C. Maximal virus release by ROD10 at the 5 hr time point was defined
699 as 100% and the remaining data points were adjusted accordingly. Data are presented as mean
700 +/- S.E.M. from two independent analyses.

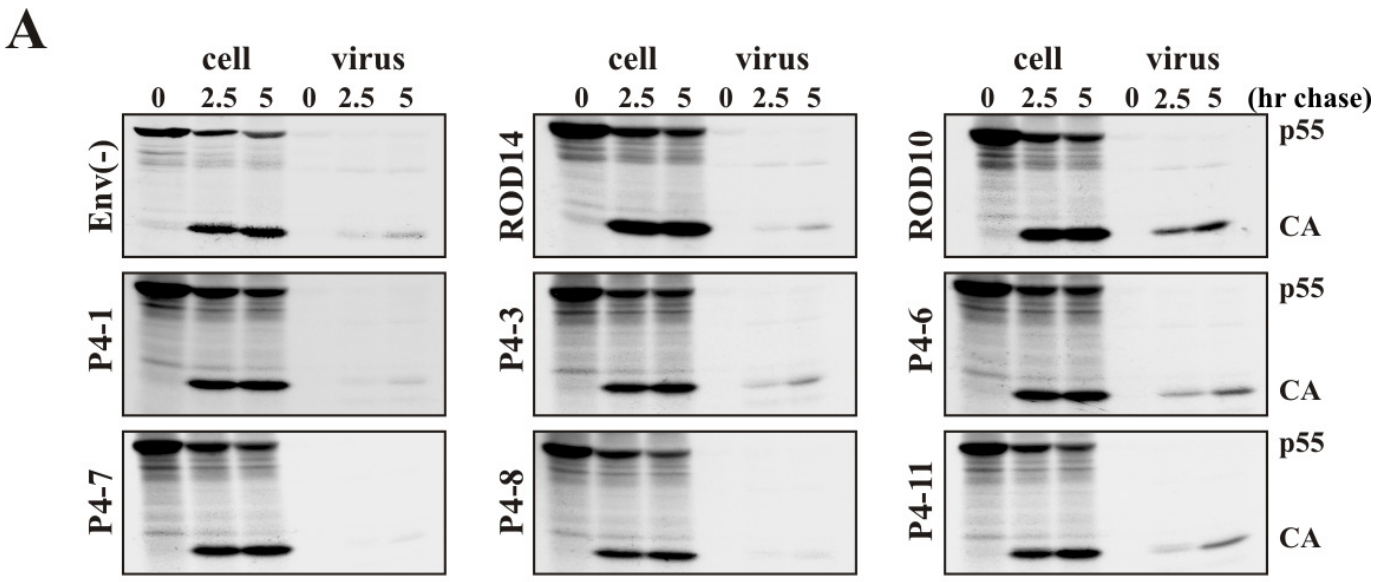
701

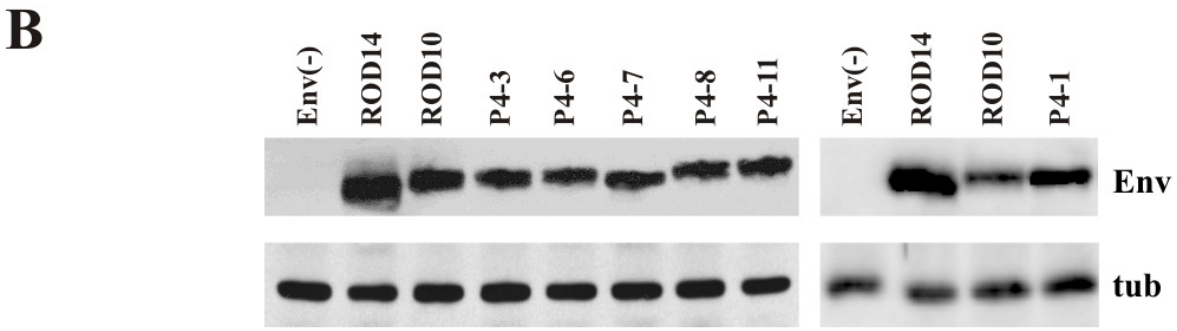
702 **Figure 7. Co-immunoprecipitation of BST-2 with HIV-2 Env. (A)** 293T cells were transfected
703 with 0.25 µg of pcDNA-BST-2 together with 4 µg of empty vector (Ctrl), or HA-tagged
704 pROD14-Env or pROD10-Env, respectively. Cell extracts were prepared 24 h later and a
705 fraction of total lysate was used as input control (top). The remaining lysate was used for
706 immunoprecipitation with anti-HA-coated beads (bottom). Samples were separated by SDS-

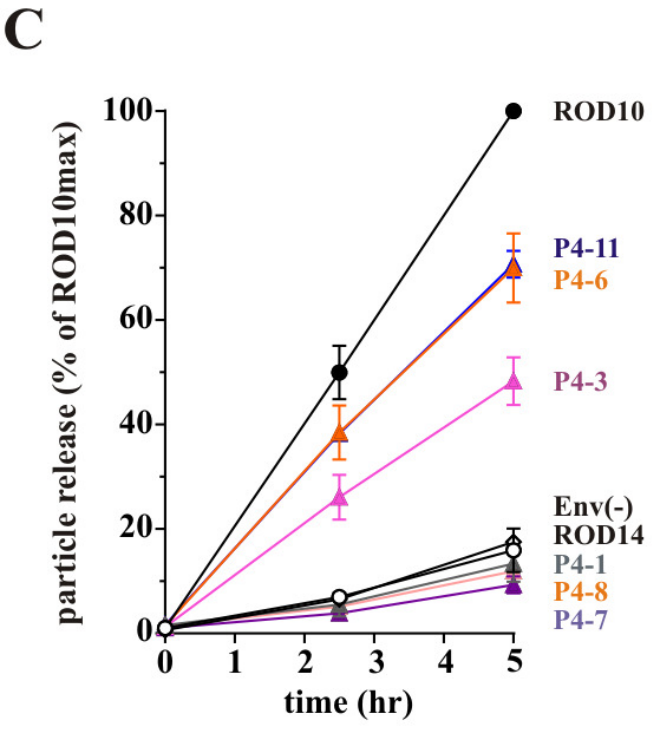
PAGE and probed with antibodies to HA (Env-HA) or BST-2. **(B)** 293T cells were transfected with 0.25 µg of pcDNA-BST-2 together with 4 µg of empty vector (Ctrl) or the indicated Env expression vectors. Samples were processed as in panel A. The experiment was performed independently three times. Shown is a representative result.

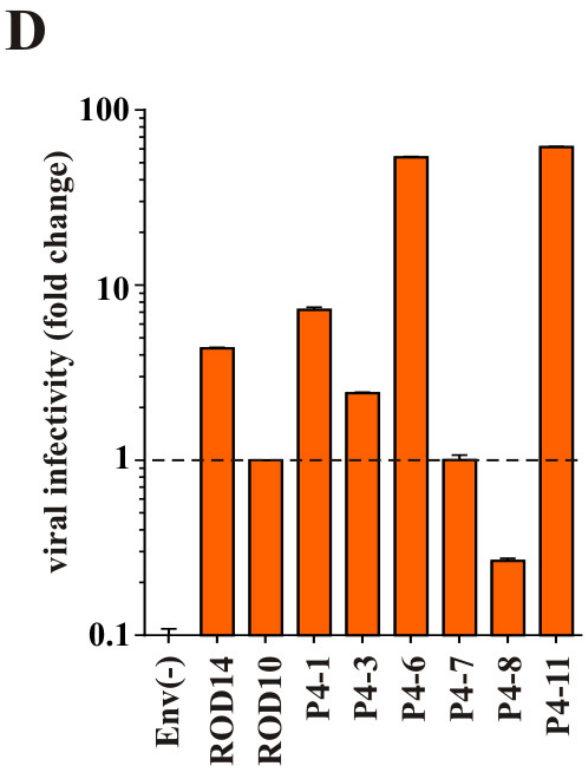
Fig. 8: Multiple changes in Env affect its Vpu-like activity. Shown is a partial amino acid alignment of four HIV-2 Env isolates. ROD10, ROD14, P4-7, and P4-11 sequences differ by deletions/insertions in the SU domain. Therefore, sequences were aligned using the transmembrane (TM) domains as reference (black box with white lettering). Amino acid positions refer to the initiation codon of each Env protein as position 1. The presumed precursor cleavage site (55) is indicated and the SU portion of the sequence is underlayed by a gray box. The boxed area downstream of the transmembrane domain delineates a tyrosine-based internalization motif (GYXXΘ) that includes a tyrosine (Y707) required for BST-2 antagonism (17, 18).

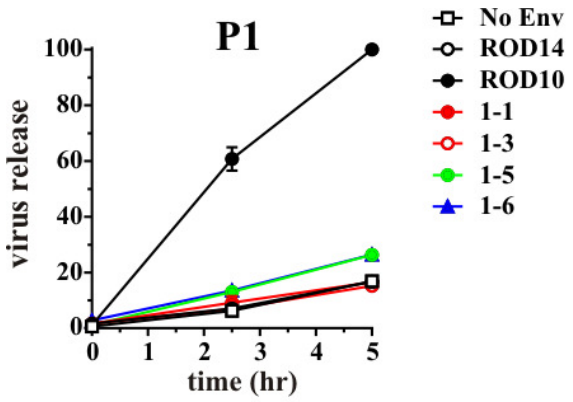


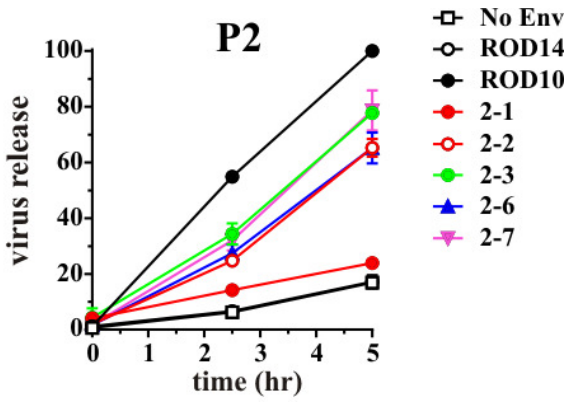


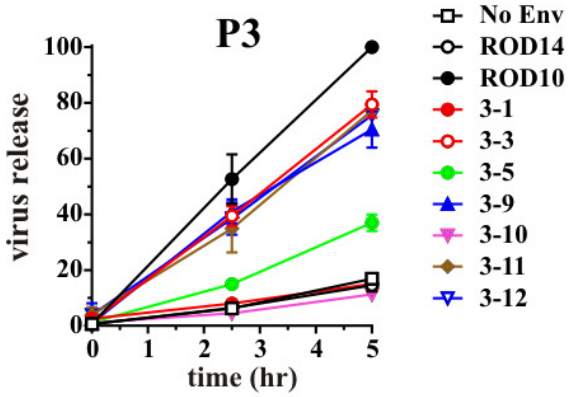


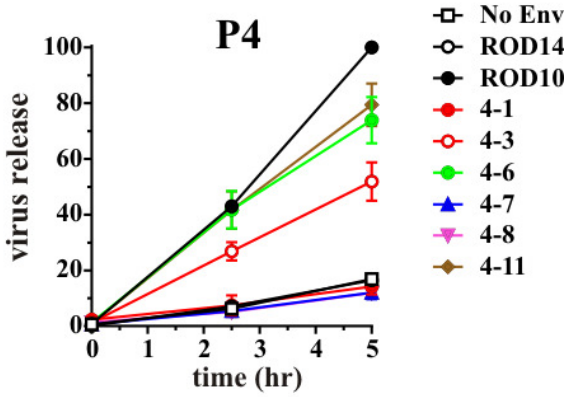


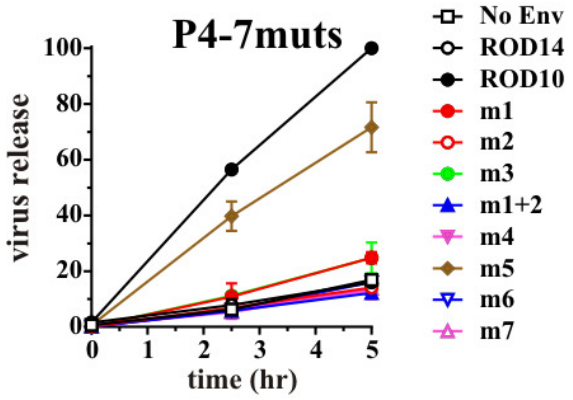


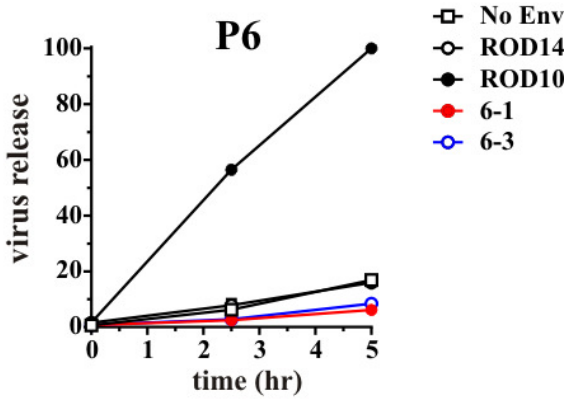


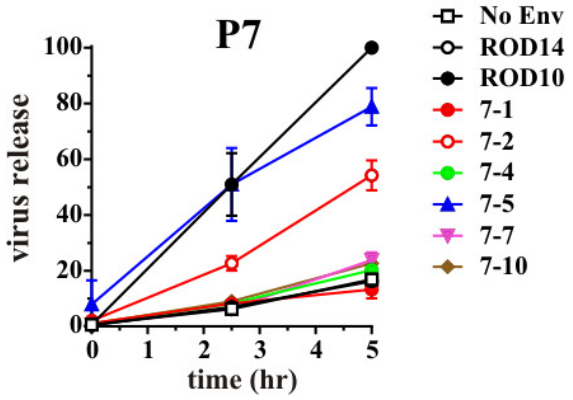


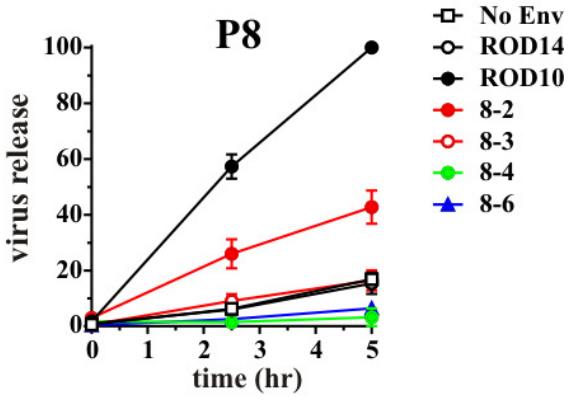


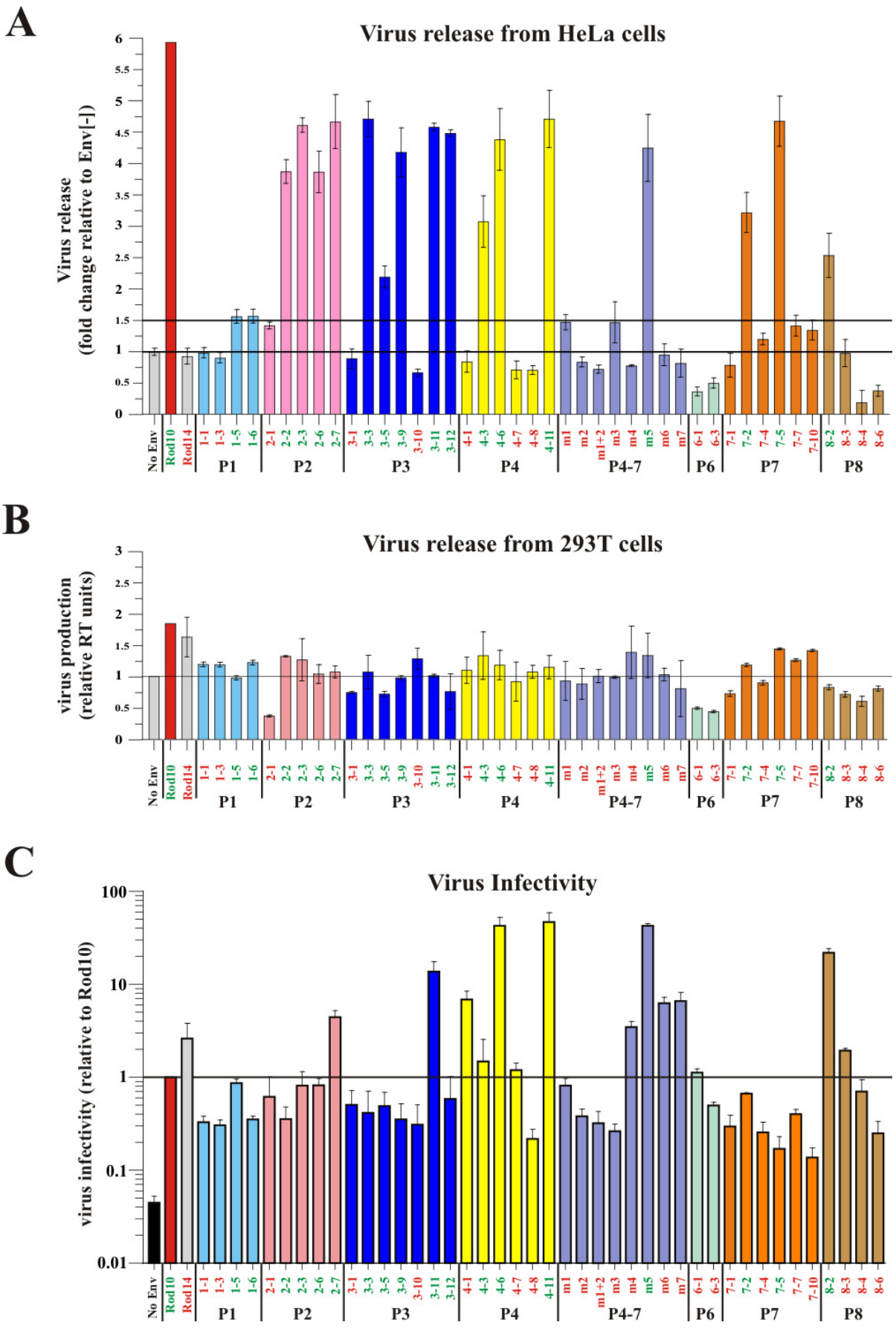


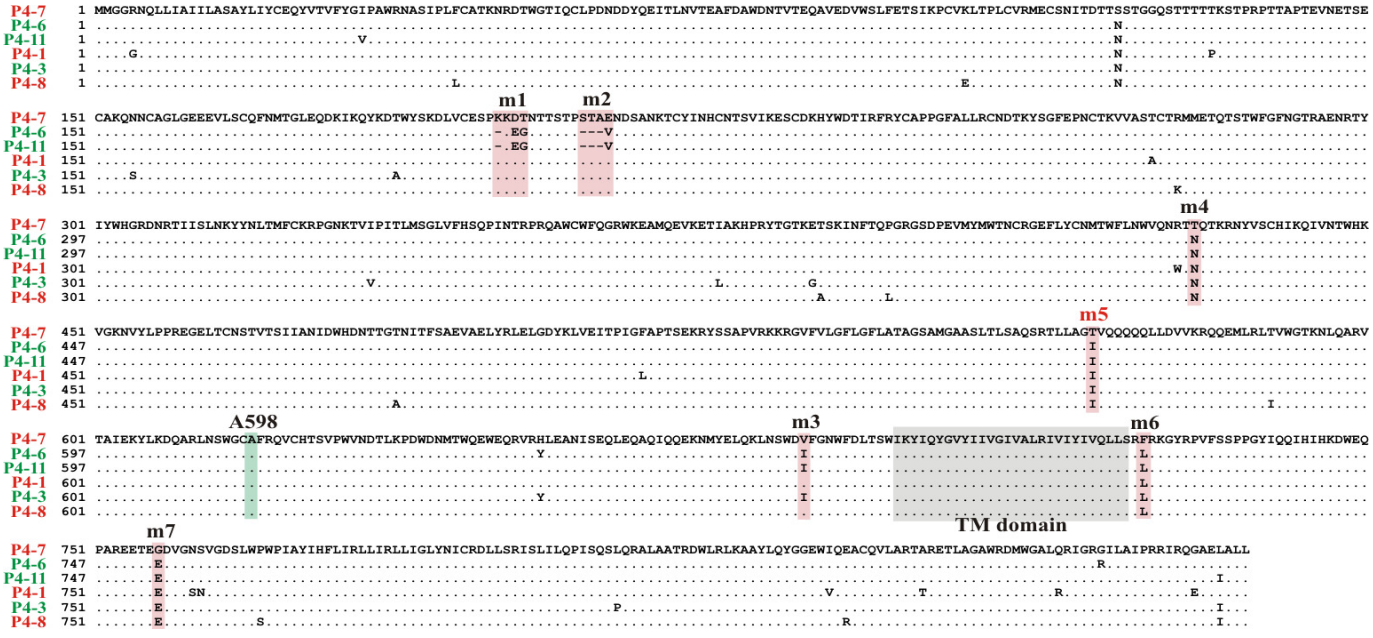


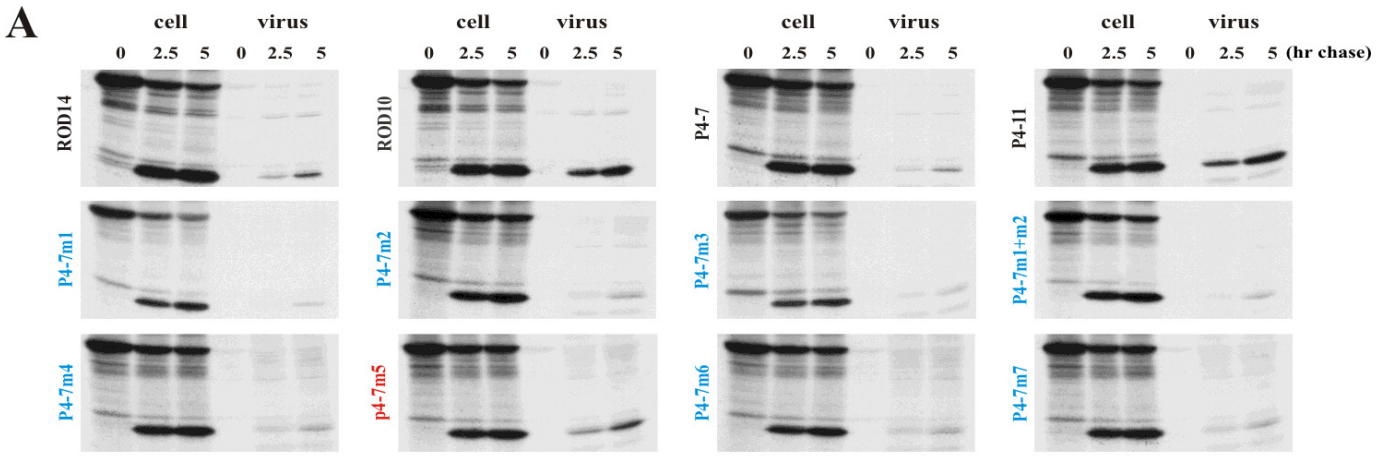


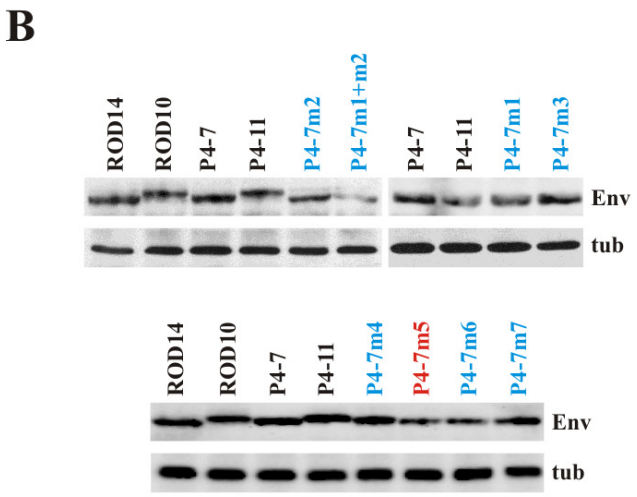


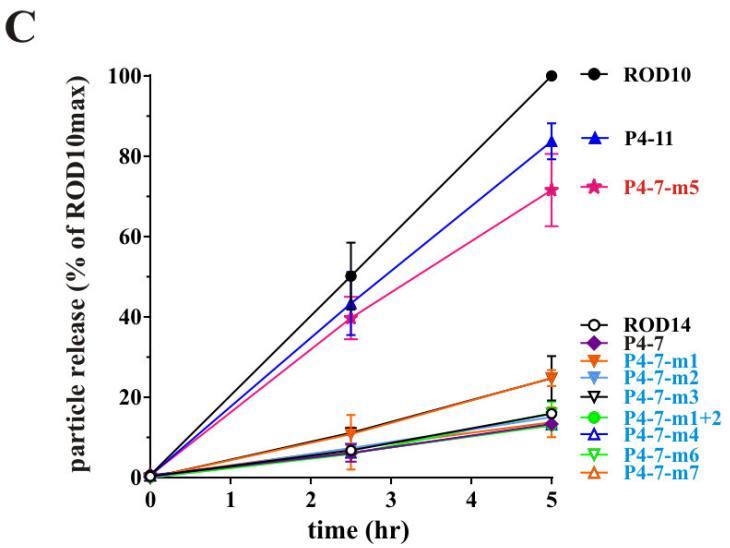


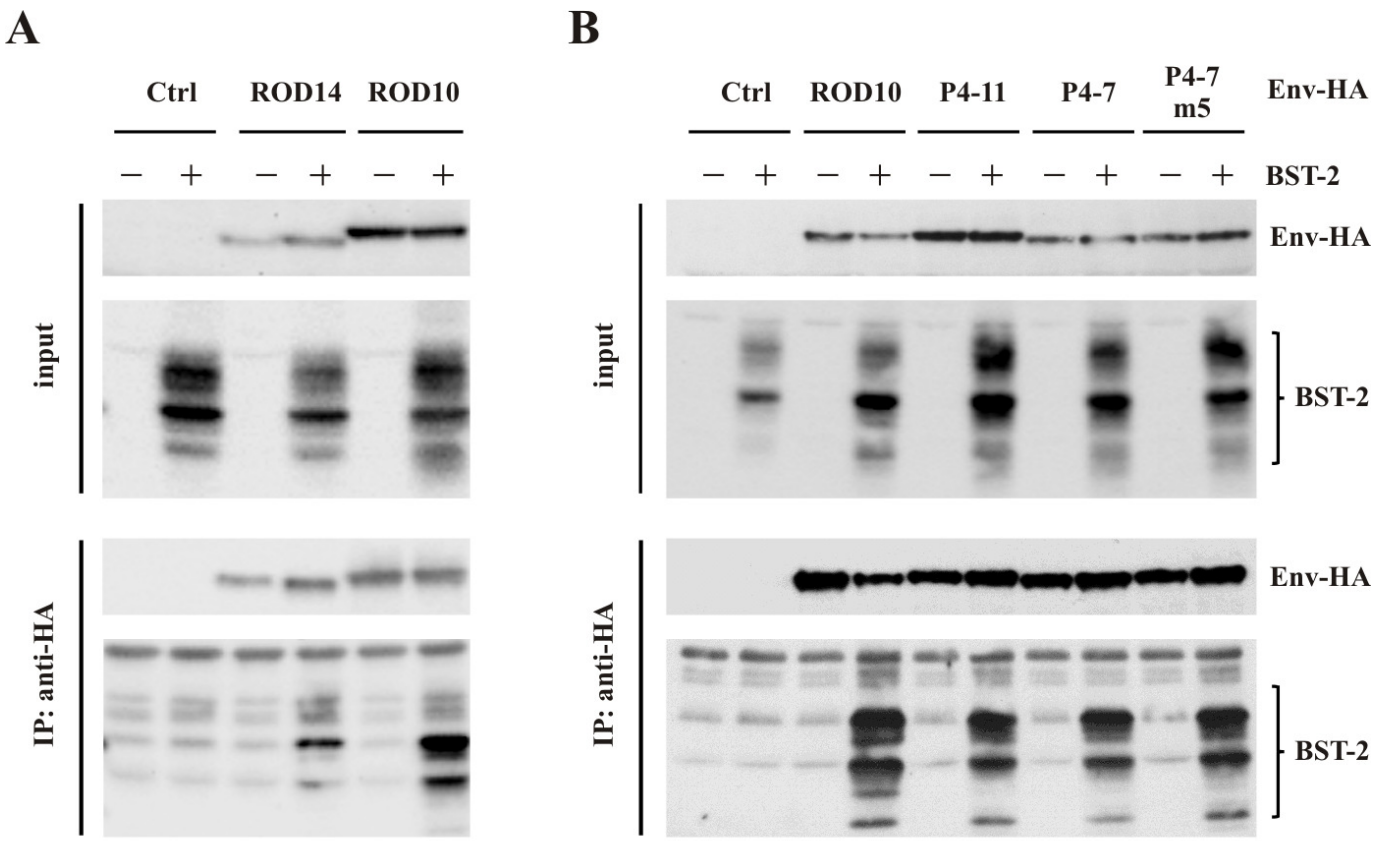












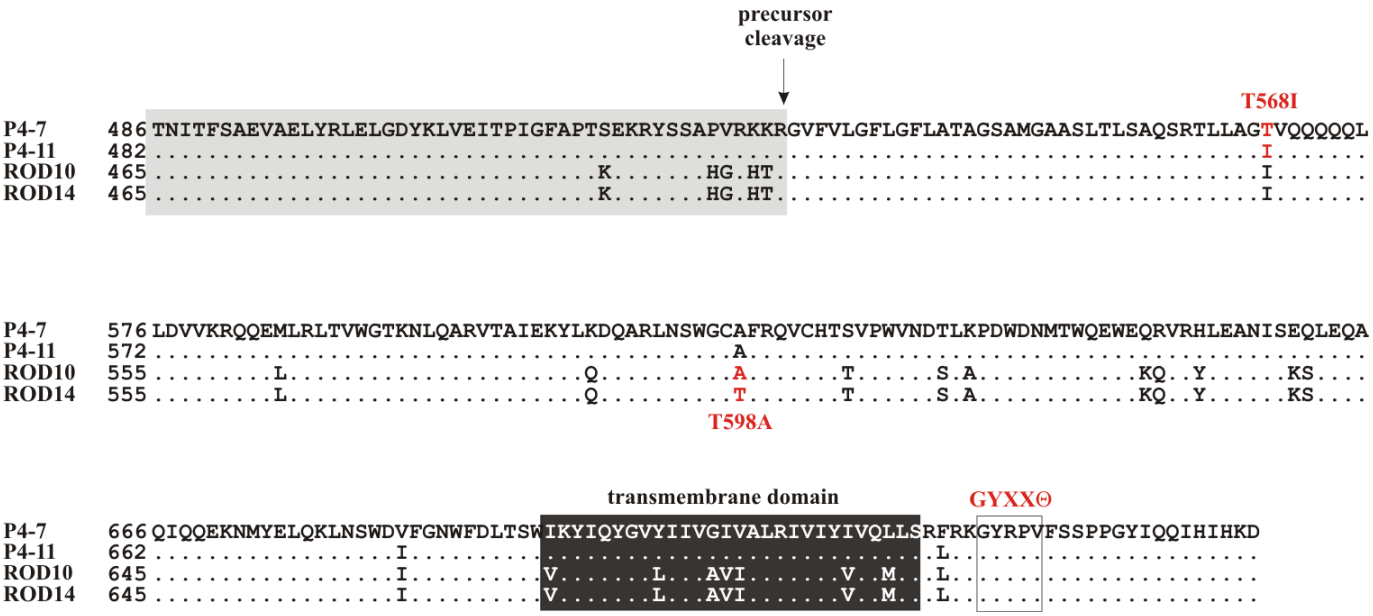


Table 1: Summary of clinical data of patients involved in this study

Patient	Sample ID a)	RNA copies/ml *)	CD4 T cell count/ul*)	Coreceptor usage	Gender	Date of sample collection	Year of diagnosis	Date of starting therapty	Therapy
P1	HCC1.03	<200	308	CCR5	F	2003	2001	2001	DDI, D4T, IDV
P2	HCC6.03	<200	615	CCR5	F	2003	1992	1996	AZT, 3TC, IDV
P3	HCC10.03	160559	48	CXCR4	M	2003	1996	1996	DDI, AZT, SQV
P4	HCC19.03	<200	175	CCR5	F	2003	2003	2005	D4T, 3TC, LPVr
P5	HCC20.03	n.a.	78	CXCR4	F	2003	1998	2005	TDF, ABC, LPVr
P6	HSM10.04	4792	265	CXCR4	F	2004	2001	2002	AZT, 3TC, NVF
P7	HSMAK.10	1793	40	dual/mixed population	F	2010	2009	no ART	no ART
P8	HSMNC.10	<200	231	CCR5	F	2010	2008	n.a.	SQV, ABC, 3TC

*) at time of sample collection
n.a. = not available

3TC Lamivudine
ABC Abacavir
AZT Zidovudine
D4T Stavudine
DDI didanosine
IDV Indinavir
LPVr Lopinavir
SQV Saquinavir

a) Marcelino et al 2010
Borrego et al 2012

Table 2. Primers used for construction and site-directed mutagenesis of HIV-2 envelope

Primer ID	Gene	Sequence (5'~3')
F3	vpr	5'-TAGACATGGAGACACCCTTGAARGMGC-3'
A1m2F	rev	5'- GCGCTCTAGAGCCACCATGAACGAAAGGGCAGACGAAGAAGGACTCC-3'
R1	nef	5'- TGTAAWACAKCCCTTCCAGTCCYCC-3'
NT5mR	env	5'- CYTCACAGGAGGGCRAKTTCTGC-3'
ROD10/14-XbaI-F	env	5'-GCGCTCTAGAATGAACGAAAGGGC-3'
ROD10/14-XhoI-HAtag-R	env	5'-GCGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATACAGGAGGGCGCT-3'
HIV-2 Rev-XbaI-F	env	5'-GCGCTCTAGAGCCACCATGAACGAAAGGGCAGACGAAGAAGGACTCC-3'
HIV-2 Rev-NheI-F	env	5'-GCGCGCTAGCGCCACCATGAACGAAAGGGCAGACGAAGAAGGACTCC-3'
1-SalI-HAtag-R	env	5'-CGCGTCGACTCAGGCGTAGTCAGGCACGTCGTAAGGATACAGGAGGGCGAGTTCTGCTCC-3'
2-XhoI-HAtag-R	env	5'-CGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATACACTATCCCGGCCAGTAAAG-3'
3-XhoI-HAtag-R	env	5'-GCGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATACAGGAGGGCGAGTTCTGCCC-3'
3-s10-XhoI-HAtag-R	env	5'-CGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATATGTCATATTGTCCCATTTAG-3'
3-s11-XhoI-HAtag-R	env	5'-CGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATATTCTATCTGCCAAGGCCAGG-3'
4-XhoI-HAtag-R	env	5'-CGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATACAGGAGGGCGAGTTCTGCTTC-3'
5/6-SalI-HAtag-R	env	5'-CGCGTCGACTCAGGCGTAGTCAGGCACGTCGTAAGGATACAGGAGGGCGATTTCTGCTCC-3'
7-XhoI-HAtag-R	env	5'-GCGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATACAGGAGGGCGAGTTCTGCCC-3'
8-s3-XhoI-HAtag-R	env	5'-CGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATATTCCTTCTCTGTCTGGCTGT-3'
8-s4-XhoI-HAtag-R	env	5'-CGCCTCGAGTCAGGCGTAGTCAGGCACGTCGTAAGGATATCGGCCAAGGCCAGGAGCTG-3'
m1-F	env	5'-GTGAATCACCTAAAGAAGGCACAAACACAACTAGCACACCTAGCACAGCTGTAAATGACA-3'
m1-R	env	5'-TGTCATTTACAGCTGTGCTAGGTGTGCTAGTTGTGTTTGTGCCTTCTTTAGGTGATTCAC-3'
m2-F	env	5'-GTGAATCACCTAAAGAAGGCAACACAACTAGCACACCTGTAAATGACAGT-3'
m2-R	env	5'-ACTGTCATTTACAGGTGTGCTAGTTGTGTTGCCTTCTTTAGGTGATTCAC-3'
m3-F	env	5'-GCAAAAACTAAATAGCTGGGATATTTTTGGCAACTGGTTTGACTTGACCT-3'
m3-R	env	5'-AGGTCAAGTCAAACCAGTTGCCAAAAATATCCCAGCTATTTAGTTTTTGC-3'
m4-F	env	5'-ACAGAACAGGACAAATCAGACAAAACGCAATTATGTGTC-3'
m4-R	env	5'-TTGCGTTTTGTCTGATTTGTCCTGTTCTGTACCCAATTG-3'
m5-F	env	5'-TTTACTGGCTGGGATAGTGCAGCAACAGCAACAGCTGTTG-3'
m5-R	env	5'-TGCTGTTGCTGCACTATCCCAGCCAGTAAAGTCCGGGAC-3'
m6-F	env	5'-AATTGTTAAGTAGACTTAGAAAGGGCTATAGGCCTGTTTTCTC-3'
m6-R	env	5'-TATAGCCCTTTCTAAGTCTACTTAACAATTGTACTATGTATATTAC-3'
m7-F	env	5'-AGAGAAGAAACAGAAGAAGACGTTGGAAACAGCGTTGGAGACAG-3'
m7-R	env	5'-TGTTTCCAACGTCTTCTTCTGTTTCTTCTCTGGCTGGCTG-3'

R = A or G
M = A or C
W = A or T
K = G or T
Y = C or T