UNIVERSITY OF CALIFORNIA, SAN DIEGO

Activities of Transmitted/Founder and Chronic Clade B HIV-1 Vpu and a Polymorphism Specifically Affecting Virion Release

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Moein Jafari

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Professor Michael David
Professor Elina I. Zuniga

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The Thesis of Moein Jafari is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2013

DEDICATION

I would like to dedicate this work to my loving and compassionate family: to my father, Ahram, and my mother, Zarangiz. I could have never accomplished this goal without your unceasing sacrifices, love, and steadfastness. Thank you for teaching me how to live. To my dearest sister, Elham, and brother, Samin. The joy and belonging that you bring to my life is priceless.

EPIGRAPH

"The tabernacle of unity hath been raised; regard ye not one another as strangers. Ye are the fruits of one tree, and the leaves of one branch. Verily I say, whatsoever leadeth to the decline of ignorance and the increase of knowledge hath been, and will ever remain, approved in the sight of the Lord of creation. Say: O people! Walk ye neath the shadow of justice and truthfulness and seek ye shelter within the tabernacle of unity."

-Baha'u'llah

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ABSTRACT OF THE THESIS

Activities of Transmitted/Founder and Chronic Clade B HIV-1 Vpu and a Polymorphism Specifically Affecting Virion Release

by

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Professor John C. Guatelli, Chair

Acute HIV-1 infection is characterized by a robust type I interferon response, resulting in the induction of several host restriction factors. HIV-1 has evolved to counteract these restriction factors, and one such adaptation, the ability of Vpu to counteract BST2/tetherin, has been proposed as having been essential for the evolution of SIVcpz into the pandemic group M HIV-1. Vpu also downmodulates the expression of CD4, counteracting a negative effect on the infectivity of virions that could in principle inhibit viral transmission. During transmission between individuals, very few or even a single virus, the "transmitted/founder (T/F) virus", gives rise to the new infection, but in the new host the selective pressure of the immune response yields the diverse "quasispecies" of chronic infection.

In this study we examine the functional characteristics of Vpu proteins encoded by T/F viruses compared to those from acute and chronic viruses from longitudinally sampled subjects. T/F Vpu proteins showed a trend towards optimized CD4 downregulation compared to chronic Vpu proteins but did not differ in their ability to downregulate BST2, although individual clones from each group were impaired in their ability to counteract BST2. Analysis of the functionally impaired clones identified a C-terminal residue, W76, as important for Vpu enhancement of virion release. Vpu clones with a W76G substitution, or site-directed mutants, are impaired in their ability to enhance virion release, but they are not defective for BST2 surface downregulation, challenging the notion that Vpu enhances virion release by removing BST2 from the cell surface.

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INTRODUCTION

Early infection with HIV-1 is characterized by a type I interferon response in an effort to control the virus. This response results in the induction of an antiviral state that is caused by the collective effect of numerous interferon stimulated genes (ISGs), including antiviral restriction factors [1-3]. One such restriction factor is BST2 (also known as tetherin) which counteracts diverse enveloped viruses by tethering them to the host cell surface and preventing their release [4, 5]. To overcome this restriction, many viruses encode countermeasures, most of which act by surface downregulation and/or targeting of BST2 for degradation (reviewed in [6]). The BST2 countermeasure encoded by HIV-1 is Vpu, which decreases BST2 on the plasma membrane [5] through the interaction between its transmembrane domain and that of BST2 and directs the degradation of BST2 through the interaction of the Vpu cytoplasmic domain with a β-TrCP (β-transducin repeat-containing protein) containing SCF (Skp-Cullin-F-box)/CRL1 (Cullin1-RING ubiquitin ligase) E3 ubiquitin ligase complex [7-10].

BST2 restriction also exists in primate lentiviruses that do not express a Vpu protein, where this function is carried out by either the envelope protein (in HIV-2 and SIV_{tan}) or the Nef protein (in $SIV_{sm/mac}$ and SIV_{agm}) in their respective hosts. SIV_{CPZ}, the immediate precursor of HIV-1, does express a Vpu protein, but the anti-BST2 activity in this lentivirus is still provided by the Nef protein, and its Vpu is devoid of this ability. The ability of Nef to counteract BST2 has been mapped to a 4 amino acid sequence in the

cytoplasmic tail of BST2. However, since human BST2 lacks this motif it is resistant to Nef antagonism. HIV-1 has been able to overcome BST2 restriction in human by adapting its Vpu protein to perform this function, while maintaining its previous functions. Notably, Vpu proteins from group O HIV-1 strains are poorly active against BST2, while those from the rare group N viruses do not degrade CD4. This anti-BST2 function of HIV-1Vpu is thought to have been a key adaptation enabling SIV_{cpz} to evolve into the pandemic group M HIV-1 [7-10]. Since Vpu's anti-BST2 activity was important for cross-species transmission of HIV-1 we hypothesize that it may be important for human-to-human transmission, particularly since BST2 is known to be upregulated during the initial interferon response to HIV-1 infection [11] and a successful founder virus must be able to counteract this.

Vpu is known to modulate the expression of other cellular membrane proteins in addition to BST2, including CD4. Vpu acts on newly synthesized CD4 by retaining it in the endoplasmic reticulum and subsequently targeting it for proteasomal degradation in a β-TrCP-dependent fashion [12, 13]. Vpu is one of the three HIV-1 proteins (the other two being Env and Nef) that target CD4, underscoring the importance of this function for the virus. As the main receptor of HIV, CD4 proteins present in the cytoplasm and cell surface can bind to the Env protein and negatively affect the virus life cycle by interfering with packaging of Env into virions and substantially reducing the infectivity of released virions by blocking the docking site of packaged Env [14-16]. Therefore, efficient enhancement of virion infectivity by Vpu through its downmodulation of CD4 might be important for viral transmission.

Vpu is a 16kDa type I transmembrane protein that consists of a single N-terminal hydrophobic TMD and a hydrophilic cytoplasmic domain [17-19]. The cytoplasmic domain contains two amphipathic α-helical domains of opposite polarity that interact with each other. These helixes are joined by an unstructured region that contains the highly conserved DSGxxS motive. Phosphorylation of the serine residues of this motive by Casein kinase 2 (CK-2) is required for binding of Vpu to β-TrCP and subsequent proteosomal degradation of CD4 [13, 20-22]. As explained above, the transmembrane domain has a critical function in promoting virus release. This is achieved through antiparallel hydrophobic binding of Vpu and BST2 TMDs helixes that is dependent on ^{10}AxxxAxxxAxxxW^{22} sequence of Vpu and ^{30}VxxxIxxLxxxL^{41} sequence of BST2 [23].

Transmission of HIV-1 to a new host has been shown to involve very few or only one virus, the "transmitted/founder" (T/F) virus, which evolves into a diverse "quasispecies" under selection by the host immune response [24-28]. Examination of the virus sequences from heterosexual, homosexual, mother-to-child, and subcutaneous transmission pairs showed that only a minor subset of circulating quasispecies in the donor are transmitted [29, 30]. In an attempt to identify the characteristics of these transmitted viruses a number of studies showed that although the transmitting subjects usually have viruses that can use both chemokine receptor 5 (CCR5) and CXC chemokine receptor 4 (CXCR4) for host cell entry, the variants that only use CCR5 (R5) are nearly exclusively transmitted [29, 31, 32].

Recent studies have shed more light on the identity of the T/F viruses. Analysis of transmission pairs showed that the early viruses found in a new host are most similar to

the virus that originally infected the donor subject [33, 34]. This finding not only confirms the existence of a severe genetic bottleneck during transmission, but also shows that among the R5 viruses circulating in the donor the variant closest to the ancestral strain is transmitted again.

About half of the forward transmission events occur during the early stages of infection when the transmitting partner is unaware of his or her HIV status and viral load is high [29], and therefore there is great interest in determining the phenotypic characteristics of T/F viruses that enable them to initiate an infection in a new host. Because of the potential implications for vaccine development, many studies of T/F viral phenotypes have focused on the viral envelope [24, 26-28], and the other proteins of these viruses remain largely uncharacterized. One recent study found that specific codon changes in Vpu were significantly associated with glycoprotein 120 (gp120) tropism signatures [35]. This finding can lead to the hypothesis that viral determinants other than envelope polymorphisms might play a role in the transmission bottleneck effect, and that specific Vpu protein characteristics might be linked to transmission fitness.

Because of its modulation of host factors involved in the immune response and its influence on virion infectivity, Vpu may contribute to successful person-to-person transmission and establishment of a new infection. We hypothesize that Vpu alleles of T/F viruses may be specifically optimized for functions important for transmission.

In present study, Vpu alleles from 10 sexually transmitted clade B group M HIV-1 Transmitted/Founder (T/F) infectious molecular clones (IMC) [24-28] were epitope-tagged and cloned into a Rev-dependent expression vector. For comparison, Vpu alleles

from NL4-3 and 4 pairs of longitudinally-acquired plasma samples from the acute and chronic phases of infection of 4 clade B HIV-1-infected patients from the San Diego Primary Infection Cohort (SDPIC) were cloned in a similar fashion. After evaluating the capacity of each allele for directing BST2/tetherin downregulation from the cell surface, enhancement of virion release, and CD4 downregulation, we found that Vpu proteins from T/F viruses trended towards optimization of their anti-CD4 function compared to Vpu from NL4-3 and the chronic clade B isolates from the SDPIC. T/F Vpu proteins as a group were no better at BST2 downregulation than Vpu from NL4-3 or the chronic isolates. These data suggest that optimization of Vpu's anti-CD4 activity may play a role in transmission and the establishment of a productive infection in a new host.

Moreover, we identified a conserved tryptophan at position 76 in the Vpu cytoplasmic tail that is critical for Vpu enhancement of virion release but dispensable for BST2 surface downregulation. This decoupling of surface downregulation of BST2 and virion release enhancement by Vpu W76 mutants challenges the prevailing notion that Vpu counteracts BST2 simply by removing it from the cell surface.

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MATERIALS AND METHODS

Cell Cultures—HeLa P4-R5 [36-38] were acquired from the NIH AIDS Research & Reference Reagent Program from Dr. Nathaniel Landau and maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin and 1 ug/ml puromycin.

Plasmids and Cloning—The Rev-dependent expression vector pcDNA-RRE was constructed by cloning the NL4-3 Rev-response element (RRE) into pcDNA3.1(-) (Invitrogen). The panel of ten full-length transmitted/founder (T/F) HIV-1 infectious molecular clones (IMC) was obtained through the NIH AIDS Research & Reference Reagent Program from Dr. John Kappes [24-27]. *Vpu* sequences were amplified from these IMCs by PCR with Vpu sequence-specific primers designed to introduce a C-terminal FLAG tag, then cloned into the pcDNA-RRE expression vector. To clone Vpu alleles from longitudinally-acquired plasma samples (Table 1) from the San Diego Primary Infection Cohort (SDPIC), HIV RNA was extracted from plasma samples using the High Pure Viral RNA Kit (Roche), reverse transcribed using the RETROscript Kit (Ambion) with random decamer primers, then amplified by nested PCR with primers targeting conserved sequences flanking the Vpu coding region. Nested PCR products were column purified using the QIAquick PCR Purification Kit (Qiagen) then bulk sequenced. Cloning primers including a C-terminal FLAG tag were designed based on the bulk sequencing, with multiple primer sets designed for samples exhibiting variation in the 5' and 3' Vpu coding sequence. These primers were used to amplify FLAG-tagged

Vpu coding sequence from the nested PCR directed mutagenesis, mutations were introduced into the relevant pcDNA-Vpu-FLAG-RRE constructs using the QuikChange kit (Stratagene) and verified by sequencing. The β-TrCP–HA expression vector in the backbone vector pCS2-HA3 was previously described [39].

Transfections—HeLa P4-R5 cells in 6-well plates were transfected with 2 μg of the Vpu expression construct (pcDNA-Vpu-FLAG-RRE) and 2 μg of the Rev- and GFP-co-expressing plasmid pRev-IRES-GFP using Lipofectamine 2000 (Invitrogen). Cells were harvested the next day for immunoblot and staining for flow-cytometry. For virion release assays, HeLa P4-R5 cells in 12-well plates were transfected with 500 ng of the Vpu expression construct (pcDNA-Vpu-FLAG-RRE) and 600 ng of proviral plasmid lacking Vpu (pNL4-3 ΔVpu). Interferon-treated cells were cultured in 1000 U/ml human interferon-α A/D (Sigma). Supernates and cells were collected 24 hours later for p24 ELISA and immunoblot, respectively.

Flow Cytometry—Transfected HeLa P4-R5 cells were stained with APC anti-human CD4 antibody (OKT4, Biolegend), APC mouse IgG2b, κ isotype control (Biolegend), Alexa Fluor 647 anti-human CD317 (BST2, Tetherin; RS38E, Biolegend) or Alexa Fluor 647 mouse IgG1, κ isotype control according to manufacturer's instructions, then fixed in 1.5% paraformaldehyde. Surface expression of CD4 and BST2 were analyzed using a BD Accuri C6 Flow Cytometer after gating on live GFP-positive (Rev-expressing) cells. For staining of intracellular p24, cells were fixed and permeabilized using the BD

Cytofix/Cytoperm kit according to manufacturer's instructions, followed by staining with FITC-conjugated anti-HIV-1 p24 antibody (KC57, Beckman Coulter).

Virion Release Assays—Supernates from NL4-3 ΔVpu provirus and Vpu expression plasmid co-transfected HeLa P4-R5 cells were collected 24 hours after transfection essentially as described [5]. Virion-associated p24 was pelleted through a 20% sucrose cushion before measurement by p24 ELISA (PerkinElmer or Advanced Bioscience Laboratories).

Infectivity Assays—Infectious center assays using HeLa P4-R5 cells were performed and analyzed as previously described [40].

Co-immunoprecipitation—For co-immunoprecipitation, HeLa P4-R5 cells in 6 well plates (2 wells per condition) were co-transfected with 1.3 μg β-TrCP-HA expression vector, 1.3 μg Rev expression vector (pRev-IRES-GFP) and 1.3 μg Vpu-FLAG expression vector (or empty pcDNA3.1(-) vector as a control). Twenty-four hours later, the cells were collected (duplicate wells were pooled) and lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5% glycerol supplemented with protease inhibitor cocktail (Roche Diagnostics) for 30 min on ice followed by 30 min at room temperature. Cell lysates were cleared by centrifugation at 16,000 x *g* at 4°C for 10 min and then incubated with anti-FLAG M2-conjugated magnetic beads (Sigma) for 2 hr at 4°C. Beads were then washed 5 times with wash buffer (50mM Tris-HCl (pH 7.4), 250 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5%

glycerol), and the precipitated proteins were eluted with Laemmli buffer and analyzed by immunoblot.

Immunoblots—Immunoblots for Vpu, BST2, gp120, actin, and HA- and FLAG-epitopes were performed as previously described [41-43].

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RESULTS

T/F Vpus are Optimized for CD4 Down-modulation— Transmitted/founder Vpu proteins (Figure 1A shows their amino acid sequence alignment) were FLAG-tagged and cloned into a Rev-dependent expression construct (Figure 1B). To test for the downregulation of CD4 and BST2 from the cell surface, we used a flow cytometric assay in which HeLa P4-R5 cells were transfected with a bicistronic plasmid co-expressing Rev and GFP as well as a Rev-dependent vector expressing FLAG-tagged Vpu proteins (Figure 1B), then surface stained with fluorophore-conjugated anti-CD4 or anti-BST2 antibodies. Transfected cells that produce enough Rev to express Vpu are also GFP-positive, and depending on the functionality of the tested Vpu protein will exhibit a decrease in surface CD4 and BST2. This is evident in the two-color plots of Figure 1C; as GFP (and Rev) expression increases, CD4 and BST2 surface expression decrease. For T/F Vpu proteins CH040 (middle panels of Figure 1C) and CH077 (right panels), BST2 downregulation (bottom row) appears similar to that of Vpu_{NL4-3}, but for CD4 downregulation (top row), CH077 appears more active than CH040 and Vpu_{NL4-3}. This is shown again in Figure 1D, where the CD4 downregulation activity of each T/F Vpu is plotted as a ratio compared to the activity of Vpu_{NL4-3}. All T/F Vpus, with the exception of CH040, exhibited relatively enhanced anti-CD4 activity, reducing the surface expression of CD4 25-60% more effectively than Vpu_{NL4-3}. The efficient counteraction of CD4 by Vpu depends primarily although not exclusively on its cytoplasmic domain (CD) [44, 45], and the variation in the anti-CD4 activity of these T/F Vpus suggests that the

tertiary structure of this domain, although apparently dynamic and tolerant of mutations, might be related to its functional efficiency (Figure 1A).

Most T/F Vpus Downregulate BST2 With the Same Potency as Vpu$_{NL4-3}$ — As BST2 is an interferon-induced antiviral restriction factor and the acute phase of HIV-1 infection is marked by a substantial type I interferon response, we hypothesized that T/F Vpu proteins might be optimized for BST2 down-modulation, since they were derived from viruses that successfully established infection under this selective pressure. To test this, HeLa P4-R5 cells were transfected with FLAG-tagged Vpu and the Rev expression constructs and analyzed for surface expression of BST2. As seen in Figures 1C and 1E, all T/F Vpus except for RHPA were similar to Vpu$_{NL4-3}$ in their BST2 surface-downregulation activity. The impaired RHPA protein notably differs from NL4-3 by an A15G polymorphism in the transmembrane domain; this is the second A in the AxxxAxxxAxxxW motif previously found to be important for the interaction between the TMDs of Vpu and BST2 [23, 46]. RHPA also encodes a variant amino acid (leucine) instead of the conserved W76 in the cytoplasmic tail, which is studied further below. The expression levels of these Vpu proteins were detected by immunoblot for the FLAG epitope (Figure 1F). While expression levels of these Vpu proteins appear to be only roughly correlated with their CD4 downregulation activities, RHPA, which is impaired for BST2 surface downregulation, is the most poorly expressed among the T/F Vpus.

T/F Vpus Enhance Viral Egress with Similar Effectiveness to Vpu$_{NL4-3}$ and are Not Relatively Resistant to IFN-α —Treatment of cells with type I interferon upregulates

BST2 and enhances its inhibition of virion release. To test whether T/F Vpus are optimized for the counteraction of BST2's inhibitory effect on viral egress, we transfected HeLa P4-R5 cells with HIV-1 provirus that lacks Vpu (NL4-3 ΔVpu) along with the Vpu-FLAG-RRE expression constructs to provide Vpu in *trans*. Cells were cultured in media with or without 1000 U/ml IFN-α and after 24 hours supernates and cells were collected. Viral supernates were pelleted through 20% sucrose cushions and p24 was measured by ELISA (Figure 2A). Cells were analyzed for Vpu, BST2 and gp120 expression by immunoblot (Figure 2B). Although variation is evident among the T/F Vpus, we found that the phenotypes of the T/F Vpus with respect to enhancement of virion release were similar to that of Vpu_{NL4-3}, except for CH040, which was substantially impaired. No Vpu protein was resistant to the effects of interferon, which as expected induced the expression of BST2 as detected by immunoblot. Notably, despite being impaired for enhancement of viral egress, CH040 Vpu was as potent as Vpu_{NL4-3} at downregulating cell surface BST2 (Figure 1E), suggesting that these two functions of Vpu are separable. As shown below, this phenotypic discordance appears largely due to a W76G polymorphism near the protein's C-terminus. The expression of these Vpu proteins as detected by immunoblot for FLAG (Figure 2B) did not reveal a clear correlation with the virion release phenotype (as was similarly the case regarding the data of Figure 1 with respect to the phenotypes of CD4 and BST2 downregulation). Notably, in the presence of IFN-α, the expression of all Vpu proteins was reduced, correlating with the attenuated enhancement of virion release under these conditions. Altogether, the data indicate that Vpu proteins from T/F viruses are not optimized for enhancement of virion

release relative to Vpu_{NL4-3}, even when the experimental conditions include exogenous IFN-α to mimic the antiviral state that occurs in response to acute infection *in vivo*.

Individual Vpu Proteins Isolated from Chronically Infected Persons Vary Substantially in Their Anti-CD4 and Anti-BST2 Effects— While T/F Vpus appear to be optimized for CD4 downregulation compared to Vpu_{NL4-3}, this Vpu is from a lab-adapted HIV-1 strain and might not be representative of Vpu proteins derived from primary, chronic clade B isolates. To compare the function of T/F Vpus to that of a selection of Vpus isolated from chronically infected persons, we cloned Vpu alleles from plasma samples acquired longitudinally from treatment-naive subjects in the San Diego Primary Infection Cohort (SDPIC). Viral RNA was extracted from four pairs of plasmas from the acute and chronic phases of infection for each subject. The isolates from the acute phase were collected 45-85 days after the estimated date of infection (Table 1), so they were likely subjected to selection pressures in the host, in particular the cytotoxic T lymphocyte response, that would induce coding changes relative to the T/F virus in that individual. Therefore, studies of the acute Vpus are most relevant in comparison to their matching chronic Vpus, where differences in function may be attributed to specific amino acid changes that developed *in vivo*. Nonetheless, the chronic Vpus also provide a comparison to the T/F Vpus, as described below.

Viral RNA extracted from plasma was reverse transcribed into cDNA, then amplicons including the Vpu sequence were cloned by nested PCR and sequenced in bulk. Based on the sequencing results, primers were designed to FLAG-tag and clone Vpu proteins into the Rev-dependent expression vector described above. The amino acid

sequence alignment of the cloned acute and chronic Vpu proteins (shown in Figure 3A) reveals that, as anticipated, the acute and chronic Vpu pairs are more similar to each other than to acute or chronic Vpu isolates from other patients. Analysis of CD4 downregulation by FACS as described above revealed that all acute and chronic Vpu pairs are as good or better at CD4 downregulation than Vpu_{NL4-3}, except for the pair from subject N528 (Figure 3B). Acute and chronic Vpu pairs were also similar to Vpu_{NL4-3} in their ability to downregulate BST2 from the cell surface (Figure 3C). While the N988 acute Vpu was most active against CD4 among the tested isolates, it was the least active for BST2 downregulation. Similar to the T/F Vpu proteins, the expression of the acute and chronic Vpus did not clearly correlate with their relative anti-CD4 or anti-BST2 activity (Figure 3D).

Acute and chronic Vpu proteins were also tested for their ability to enhance viral egress as described above (Figure 3E), but in the absence of interferon treatment. Although the ability to enhance virion release was highly variable among these Vpus, N528 acute and N988 chronic Vpus were substantially impaired for this phenotype. For N528 acute Vpu, which is also less active for BST2 surface downregulation, this phenotype might be related to the presence of two hydrophilic residues at positions 7 and 8 (serine-threonine); this could reduce the efficiency of interaction between the transmembrane domains (TMDs) of Vpu and BST2 by altering the tilt angle of the Vpu TMD helix in the lipid bilayer as a result of hydrophobic mismatch [23]. N988 chronic Vpu was slightly impaired for BST2 surface downregulation but was nearly defective for enhancement of virion release. Notably, the N988 acute Vpu was not markedly impaired

for enhancement of virion release, although it was similar to N988 chronic Vpu in its ability to downregulate BST2 from the cell surface. These two paired proteins differ by only three amino acids: N988 acute Vpu has the conserved proline at amino acid 3 while N988 chronic has a serine; it has a glutamic acid at amino acid 5 while N988 chronic has a lysine; and it has the conserved tryptophan at position 76 while N988 chronic has a glycine. This W76G polymorphism near the C-terminus of the Vpu cytoplasmic tail is, as noted above, also present in T/F Vpu CH040, a Vpu that is characterized by the same discordance between the phenotypes of BST2 downregulation and enhancement of virion release (Figures 1 and 2). The role of this residue in enhancing virion release is studied by site-directed mutagenesis below.

T/F Vpu Alleles as a Group Trend Toward Better CD4 Downregulation Activity Compared to Chronic Vpus— CD4 downregulation activity of T/F Vpu proteins as a group was compared to that of chronic Vpu proteins (comparing values from two experiments for each in duplicate). While a trend toward better CD4 downregulation by the T/F alleles is apparent (Figure 4A), analysis by Mann-Whitney test revealed that this difference is not statistically significant, yielding a p-value of 0.0759. This might be due to the small sample size and the variation among the chronic Vpus. The comparison of the CD4 downregulation activity of T/F Vpus to that of the acute proteins yielded a p-value of 0.1878. The BST2 downregulation activities of the T/F, acute, and chronic proteins were not different (Figure 4B): the T/F vs. acute Vpu comparison yielded a p-value of 0.6354 and the T/F vs. chronic Vpu comparison yielded a p-value 0.9451. Notwithstanding the lack of statistical significance, the trend toward a hierarchy of

optimal CD4-downregulation among the T/F Vpus (with lesser activity among the acute Vpus and the lowest activity among the chronic Vpus) is absent in the case of the BST2 downregulation phenotype.

Differences in Clade B Consensus and NL4-3 Vpu Cytoplasmic Tail Residues that Affect Vpu Function. **Although Vpu_{NL4-3}**— Vpu is often studied as the prototype for Vpu function, obvious differences in amino acid sequence exist between NL4-3 and the clade B consensus Vpus (Figure 1A), particularly in the cytoplasmic domain. We hypothesized that these sequence anomalies might account for differences between Vpu_{NL4-3} function and that of the tested primary clade B isolates. For instance, while the clade B consensus Vpu (and that of most primary alleles tested here) has DQE at residues 60-62, this is deleted in NL4-3. Also, Vpu_{NL4-3} has a GVEM duplication at residues 67-70 that is not present in the primary clade B isolates. To test the effects of these variations, we mutated the Vpu of the T/F isolate CH077, which is superior to Vpu_{NL4-3} for CD4 downregulation, equally potent for BST2 downregulation, and slightly less effective at enhancing virion release. We generated CH077 Vpu mutants that lacked DQE at residues 60-62 or harbored the GVEM duplication at residue 71. CH077 ΔDQE was no longer better than Vpu_{NL4-3} at CD4 downregulation (Figure 5A), although this mutation had no effect on the ability of CH077 to downregulate BST2 (Figure 5B) and slightly improved its ability to enhance virion release (Figure 5C). Just downstream of the β-TrCP binding site, the ΔDQE mutation might alter the conformation of the cytoplasmic tail, differentially affecting its affinity for cofactors involved in CD4 downregulation and the enhancement of virion release. In contrast, the GVEM duplication when inserted into the

CH077 Vpu amino acid sequence had no effect on CD4 downregulation (Figure 5A), BST2 downregulation (Figure 5B), or enhancement of virion release (Figure 5C). These data suggest that while the GVEM duplication might in principle provide compensation for the DQE deletion in Vpu_{NL4-3}, its insertion does not compromise the function of another Vpu protein. Apparently, Vpu-function is not highly sensitive to the length of the C-terminal portion of the Vpu cytoplasmic domain.

Site-Directed Mutation of Tryptophan 76 Reveals Its Specific Importance for Vpu-mediated Enhancement of Virion Release— Among the primary Vpu proteins analyzed here, the two most impaired for enhancement of virion release, the T/F Vpu CH040 and the chronic Vpu from subject N988, both have a tryptophan-76-to-glycine (W76G) polymorphism. Despite their impaired ability to counteract BST2-mediated restriction of virion release, these Vpu proteins were remarkably effective at downregulating cell surface BST2 (Figures 1-3). To investigate whether the W76G substitution is sufficient to impair the enhancement of virion release, site-directed mutations encoding it were introduced in NL4-3 Vpu and the Vpu of the high-performing and well-expressed T/F Vpu CH077 (Figure 6). As noted above, the T/F Vpu RHPA also has a polymorphism involving this residue: W76L. However, while RHPA Vpu is modestly impaired for enhancement of virion release compared to VpuNL4-3, it was also impaired for BST2 surface downregulation, unlike the Vpus from CH040 and chronic N988, which have the W76G polymorphism (Figures 1-3). Site-directed mutagenesis was therefore also used to introduce the W76L substitution into Vpu_{NL4-3}. All of these mutants were tested for CD4 and BST2 surface downregulation (Figure 6) as well as for enhancement of viral egress

(Figure 7). To insure a direct comparison between the phenotypes of BST2 downregulation and enhancement of virion release, we measured cell surface BST2 on the cells used for the virion release assays (Figure 7A), as well as independently when Vpu was expressed in the absence of viral assembly (Figure 6). As expected (Figure 6A), the W76G and W76L mutations had no effect on the downregulation of CD4 by NL4-3 or CH077 Vpu. BST2 downregulation (Figures 6B and 7A) was likewise minimally or unaffected by the W76G or W76L substitutions. This suggests that the impaired downregulation of surface BST2 by RHPA Vpu (which has L76) is not due to this polymorphism. In contrast to their minimal impact on the downregulation of surface BST2, both the W76G and W76L substitutions substantially decreased the ability of NL4-3 Vpu to enhance virion release (Figure 7B). The W76G substitution also impaired virion release enhancement by CH077 Vpu. Together, these data indicate the substitution of W76 near the C-terminal end of the Vpu cytoplasmic tail with glycine or leucine substantially impairs Vpu's ability to enhance virus release without markedly affecting its ability to reduce the total amount of BST2 at the cell surface. This phenotypic discordance is especially evident in the case of the W76G substitution in the context of the T/F CH077 Vpu.

We examined whether the defect in anti-BST2 activity conferred by the W76G substitution relates to Vpu's ability to interact with β-TrCP, which recruits a SCF (Skp-Cullin 1-F box) E3 ubiquitin ligase to direct ubiquitination and degradation of BST2 [41, 47-49]. Mutations of the serines in the β-TrCP binding motif of Vpu (S52,56N) abrogate β-TrCP binding, not only abolishing Vpu-mediated downregulation of CD4 but also

substantially impairing its ability to downregulate BST2 and enhance virion release [13, 49]. To determine whether the W76G substitution affected the Vpu-β-TrCP interaction or instead acted by a different mechanism to disrupt Vpu's anti-BST2 effects, mutations encoding the W76G and S52,56N substitutions were introduced separately and in combination into the T/F CH077 Vpu allele. These mutants were tested for CD4 and BST2 surface downregulation and for enhancement of virion release. As expected, CH077-S52,56N was markedly impaired for CD4 downregulation while CH077-W76G was not (Figure 6C). When combined, the W76G and S52,56N mutations had an additive effect, abrogating the ability of CH077 Vpu to downregulate CD4. Similarly, CH077-W76G was not impaired for BST2 surface downregulation (Figure 6B); CH077- S52,56N was very slightly impaired. The combination mutant CH077 S52,56N-W76G was most impaired for BST2 downregulation, despite the lack of effect of the W76G single mutation, although all of these effects were modest. More strikingly, substitution of W76G and mutation of the β-TrCP binding site caused independent and additive impairments of Vpu's ability to enhance virion release (Figure 7B): Vpu CH077-W76G was more impaired for enhancement of virion release than was Vpu CH077-S52,56N, and the Vpu with both S52,56N and W76G substitutions had virtually no activity. These additive effects suggested that these mutations are acting at different steps through which Vpu counteracts the restriction imposed by BST2. Moreover, the data suggest that W76 is important for an aspect of BST2 antagonism that is disassociated mechanistically from surface downregulation and degradation.

We further excluded that the W76G mutation affects the Vpu-β-TrCP interaction by co-immunoprecipitation (Figure 7C). HeLa P4-R5 cells were transfected with Vpu-FLAG-, Rev-, and HA-tagged β-TrCP-expression plasmids, and the cell lysates were incubated with anti-FLAG-magnetic beads. Despite robust expression levels, CH077-S52,56N did not pull down β-TrCP, as previously demonstrated [48]. CH077-W76G, however, was able to interact with β-TrCP to the same extent as CH077 Vpu, indicating that the defects in anti-BST2 activity conferred by the W76G mutation were, as expected, not related to its capacity to interact with β-TrCP. Importantly, these data also indicated that the relatively greater ability of the T/F Vpus to downregulate CD4 relative to Vpu_{NL4-3}, exemplified here by Vpu CH077, is not likely due to improved binding to β-TrCP.

This section, in part, has been submitted for publication of the material as it may appear in Jafari, Moein; Guatelli, John C.; Lewinski, Mary K. "Activities of Transmitted/Founder and Chronic Clade B HIV-1 Vpu and a Polymorphism Specifically Affecting Virion Release." Journal of Virology, 2013. Moein Jafari was the primary investigator and author of this material.

DISCUSSION

Despite facing the robust type I interferon response of acute infection and the associated upregulation of BST2 [11], the transmitted/founder Vpu proteins tested here are no better at BST2 downregulation or enhancement of virion release compared to the "prototype" clade B Vpu_{NL4-3} or the clade B Vpus obtained from a cohort of chronically infected patients. These results suggest that the recent observation that clade B T/F viruses are relatively more resistant to interferon than chronic clade B controls [50] is not likely related to an optimized anti-BST2 activity of Vpu. However, we observed a trend, although not achieving statistical significance, toward optimization of T/F Vpu proteins for CD4 downregulation compared to Vpu_{NL4-3} and the chronic Vpus. This raises the possibility that the anti-CD4 activity of Vpu plays a role in transmission, possibly through consequent enhancement of the infectivity of newly produced virions. This hypothesis is consistent with recent results indicating that T/F viruses exhibit enhanced cell-free infectivity and greater virion-incorporation of envelope glycoprotein [50].

Examination of the amino acid sequences of primary Vpu isolates with differential anti-BST2 activities suggested that the conserved tryptophan at position 76 near the protein's C-terminus is important for the Vpu-mediated enhancement of virion release but is less critical for the downregulation of BST2 from the cell surface. This conclusion was directly supported by site directed mutagenesis of both NL4-3 and a highly functional T/F Vpu protein. Specifically, the naturally occurring W76G polymorphism is associated with impaired enhancement of virion release but preserved BST2 surface-downregulation. Site-directed mutants encoding W76G are similarly

impaired. Co-immunoprecipitation experiments showed that the W76G mutation does not affect the binding of Vpu to β-TrCP. Moreover, evaluation of the combination mutant S52,56N (which abrogates binding to β-TrCP) plus W76G indicated that these mutations are additive, and thus likely mechanistically distinct.

The mechanism by which W76 specifically contributes to the enhancement of virion release by Vpu is unclear, but it appears to be independent of the downregulation of BST2 from the cell surface, and given the additive nature of its contribution to that of β-TrCP, it is likely also independent of the degradation of BST2. The contribution of W76 might involve the recently described ability of Vpu to displace BST2 from virion budding sites in the plane of the plasma membrane [51]. This phenotype mapped to the C-terminal α-helix in the Vpu cytoplasmic domain, which is adjacent the unstructured extreme C-terminus in which W76 resides. Interestingly, the displacement-effect is phenotypically additive with the effect of β-TrCP, reminiscent of the additive effect of the W76G and S52/56N mutations herein. But rather than accounting for the residual activity of a Vpu mutant that lacks the ability to bind β-TrCP and is essentially defective in downregulating BST2 [51], the phenotype of the W76G variants shown here suggests that the overall downregulation of cell surface BST2 is not sufficient for the antagonism of restricted virion release. Currently, we hypothesize that despite the downregulation of total cell surface BST2 by Vpu W76G, sufficient BST2 likely remains at virion budding sites to preserve the restriction of release. How W76 would specifically enable Vpu to include virion-budding-site associated BST2 in the downregulated population remains to be elucidated. Nonetheless, we speculate that Vpu might require such an activity due to

the timing of its expression. We note that Vpu, like Gag, is a Rev-dependent protein that is expressed relatively late in the virus replication-cycle. Vpu has no "head start" to remove BST2 from the cell surface before the onset of viral assembly, as might be the case for the SIV Nef proteins that antagonize BST2 (reviewed in [6]). Thus, optimal Vpu-activity with respect to the enhancement of virion release might require a mechanism that enables the inclusion of BST2 that is already associated with assembling virions to be downregulated, and we hypothesize that this mechanism requires W76. Notably, W76 is a conserved feature of clade B Vpu proteins such as those studied here, but it is not conserved within other clades of group M HIV-1, including the most prevalent clade, C. Whether this lack of conservation can be explained by alternative sequences at the extreme C-terminus of Vpu proteins that provide a functional contribution similar to W76, or whether the downregulation of BST2 from the cell surface, an activity for which W76 is dispensable, is actually a more robustly conserved phenotype than the enhancement of virion release, remains to be clarified.

In summary, T/F Vpu proteins as a group do not seem superior to Vpu proteins from chronically infected persons with respect to their anti-BST2 activity, although they may be optimized for CD4 downregulation. Whether the enhanced anti-CD4 activity of T/F Vpus translates into enhanced viral infectivity, and whether it plays a role in transmission are key questions that remain to be answered. In addition, the study of Vpu proteins encoded by primary isolates lead us to identify C-terminal residue W76 as important for enhancement of virion release by clade B Vpu by a mechanism that is not associated with the removal of BST2 from the cell surface.

More work needs to be done to fully unravel any potential phenotypic differences among T/F Vpu proteins. The phenotypic characteristic of T/F Vpu proteins studied here are the known phenotypes that the virus uses to directly ensure its propagation. However, at least three other Vpu phenotypes fulfill this purpose indirectly: suppression of BST2 induced NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling, downregulation of NTB-A, a co-stimulator of NK cell killing, and interference with CD1d recycling on infected dendritic cells.

Unlike other aspects of HIV-1, like the Tat and Nef proteins, which have been shown to activate NF-κB pathway [52, 53], Vpu inhibits BST2 induced activation of NF-κB [42]. NF-κB is a rapid-acting primary transcription factor that is activated under variety of cellular stresses, including viral infection. Activation of the NF-κB transcriptional program is a crucially important early step of immune activation and targets over one hundred genes. As a result, interference with NF-κB signaling is one of the tools widely employed by viruses (reviewed in [54]). Interestingly, unlike downregulation of BST2, which is completely dependent on direct interaction of the Vpu and BST2 TMDs and the removal of BST2 from the cell surface, inhibition of NF-κB is mostly independent of this interaction. Moreover, it has been suggested that multimerization of BST2 at the cell surface or its localization to the ER may be key to its ability to act as a viral sensor and activate the NF-κB pathway. Vpu may act by disrupting proper multimerization and localization of BST2 and/or by saturating available β-TrCP, which is required for the release of NF-κB inhibition via the ubiquitination and degradation of IκB [42, 55].

This tactic of inhibiting NF-κB is paradoxical because HIV-1 employs binding sites for NF-κB in its promoter and uses these to increase the rate of transcriptional initiation. This paradox lead to the hypothesis that fine-tuning of NF-κB in different stages of viral life cycle might be what the virus would benefit from, rather than mere activation or suppression of this pathway. Moreover, differential manipulation of this signaling pathway might be important during the different stages of HIV-1 infection. Therefore, study of the NF-κB modulation phenotype of T/F Vpus might reveal distinct features of these proteins that help T/F viruses establish new infections.

Another phenotype of Vpu that was recently identified is downregulation of NK-T- and -B cell antigen (NTB-A) from surface of the infected cell [56-58]. Natural Killer (NK) cells are a part of the innate immune system and their response to infected cells does not require prior exposure to the pathogen. Hence, they are instrumental in fighting viral infections during early stages of the disease, before the emergence of specific adaptive immune responses. The response of a NK cell to a target cell depends on the interaction of NK cell receptors with their specific ligands on the target cell. These receptor-ligand interactions are of three kinds: inhibitory, activating, and coactivating (reviewed in [59-61]). NTB-A is a coactivating transmembrane protein that is expressed on NK cells and is characterized by its -homotypic interaction with molecules present on the target cell [62, 63]. This coactivating surface molecule appears to work in conjunction to NKG2D activating receptor on NK cells. In the absence of NTB-A binding the activation of NK cells through NKG2D signaling seems to become less effective at inducing degranulation. Thus, Vpu antagonizes lysis by NK cells by reducing NTB-A

molecules from the cell surface [57]. This function might be instrumental in early HIV-1 infection, since it promotes survival of virus-producing cells and spread of infection. It can, therefore, be speculated that T/F Vpu proteins may possess enhanced anti-NTB-A activity to ensure establishment of a productive infection.

Downregulation of CD1d from the surface of Professional Antigen Presenting Cells (APCs) is yet another function of Vpu [64]. CD1d is a member of CD1 family of non-polymorphic MHC-like molecules, which are typically expressed by APCs and present foreign lipid-based antigens. A subtype of T cells, known as Natural Killer T (NKT) cells, are functionally distinct from MHC-restricted T cells and express many of the NK cell markers [65]. Many of these NKT cells express a special type of T cell Receptor (TCR) that is specific for lipid-based antigens presented in the context of CD1d. NKT cells are essential in early response to pathogens because they can produce large amounts of cytokines, and are able to promote or suppress adaptive immunity without the need for clonal expansion [66, 67]. Therefore, since the T/F HIV-1 viruses must remain undetected by NKT to be able to establish a productive infection, efficient surface downregulation of CD1d by T/F Vpu proteins seems to be important in early stages of infection. The scope of phenotypic analysis of T/F Viruses (and their Vpu protein in particular) can, therefore, be extended to counteraction of NKT cells.

In conclusion, exploration of functional properties of T/F Vpus may reveal valuable information about the requirements the virus must possess to overcome early host restrictions. Moreover, other aspects of T/F HIV-1 viruses need to be elucidated to

uncover possible unique features of these viruses, which can ultimately be used to develop preventative measures against this pandemic disease.

This section, in part, has been submitted for publication of the material as it may appear in Jafari, Moein; Guatelli, John C.; Lewinski, Mary K. “Activities of Transmitted/Founder and Chronic Clade B HIV-1 Vpu and a Polymorphism Specifically Affecting Virion Release.” Journal of Virology, 2013. Moein Jafari was the primary investigator and author of this material.

FIGURES AND TABLES

Figure 1. CD4 and BST2 downregulatory activity and expression of transmitted/founder Vpu proteins. A. Amino acid sequence alignment of T/F Vpu alleles compared to NL4-3 and the clade B consensus sequence. B. Construction of Vpu-FLAG expression vectors. Vpu alleles from transmitted/founder HIV-1 infectious molecular clones were PCR amplified using allele-specific primers, with a FLAG-tag included in the 3' (reverse) primer, and cloned into the MCS of the pcDNA-RRE vector, which includes a downstream Rev Response Element (RRE). C. Plasmids encoding Vpu-FLAG-RRE and Rev-IRES-GFP were used to co-transfect HeLa cells. The next day, the cells were stained for either CD4 or BS2 and analyzed by flow cytometry. Two-color plots showing surface downregulation of CD4 (y-axis, upper panels) or BST2 (y-axis, lower panels) vs, GFP (x-axis) by NL4-3 Vpu and two selected T/F Vpus (CH040 and CH077). D. Relative CD4 downregulation by T/F Vpu alleles. Samples were gated on live GFP+ (Rev+) cells. The mean fluorescence intensity (MFI) for the isotype control was subtracted from the MFI of each anti-CD4-stained sample. The value for each T/F Vpu was then subtracted from that of the no Vpu control, and this value was divided by the same calculation made for NL4-3 Vpu to derive the relative activity. The values shown are means from two experiments performed in duplicate. Error bars are the standard deviations. E. Relative BST2 downregulation by T/F Vpu alleles. Samples were gated on live GFP+ (Rev+) cells, and the activity relative to NL4-3 Vpu was calculated as described above for CD4. The values shown are means from two experiments performed in duplicate. Error bars are the standard deviations. F. Immunoblot analysis of the expression of T/F Vpu alleles. Cell lysates were analyzed for Vpu (FLAG), BST2, and actin.

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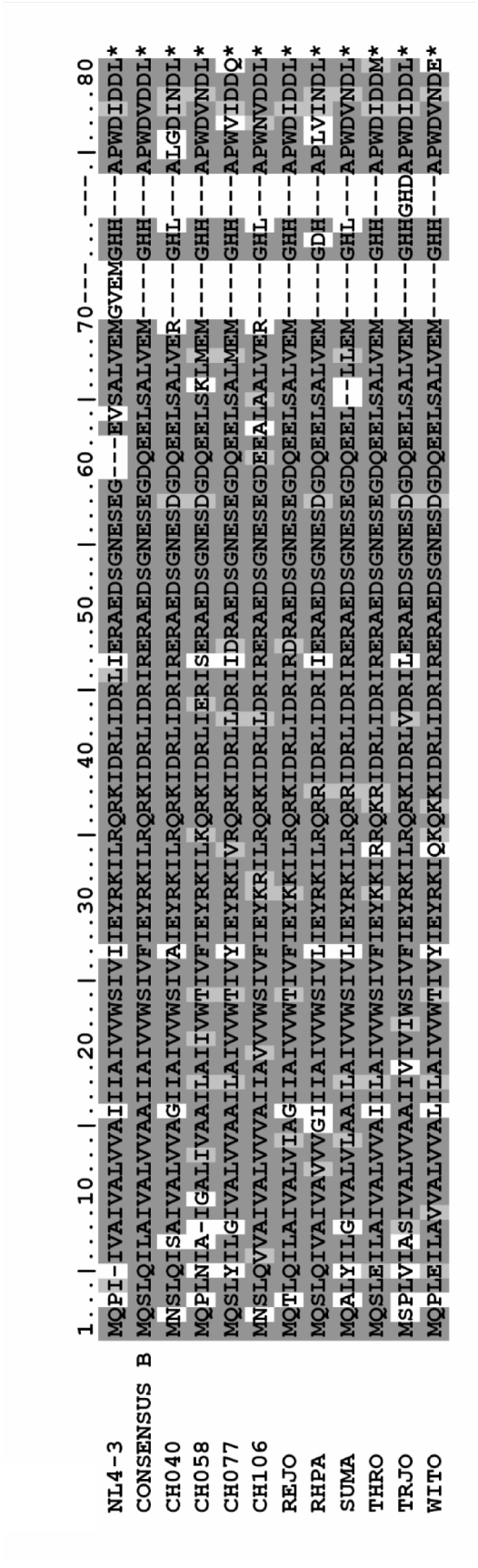
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CONSENSUS B MQSLQILAIVALVVAAIIAIVVWSIVFIEYRKILRQRKIDRLIDRIRERAEDSGNESEGDQEELSALVEM---GHH---APWDVDDL*
CH040 MNSLQISAIVALVVAGIIAIVVWSIVAIEYRKILRQRKIDRLIDRIRERAEDSGNESDGDQEELSALVER---GHL---ALGDINDL*
CH058 MQPLNIA-IGALIVAAILAIIVWTIVFIEYRKILKQRKIDRLIERISERAEDSGNESDGDQEELSKLMEM---GHH---APWDVNDL*
CH077 MQSLYIIGIVALVVAAILAIVVWTIVYIEYRKIVRQRKIDRLLDRIIDRAEDSGNESEGDQEELSALMEM---GHH---APWVIDDQ*
CH106 MNSLQVVAIVALVVVAIIAVVVWSIVFIEYKRILRQRKIDRLLDRIRERAEDSGNESEGDEEALAALVER---GHL---APWNVDDL*
REJO MQTLQILAIVALVIAGIIAIVVWTIVFIEYKKILRQRKIDRLIDRIRDRAEDSGNESEGDQEELSALVEM---GHH---APWDIDDL*
RHPA MQSLQIVAIVAVVVGIIIAIVVWSIVLIEYRKILRQRRIDRLIDRIIERAEDSGNESDGDQEELSALVEM---GDH---APLVINDL*
SUMA MQALYILGIVALVLAAILAIVVWSIVLIEYRKILRQRRIDRLIDRIRERAEDSGNESEGDQEEL--LLEM---GHL---APWDVNDL*
THRO MQSLEILAIVALVVAIILAIVVWSIVFIEYKKIRRQKRIDRLIDRIRERAEDSGNESEGDQEELSALVEM---GHH---APWDIDDM*
TRJO MSPLVIASIVALVVAAIIVIVIWSIVFIEYRKILRQRKIDRLVDRILERAEDSGNESDGDQEELSALVEM---GHHGHDAPWDIDDL*
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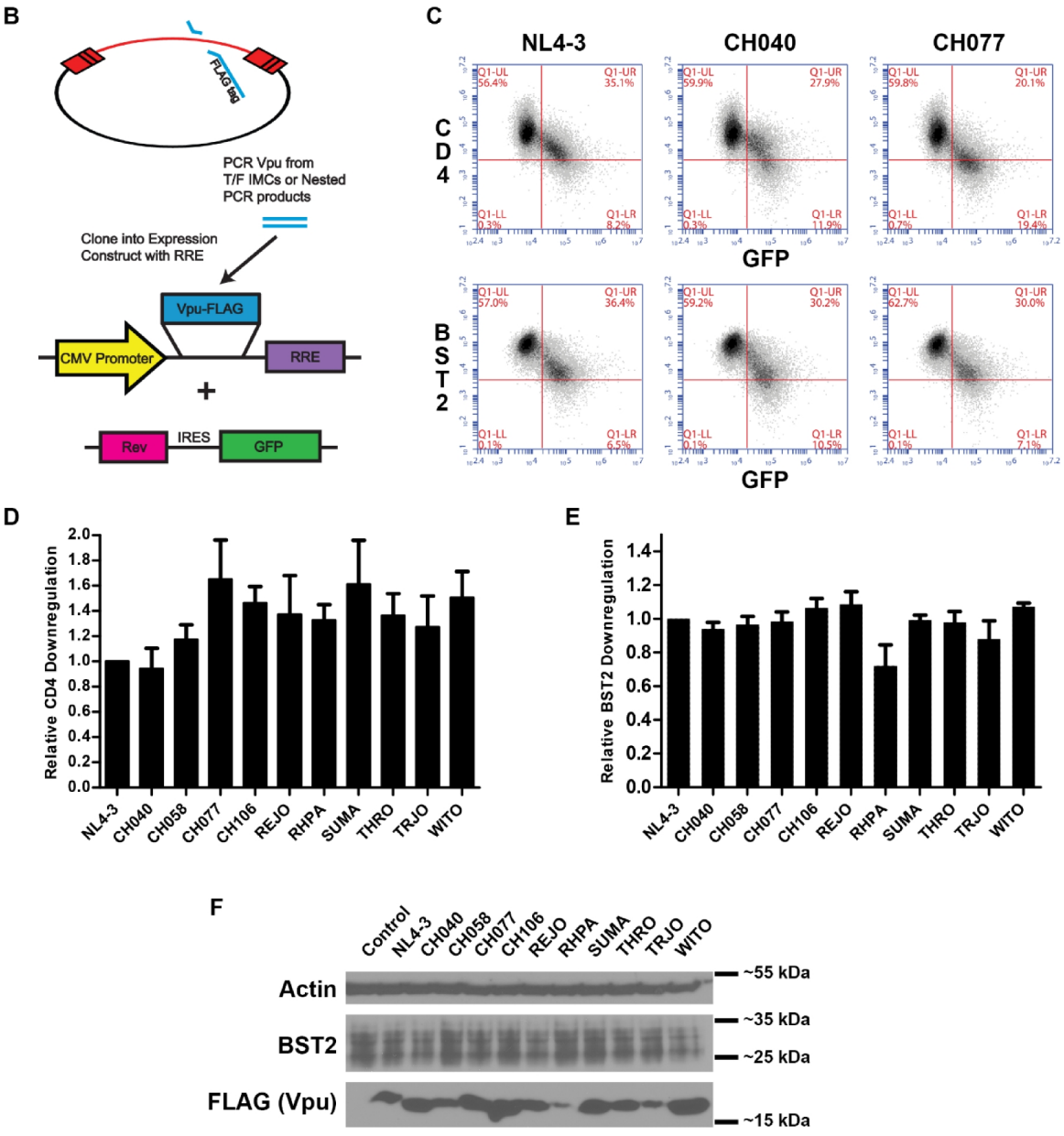

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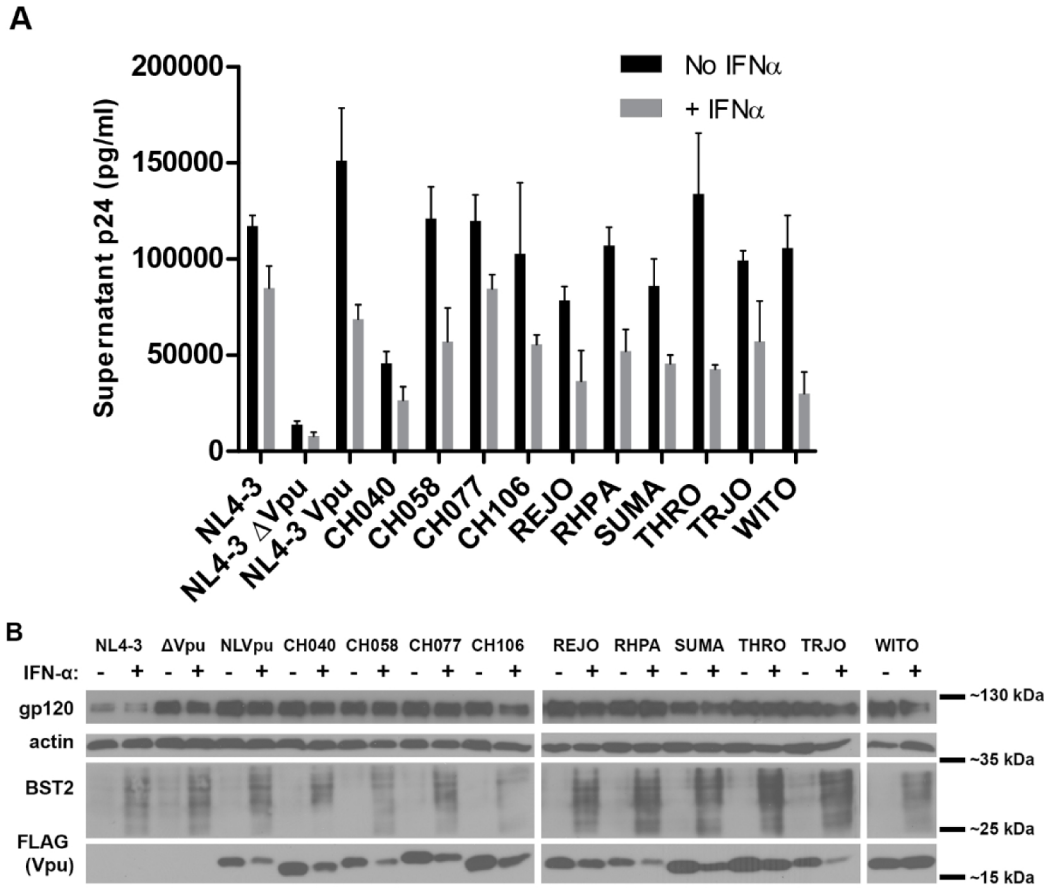

Figure 2. Enhancement of virion release by transmitted/founder Vpu proteins. A. Virion release enhancement by T/F Vpu alleles in the presence or absence of IFN-α. Cells were co-transfected with the indicated T/F Vpu expression plasmids and a provirus that does not express Vpu (NL4-3 ΔVpu) then cultured either without (black bars) or with (gray bars) IFN-α (1000U/ml) overnight. The concentration of pelleted p24 antigen in the culture supernates was measured by ELISA. Error bars are the standard deviation of quadruplicates. B. Immunoblot analysis of cell lysates showing the induction of BST2 following IFN-α treatment as well as actin, gp120 and Vpu-FLAG.

Figure 3. Activity and expression level of Vpu proteins isolated from the acute and chronic phases of infection. A. Amino acid sequence alignment of acute and chronic Vpu proteins compared to NL4-3 and the clade B consensus sequence. B. Relative CD4 downregulation by acute and chronic Vpu alleles. The experiment was done and the samples were analyzed as described in the legend of Figure 1. The values shown are means from two experiments performed in duplicate. Error bars are the standard deviations. C. Relative BST2 downregulation by acute and chronic Vpu alleles. The experiment was done and the samples were analyzed as described in the legend of Figure 1. The values shown are means from two experiments performed in duplicate. Error bars are the standard deviations. D. Immunoblot analysis of the expression of acute and chronic Vpu alleles. Cell lysates were analyzed for Vpu (FLAG) expression, BST2, and actin. E. Virion release enhancement by acute and chronic Vpu alleles. The experiment was done and the samples analyzed as described in the legend of Figure 2, except that IFN was not used. Error bars are the standard deviation of quadruplicates.

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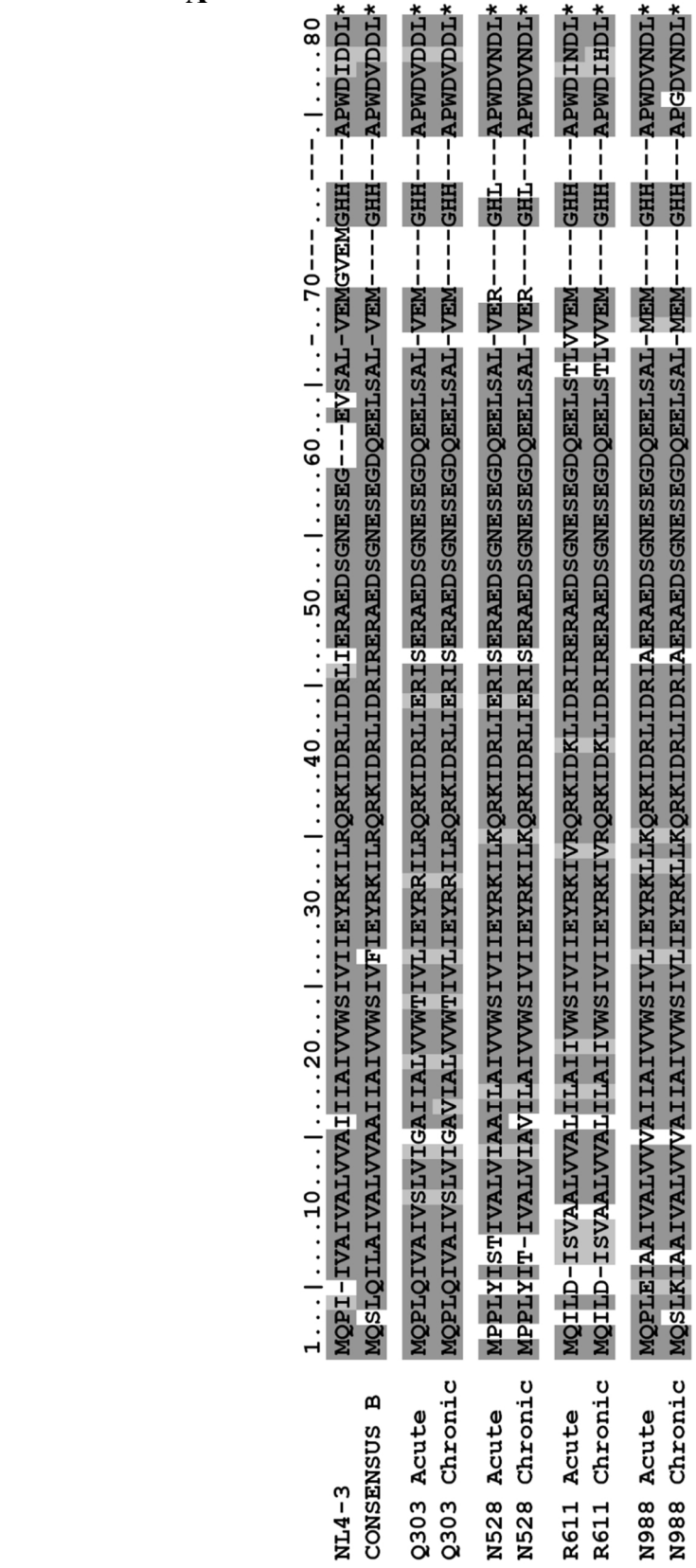
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NL4-3 MQPI-IVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEG---EVSAL-VEMGVEMGHH---APWDIDDL*
CONSENSUS B MQSLQILAIVALVVAAIIAIVVWSIVFIEYRKILRQRKIDRLIDRIRERAEDSGNESEGDQEELSAL-VEM----GHH---APWDVDDL*
Q303 Acute MQPLQIVAIVSLVIGAIIALVVWTIVLIEYRRILRQRKIDRLIERISERAEDSGNESEGDQEELSAL-VEM----GHH---APWDVDDL*
Q303 Chronic MQPLQIVAIVSLVIGAVIALVVWTIVLIEYRRILRQRKIDRLIERISERAEDSGNESEGDQEELSAL-VEM----GHH---APWDVDDL*
N528 Acute MPPLYISTIVALVIAAILAIVVWSIVIIEYRKILKQRKIDRLIERISERAEDSGNESEGDQEELSAL-VER----GHL---APWDVNDL*
N528 Chronic MPPLYIT-IVALVIAVILAIVVWSIVIIEYRKILKQRKIDRLIERISERAEDSGNESEGDQEELSAL-VER----GHL---APWDVNDL*
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R611 Chronic MQILD-ISVAALVVALILAIIVWSIVIIEYRKIVRQRKIDKLIDRIRERAEDSGNESEGDQEELSTLVVEM----GHH---APWDIHDL*
N988 Acute MQPLEIAAIVALVVVAIIAIVVWSIVLIEYRKLLKQRKIDRLIDRIAERAEDSGNESEGDQEELSAL-MEM----GHH---APWDVNDL*
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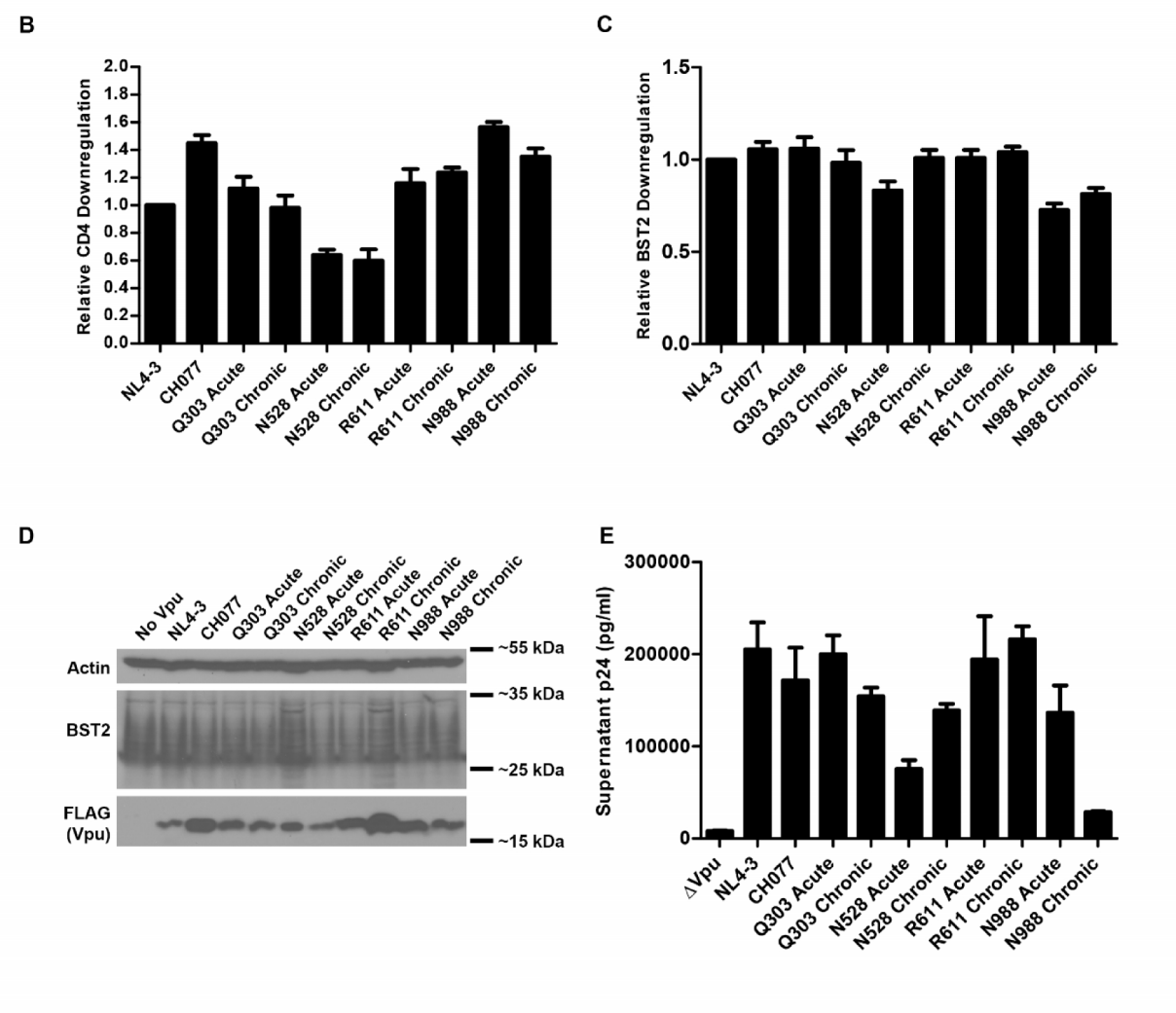

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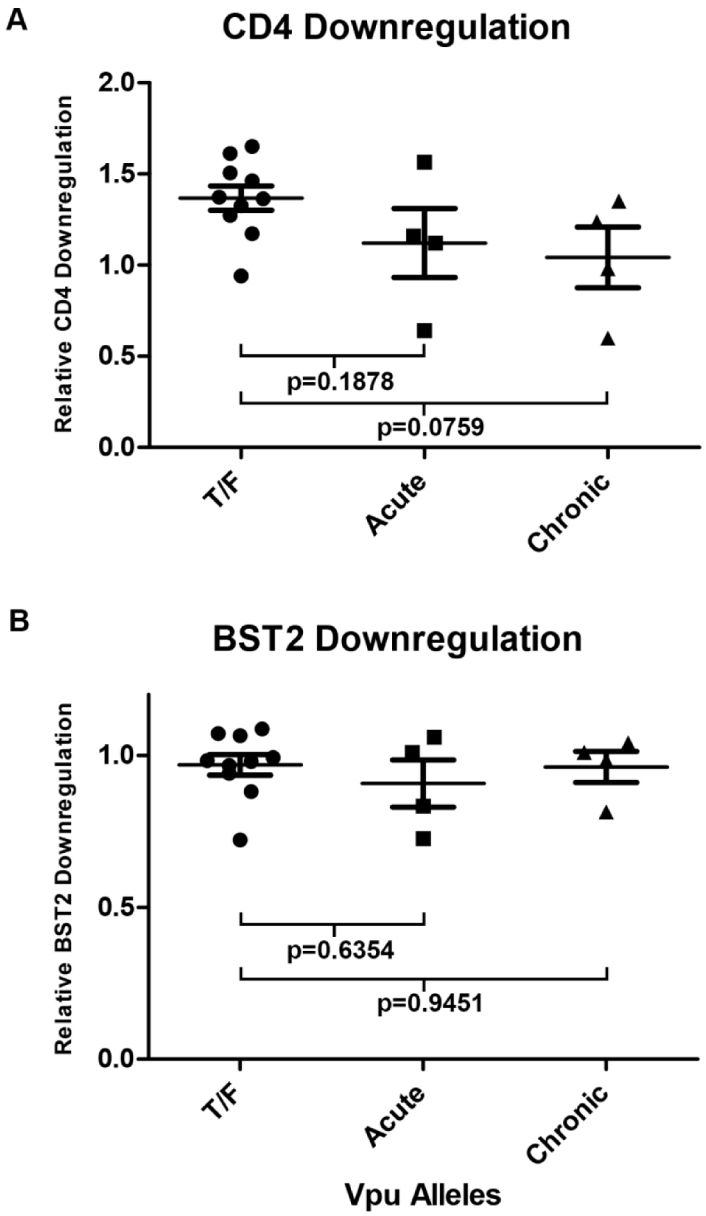

Figure 4. Comparative activities of Vpu proteins from transmitted/founder, acute, and chronic Vpu proteins. A. Aligned dot plots of mean relative CD4 downregulation ratios for T/F, acute and chronic Vpu proteins. Whiskers show standard error of the mean (SEM). Analysis by Mann-Whitney test yielded the indicated p-values. B. Aligned dot plots of mean relative BST2 downregulation ratios for T/F, acute and chronic Vpu alleles. Whiskers show SEM. Analysis by Mann-Whitney test yielded the indicated p-values.

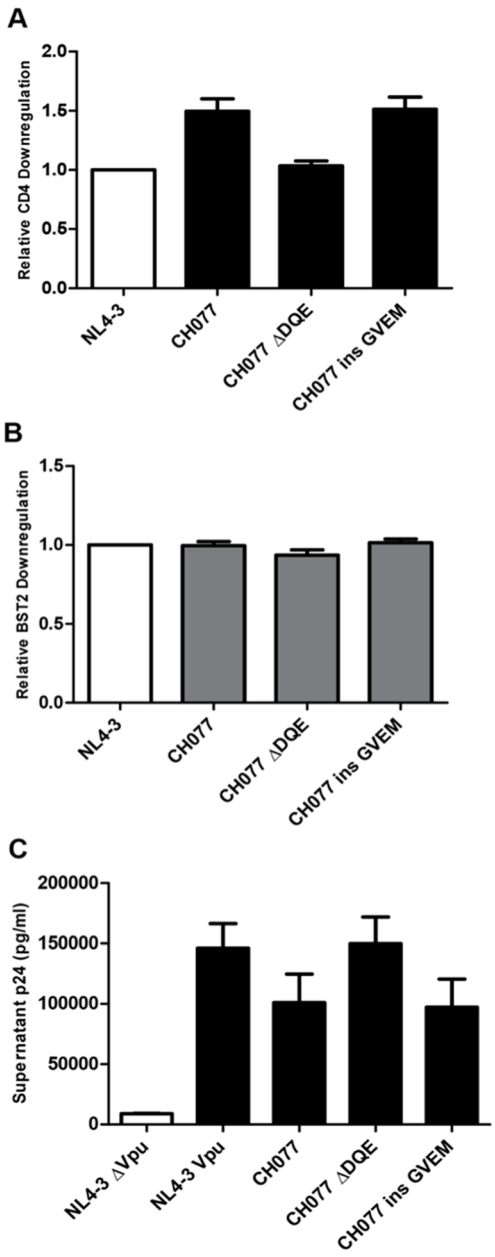

Figure 5. Effects of insertions and deletions in the cytoplasmic domain of Vpu. A. Effects of NL4-3-like cytoplasmic domain mutations on CD4 downregulation. CH077 Vpu mutants that lacked DQE at residues 60-62 or harbored the GVEM duplication at residue 71 were generated and tested as described above. For this analysis, CD4 MFI ratios were calculated as in Figures 1D and 3B. B. Effects of NL4-3-like cytoplasmic domain mutations on BST2 downregulation. For this analysis, BST2 MFI ratios were calculated as in Figures 1E and 3C. C. Effects of NL4-3-like cytoplasmic domain mutations on the enhancement of virion release. Error bars are the standard deviation of quadruple.

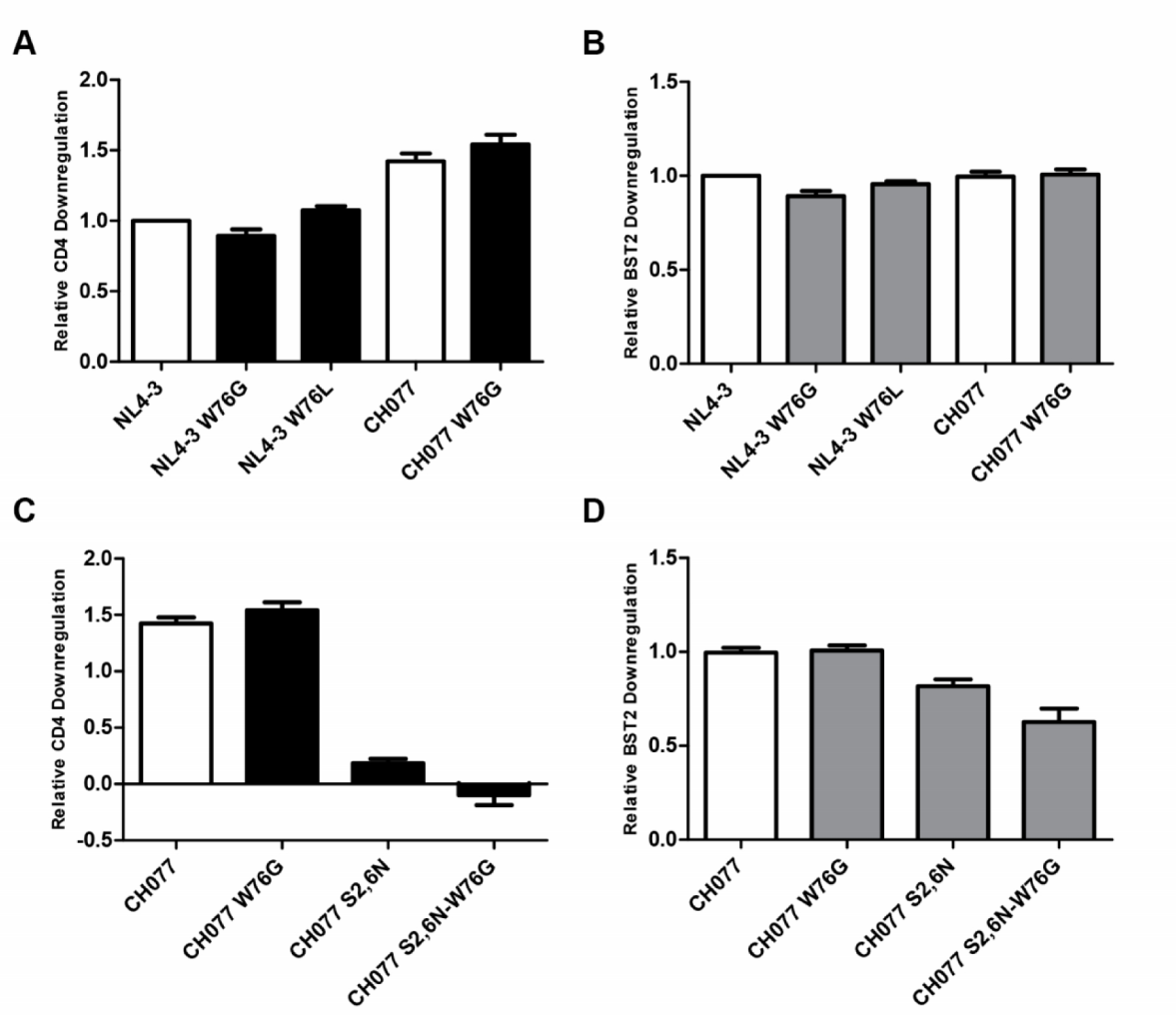

Figure 6. Site-directed mutagenesis of Vpu W76: effects on the downregulation of CD4 and BST2, and the interaction with β-TrCP. A and C. Relative CD4 downregulation by the site-directed mutant Vpus: A. Cytoplasmic domain W76 mutants of NL4-3 or CH077 Vpus. Data are expressed relative to NL4-3 Vpu. C. Additive effects of the W76G and the S52,56N β-TrCP-binding mutations on CD4 downregulation. For this analysis, CD4 MFI ratios were calculated as in Figures 1D, 3B and 5A. The values shown are means from two experiments performed in duplicate. Error bars are the standard deviations. B and D. Relative BST2 downregulation by the site-directed mutant Vpus: B. Effects of site-directed W76 mutants. D. Additive effects of W76G and the S52,56N β-TrCP-binding mutations on BST2 downregulation. For this analysis, BST2 MFI ratios were calculated as in Figures 1E, 3C and 5B. The values shown are means from two experiments performed in duplicate. Error bars are the standard deviations.

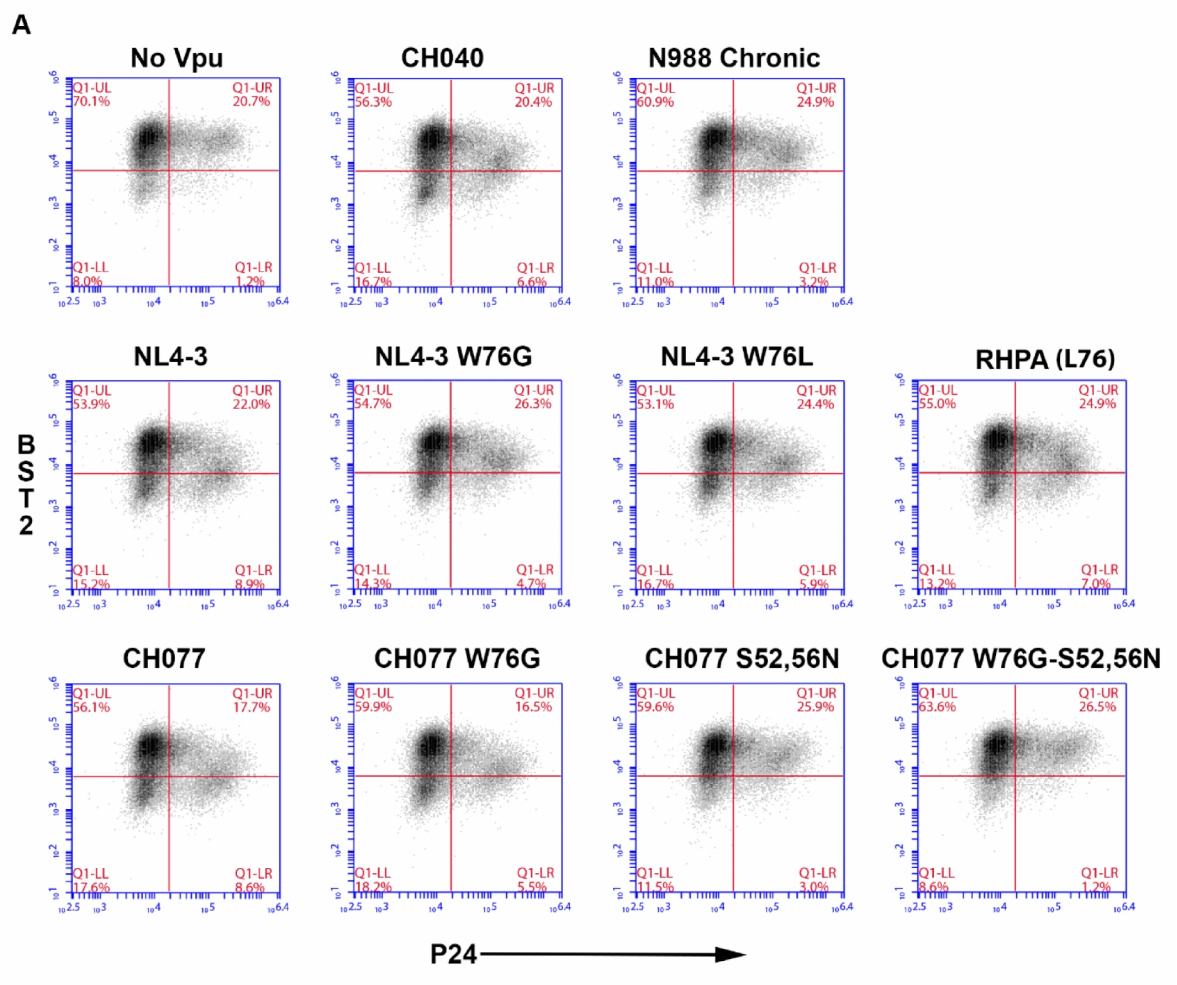

Figure 7. Role of Vpu residue W76 in cell surface BST2 downregulation and virion release measured concurrently and its interaction with β-TrCP. A. Surface downregulation of BST2 in cells co-transfected with provirus lacking Vpu (NL4-3 ΔVpu) and the indicated Vpu-FLAG expression constructs. The day after transfection, the cells were analyzed by flow cytometry. The two-color plots show BST2 (y-axis) vs. intracellular p24 expression (x-axis). Results are representative of two experiments performed in duplicate. B. Virion release enhancement by the Vpus: pelleted p24 antigen was measured by ELISA in supernates from cultures of the same cells analyzed in panel A. Error bars are the standard deviation of quadruplicates. C. Co-immunoprecipitation of FLAG-tagged Vpu and β-TrCP. HeLa P4-R5 cells were co-transfected with plasmids expressing HA-tagged β-TrCP, Rev, and FLAG-tagged Vpu or an empty vector. Cell lysates ("input") and immunoprecipitates ("IP-FLAG") were probed for HA (β-TrCP), actin, and FLAG (Vpu). While Vpu harboring the S52,56N mutation of the β-TrCP binding motif did not pull down β-TrCP, Vpu-W76G interacted with β-TrCP as well as NL4-3, as did the T/F Vpu proteins of CH040 and CH077.

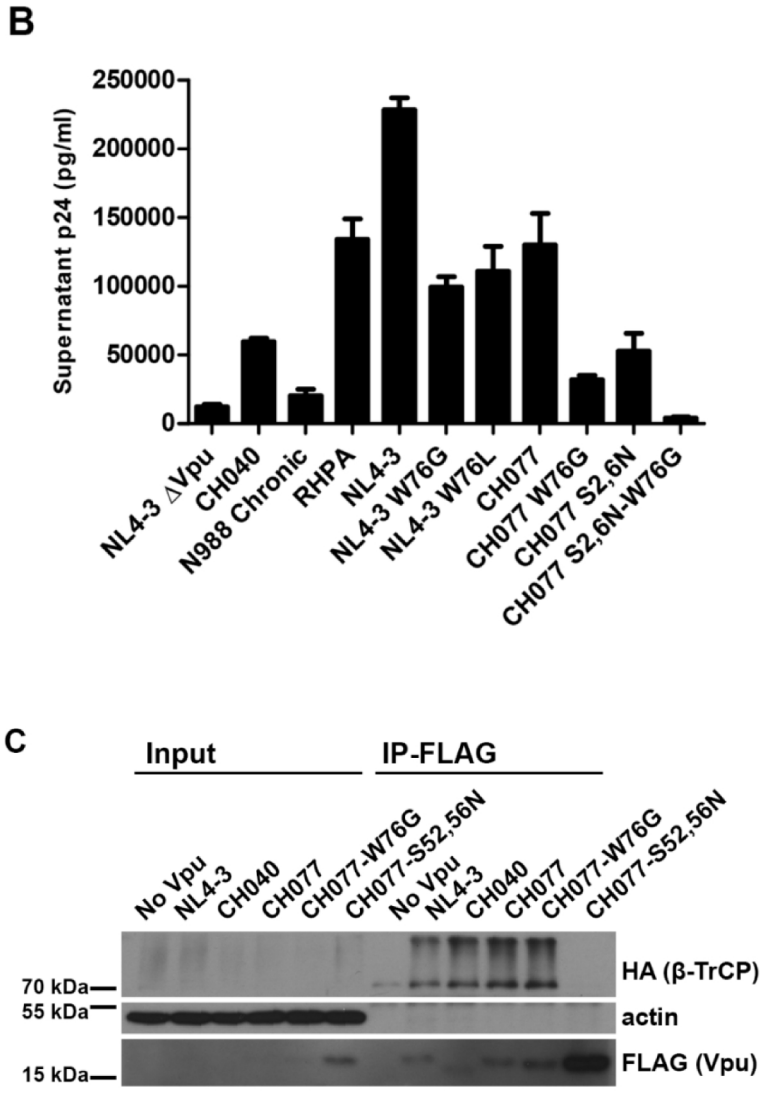

Figure 7. Continued

Table 1. Patient sample information acquired from SDPIC. Shown here are CD4+ T-cell counts and viral loads from longitudinal sampling of patients in acute and chronic phases of HIV-1 infection, which were used to clone Acute and Chronic Vpu proteins studied here. There appears to be no direct correlation between tested activities of these Vpu proteins and the time of sampling, CD4+ T-cell count, or viral load of the corresponding patients.

Sample Name	Collection Time (days from EDI)	CD4 (cells/μL)	Viral Load (copies/mL)
Q303 Acute	45	642	89,900
Q303 Chronic	545	522	3,910
N528 Acute	85	813	7,200
N528 Chronic	1069	224	57,500
R611 Acute	85	418	17,600
R611 Chronic	456	704	13,100
N988 Acute	45	959	109,000
N988 Chronic	1777	408	2,270

This section, in part, has been submitted for publication of the material as it may appear in Jafari, Moein; Guatelli, John C.; Lewinski, Mary K. “Activities of Transmitted/Founder and Chronic Clade B HIV-1 Vpu and a Polymorphism Specifically Affecting Virion Release.” Journal of Virology, 2013. Moein Jafari was the primary investigator and author of this material.

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