Abstract

Background: Genetic studies reveal that vpu is one of the most variable regions in HIV-1 genome. Functional studies have been carried out mostly with Vpu derived from laboratory adapted subtype B pNL 4-3 virus. The rationale of this study was to characterize genetic variations that are present in the vpu gene from HIV-1 infected individuals from North-India (Punjab/Haryana) and determine their functional relevance.

Methods: Functionally intact vpu gene variants were PCR amplified from genomic DNA of HIV-1 infected individuals. These variants were then subjected to genetic analysis and unique representative variants were cloned under CMV promoter containing expression vector as well as into pNL 4-3 HIV-1 virus for intracellular expression studies. These variants were characterized with respect to their ability to promote virus release as well as cell death.

Results: Based on phylogenetic analysis and extensive polymorphisms with respect to consensus Vpu B and C, we were able to arbitrarily assign variants into two major groups (B and C). The group B variants always showed significantly higher virus release activity and exhibited moderate levels of cell death. On the other hand, group C variants displayed lower virus release activity but greater cell death potential. Interestingly, Vpu variants with a natural S61A mutation showed greater intracellular stability. These variants also exhibited significant reduction in their intracellular ubiquitination and caused greater virus release. Another group C variant that possessed a non-functional β-TrcP binding motif due to two critical serine residues (S52 and S56) being substituted with isoleucine residues, showed reduced virus release activity but modest cytotoxic activity.

Conclusions: The natural variations exhibited by our Vpu variants involve extensive polymorphism characterized by substitution and deletions that contribute toward positive selection. We identified two major groups and an extremely rare β-TrcP binding motif mutant that show widely varying biological activities with potential implications for conferring subtype-specific pathogenesis.

Introduction

The pathogenesis of HIV infection is remarkably different from other primate lentiviruses in many ways. It manifests immune-deficiency, the pathological hallmark of HIV infection caused by severe depletion of infected as well as uninfected lymphocyte populations [1–2]. HIV-1 is remarkably successful in overcoming various cellular restriction factors by exploiting the properties of its accessory proteins [3–4]. It displays extreme genetic variability due to error prone process of reverse transcription, its recombinogenic nature and rapid rate of replication [3–7]. Moreover quasispecies are continuously generated in infected individuals as they are subjected to various selection pressures such as drugs, immune response, genetic factors as well as anti-retroviral restriction factors. Therefore, it is reasonable to suggest that multiple mechanisms govern continuous generation of these genetic variants in an infected individual. Previously Yusim et al. (2002) studied relative variability of HIV-1 proteins and there analysis showed that Vpu displayed highest entropy of variation in infected individual [8].

Vpu is an exclusive feature of HIV-1 proteome not present in HIV-2 and in most Simian immunodeficiency viruses (SIVs) [9–11]. Furthermore, functionally competent (with respect to BST-2 degradation) Vpu protein is encoded only by pandemic M group of viruses but not by other non-pandemic groups (O and P) of HIV-1 [12]. For these reasons, Vpu seems to be most recent evolutionary adaptation conferring pandemic potential to HIV-1. Vpu facilitates efficient virus release by overcoming restriction imposed by host factors e.g. CD317 (also called BST-2 or Tetherin) and degradation of CD4 receptor [13–14]. The key to
biological function of Vpu is the fact that it can act as an adaptor linking its target proteins to SCF β-TrCP ubiquitin ligase complex [14]. This interaction involves highly conserved and constitutively phosphorylated DS\textsubscript{52}GNES\textsubscript{56} motif (β-TrCP binding motif) of HIV-1 Vpu and WD\textsubscript{40} repeat domain of β-TrCP which leads to ubiquitination and subsequent degradation of CD4 as well as BST-2 [13–15]. Vpu is known to inhibit NF-κB activation and very recently we reported a novel role of Vpu in induction of β-TrCP dependent apoptosis via stabilization of tumor suppressor protein p\textsubscript{53} [16–17].

Vpu from different genetic subtypes as well as primary isolates display high variation in their amino acid sequences that may explain subtype-specific differences with respect to their biological activities [8,18–22]. Subtype B Vpu was earlier shown to be more efficient in CD4 down regulation than Vpu C but similar comparative data for other specific functions of Vpu i.e apoptosis, virus release is lacking [23]. Interestingly, replacement of subtype B vpu with subtype C vpu gene was earlier reported to severely modulate pathogenesis and kinetics of depletion of circulating CD4\textsuperscript{+} lymphocytes in simians infected with chimeric SHIV [23]. Till date, most of the functional characterization of Vpu has been carried out with T-cell adapted HIV-1 pNL4-3 [24]. Genetic diversity among Vpu subtypes and isolates have been reported earlier but functional implication of natural variations have not been previously characterized [19–23]. Since subtype C is dominant in Asia and South Africa (>50%), it is imperative to study biological consequences of the natural variation in vpu C gene.

In this study, we wanted to study the nature of natural variations exhibited by vpu from infected individuals in Northern India and explore their functional consequences. Based on phylogenetic analysis and their comparison with consensus subtype B and C Vpu sequences, these variants were divided arbitrarily into two groups (B and C). We observed significant variations between group B and C variants. We report novel functional impact of mutations harbored by all group B but not group C Vpu variants. We also found evidence for positive selection among group B variants. All of them showed a conserved S61A substitution that led to enhanced intracellular stability and higher virus release activity. These variants retained moderate apoptotic potential associated with prototype subtype B Vpu. We also report functional implication of a natural Vpu variant that harbored a dysfunctional β-TrCP binding motif. These studies indicate that there are subtype-specific differences with respect to various biological activities attributed to Vpu.

Results

Genetic Variation in HIV-1 vpu Gene from North Indian HIV-1 Infected Individuals

To explore the genetic and functional implications of natural variations displayed by vpu alleles in HIV-1 infected patients (Clinical parameters summarized in Table 1), we PCR amplified complete open reading frames (ORFs) corresponding to the vpu locus of HIV-1 genome. The vpu variants were PCR amplified with consensus Vpu B and C-specific primers using DNA isolated from peripheral blood mononuclear cells (PBMCs) of infected individuals as reported by us earlier [17]. Vpu variants were sequenced and the unique representative sequences were subjected to phylogenetic analysis which revealed that 70% of sequences clustered with subtype B sequences (Figure 1A, filled triangles) and 30% with subtype C sequences (Figure 1A, filled circles). These sequences were compared with reference sequences obtained from Los Alamos HIV sequence database (www.hiv.lanl.gov). This analysis establishes the co-circulation of subtypes B and C among North Indian population. These sequences were also subjected to recombination analysis using Simplot and RIP (recombination identification program) but no evidence for vpu B/C recombinants was found. The Indian vpu B variants showed close resemblance with American, Japan, Brazil and Taiwan subtypes B and the Indian vpu C variants showed close resemblance with Zambia, Botswana and South African subtype C strains. To determine the nature of evolutionary selection that occurred as a consequence of divergence of vpu sequences, the average d\textsubscript{N}/d\textsubscript{S} values (the ratio of substitution rates at non-synonymous and synonymous sites) were determined. The ratio of d\textsubscript{N}/d\textsubscript{S} values ranged from 0.9 to 2.9 fold. The d\textsubscript{N}/d\textsubscript{S} ratios less than one is suggestive of the purifying selection whereas ratios greater than one is indicative of the positive selection. Three out of ten sequences showed values less than one while the remaining seven sequences showed values greater than one. To determine further the nature of selection that occurred between the two groups, the average divergence between subtypes B and C sequences were generated for each variant. The d\textsubscript{N}/d\textsubscript{S} values between B and C subtypes ranged from 1.3 to 2.4 fold suggestive of even greater positive selection of these variants (Table 2). The sequence comparisons of representative Vpu variants with either consensus B or C subtypes are shown in Figure 1B. The predicted functional domains are indicated at the top of the sequences. The reference sequences were retrieved from Los Alamos HIV-1 database. Group B variants displayed higher degree of genetic variations in all topological domains and some novel mutations with high allelic frequency as compared with group C variants which displayed comparatively less variation (analysis based on unique representative samples). It is noteworthy that the group B variants showed maximum variation in cytoplasmic helix-2 region as compared to group C variants. One common feature between the two groups was the presence of extensive deletions in the transmembrane region. We also report positive selection among group B Vpu variants that possessed a unique S61A substitution. Site directed mutagenesis studies with S61A substitution in Vpu B was earlier reported to confer higher intracellular stability that correlated with enhanced rate of virus replication [25]. Interestingly, we found one rare mutant (Vpu 24) among group C variants which had lost its functional β-TrCP binding motif due to substitution of two serine residues (Ser 52 & 56) with isoleucine. It is noteworthy that the size of the ORF of Vpu variants was different due to the extensive polymorphisms displayed by them. Most of group C variants (Vpu 4, 41 and 24) and two group B variants (Vpu S2 and 7) showed unique transmembrane deletions (four to eight amino acids) which may have functional implications with respect to various Vpu mediated functions. Beside these interesting changes, the specific determinants for CD4, BST-2 and β-TrCP binding remained highly conserved (Figure 1B) among all the variants [14–15,26–28].

The S61A Mutation Conferred Enhanced Intracellular Stability

We first wanted to test the functional implication of natural S61A substitution and other variations with respect to intracellular stability. HEK-293T cells were transfected with mammalian expression vectors encoding S61A mutants and wild type (wt) Vpu variant. Forty eight hours after transfection cell lysates were prepared and probed for relative expression of Vpu variants by immunoblotting. Representative group B or C Vpu variants (Figure 2A and B) were tested for their intracellular expression. They displayed differential migration pattern (owing to difference in overall length, phosphorylation and possibly other post-translational modifications) upon extended run on a high
Figure 1. Genetic variation in HIV-1 vpu gene from North Indian HIV-1 infected individuals. A) The phylogenetic tree was constructed using the Neighbor Joining (NJ) method with the Kimura two-parameter distance matrix. The first letter of the reference sequence denotes the type or subtype or CRF, the second letter denotes the country from sequence sampled and the third letter denotes the accession number. Filled triangle mark the subtype B variants and filled circles mark the subtype C variants sampled from North India. The accession numbers for Indian samples (NII-PGI-IND-vpu sequences) were marked within the brackets. The main supported clades were marked with asterisk (*) along the branch represents the bootstrap support >70%. The scale bar represents the evolutionary distance of 0.1 nucleotides per position in the sequence. B) Multiple sequence alignment of primary isolates of HIV-1 Vpu collected from HIV-1 infected individuals from North India. The color coding generated by software represents difference in color for amino acid with different physiochemical properties. Identical residues are represented as dots.

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percentage acrylamide gel (15% SDS-PAGE). The variants also
displayed substantial differences with respect to intracellular levels.
Notably, S61A variants (Figure 2A, Lanes 4 and 5) showed higher
expression levels than S61 wt Vpu alleles (Figure 2A, Lanes 2 and
3). Also, Cycloheximide (CHX) chase assay was performed to
study the effect of S61A mutation (Figure 2C) on the intracellular
turnover and kinetic stability of Vpu protein. After eight hours of
chase period the level of wt Vpu B and C proteins were reduced to
undetectable levels in transfected HEK-293T cells (Figure 2C
panels 1 and 2). In contrast, a comparative analysis of two Vpu
alleles (with or without S61A mutation) revealed that S61A mutant
allele (Figure 2C, panel 3) showed almost no reduction in protein
levels when compared with S61 variants (Figure 2C, panel 4)
which was comparable to wt subtype B and C Vpu protein
(Figure 2C, panels 1 and 2). We then proceeded to study the
mechanistic details of Vpu stabilization in selected variants. The
ubiquitination profile of Vpu alleles were tested following
intracellular expression in transfected HEK-293T cells. As
expected no Vpu-specific poly-ubiquitinated species were observed
in control cells (Figure 2D lane 1) or cells treated with MG132
alone (Figure 2D lane 2). Interestingly, all the three S61A variants
tested (Vpu 1, 7 and S2) showed marked inhibition of Vpu-specific
ubiquitination (Figure 2D, Lanes 7, 8 and 9) as compared to S61
wt alleles (Figure 2D, Lanes 4, 5 and 6). These results clearly show
that acquisition of S61A mutation inhibits Vpu ubiquitination
conferring increased intracellular stability and responsible for
relative protein abundance.

Phenotypic Characterization of Natural Vpu Variants with
Respect to Viral Replication

We further analyzed the functional impact of these natural
variants of Vpu alleles on virus release potential. All the vpu alleles
that were cloned in a pNL backbone were used to study biological
activities of Vpu. HeLa cells (vpu sensitive phenotype) were
transfected with equal amounts of various proviral DNA
constructs. Forty eight hour post transfection cell culture
supernatants were collected and used to infect HIV indicator
Tzmbl cells. Relative infectious viral yield associated with each
Vpu allele was measured by counting the number of blue cells in
Tzmbl-indicator cells present in identical area using an inverted
microscope. As expected compared with Vpu null virus
(pNLdVpu) (Figure 3A, panel 2), all Vpu variants displayed
higher virus release potential (Figure 3A, panels 3–10). Two of the
natural S61A variants were most potent in their ability to cause
virus release (Figure 3A, panels 5, 6 and quantitation showed in
Figure 3C). However, one S61A variant allele, Vpu 7, containing a
transmembrane deletion of eight amino acids (Figure 3C, panel 8),
when compared with other S61A alleles, showed moderate
enhancement of viral release (Figure 3A, panel 8 and Figure 3C).
All the group C variants (Vpu 4, 24 and 41 panels 7, 9, 10 and
Figure 3C) showed moderate virus release activity in comparison
to group B Vpu variants (Figure 3A, panels 5, 6, 8 and Figure 3C).
Interestingly, one Vpu C variant (Vpu 24) possessing a non-
functional β-TrcP binding motif, showed modest viral release
activity comparable to other S61 and β-TrcP motif containing wt

### Table 1. The clinical data for HIV-1 infected individuals from North India.

| Subject Code     | Age | Sex | Mode of Transmission | CD4 Count | WHO clinical stage |
|------------------|-----|-----|----------------------|-----------|-------------------|
| NII-PGI-IND-1    | 32  | M   | Heterosexual         | 169       | 1                 |
| NII-PGI-IND-2    | 40  | M   | Heterosexual         | 62        | 2                 |
| NII-PGI-IND-4    | 35  | M   | Heterosexual         | 37        | 3                 |
| NII-PGI-IND-7    | 30  | M   | Heterosexual         | Not Defined | Not Defined |
| NII-PGI-IND-24   | 23  | M   | Heterosexual         | 458       | 1                 |
| NII-PGI-IND-41   | 25  | M   | Heterosexual         | 82        | 2                 |
| NII-PGI-IND-52   | 29  | M   | Heterosexual         | 111       | 1                 |
| NII-PGI-IND-42   | 40  | M   | Heterosexual         | 69        | 3                 |

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### Table 2. The rate of accumulation of non-synonymous and synonymous substitution.

| Samples | Predicted subtypes | dN/dS ratio based on predicted subtypes | dN/dS ratio between B and C subtypes | Evolutionary selection type |
|---------|--------------------|-----------------------------------------|--------------------------------------|-----------------------------|
| Vpu1    | B                  | 2.9450                                  | 2.4582                               | Positive selection          |
| Vpu42   | B                  | 2.9450                                  | 2.4455                               | Positive selection          |
| VpuA4   | B                  | 0.9410                                  | 1.2964                               | purifying selection         |
| VpuA5   | B                  | 0.9078                                  | 1.3319                               | purifying selection         |
| VpuS2   | B                  | 1.7245                                  | 2.2352                               | positive selection          |
| Vpu7    | B                  | 1.9460                                  | 2.1040                               | Positive selection          |
| Vpu2    | B                  | 2.8672                                  | 2.3903                               | Positive selection          |
| Vpu24   | C                  | 0.9853                                  | 1.3374                               | purifying selection         |
| Vpu41   | C                  | 1.4074                                  | 1.6770                               | Positive selection          |
| Vpu4    | C                  | 1.3609                                  | 1.6537                               | Positive selection          |

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Vpu alleles (Figure 3A, panel 9 and Figure 3C). We conclude that natural variations confer higher virus release activity to group B variants than group C variants. Also the Vpu variant with non-functional $\beta$-TrcP binding motif still retained modest virus release activity.

**Induction of Cell Death by Vpu Variants Upon HIV-1 Infection**

Finally, we investigated whether the varying degree of virus release activity and kinetic stability correlated with subtype-specific differences with respect to apoptotic potential of HIV-1 during infection. To address this question, MOLT-4 T-cells were infected with an equal MOI (1) of VSV-G pseudotyped HIV-1-Vpu variants for forty eight hours and cell death was determined by PI staining. In comparison to Vpu null (pNLdVpu) HIV-1 (Figure 4A, panel 2), all Vpu variants induced higher cell death (Figure 4A, panels 3–10) in infected cells which is in agreement with the previous reports [16–17,23]. When analyzed for their relative potential to induce cell death, group B S61A variants (Figure 4A, panels 5, 6 and 7) caused cell death as comparable to wt subtype B Vpu (Figure 4A, panel 3). The group C S61+ variants, (Figure 4A, panels 8 and 9) on the other hand, showed higher apoptotic potential than group B variants. The Vpu 24 (possessing a non-functional $\beta$-TrcP binding motif) variant (Figure 4A, panel 10) induced moderate cell death (less than subtype C Vpu but comparable to pNLdVpu variant). Immunoblotting performed with equivalent amounts of lysates from infected cells (Figure 4B) also confirmed higher expression levels associated with group B S61A variants (Figure 4B, lanes 7, 8 and 9) as compared to S61+ variants (Figure 4B, lanes 4, 5 and 6). From this data we conclude that group B and C variants differ substantially in their ability to cause cell death.

**Discussion**

HIV is exceptional in its ability to accumulate mutations in all its genes in the infected individuals and escape mutants are continuously generated [5–7]. It is therefore important to know if these variations confer any survival advantage to the virus. Vpu locus is considered one of the most variable regions in HIV-1 genome [8]. It is not known whether these variations have any role in viral replication or disease progression. Vpu plays a major role in controlling viral release as well as apoptosis, both of which are important for HIV-1 pathogenesis.

Earlier studies related to characterization of natural vpu gene variants were limited mainly to genetic analysis but their functional impact with respect to virus release and apoptosis was not systematically addressed [21–22]. Site directed mutagenesis
studies performed earlier with prototype Vpu B attributed a number of amino acid residues important for virus release and apoptotic activity of Vpu [19,25–27]. We observed extreme heterogeneity in the \textit{vpu} locus of HIV-1 genome with respect to both sequences and length in accordance with previous studies on isolates from different geographical locations [21–22]. Based on phylogenetic analysis \textit{vpu} sequences were arbitrarily assigned into two groups (B and C). Group B variants displayed higher degree of

Figure 3. Phenotypic characterization of natural Vpu variants with respect to viral replication. A) HeLa cells were transfected with an equal amount of various proviral DNA constructs and total virus released in culture supernatant was quantitated using HIV indicator Tzmbl cells by β-galactosidase staining. B) Relative number of infected cells was counted for each sample and C) plotted. These results are representative of three independent experiments.

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variations in all topological domains with some novel conserved mutations when compared to group C variants. These observations suggest differences in the rate of evolution and pattern of variations in group B and C HIV-1 \textit{vpu} variants. Another interesting observation was the substitution of a phosphorylatable serine residue to alanine in cytoplasmic helix-2 in all of the group B variants (S61A). Artificially incorporating such a mutation in HIV-1 pNL4-3 Vpu was earlier reported to boost viral replication rate as well as confer stability to Vpu protein [25]. We therefore verified whether our natural S61A mutants possessing additional numerous variations (including transmembrane deletions) in cytoplasmic regions, displayed greater kinetic stability. It is noteworthy that despite exhibiting extensive polymorphisms (deletion in transmembrane domain as well as numerous point mutations), S61A mutants from group B Vpu variants showed enhanced intracellular expression and intracellular stability. The intracellular stability of S61A mutants correlated with their

\textbf{Figure 4. Phenotypic characterization of natural Vpu variants with respect to induction of cell death upon infection.} A) Infected MOLT-4 T Cells were harvested, stained with Propidium iodide (10 µg/ml) and analyzed by flow cytometry to determine the extent of cell death. The extent of cell death is indicated in upper right corner of each panel. The FACS data were analyzed by WinMDI 2.9 software. B) Equal amounts of lysate from infected cells were subjected to immunoblotting to measure Vpu expression. These results are representative of three independent experiments.

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ubiquitination profile as S61A mutants exhibited significantly less ubiquitination than S61T variants.

Since, Vpu is known to act like a molecular motor that facilitates virus release as well as cause cell death, it was logical to study the complex implication of this finding with respect to viral replicative fitness. We therefore developed an HIV-1 based system for expression of Vpu variants upon infection in various cell lines. These studies showed that group B variants exhibit superior viral release activity and moderate cell death potential comparable to Vpu C variants. All group C variants as well as a β-Trcp motif mutant showed comparable viral release activity when compared with group C variants. The viral release activity observed in Vpu variant possessing mutant β-Trcp binding motif (Vpu 24) may be due to sequestration of endogenous BST-2 as suggested in previous reports of BST-2 degradation-independent enhancement of virus release activity [29]. The reduced viral release activity observed in S61A variant Vpu 7 on the other hand could be explained because of the presence of long transmembrane deletion that seriously affected viral release process. It is noteworthy that presence of two alanine residues (A11 and A15) in Vpu transmembrane domain was earlier shown to be important for virus release activity [30]. Our data suggests that our group B variants exhibit superior viral release, show relatively higher intracellular expression levels and retain moderate cell death potential compared to Vpu C.

In summary, natural variations displayed by HIV-1 within infected individuals can give rise to Vpu variants with selective advantages with respect to virus release process or induction of cell death. Since apoptosis and viral release are two intricate and crucial determinants of viral fitness and pathogenesis, it must be exploited by virus for its maximum advantage. Cell death, although absolutely essential for pathogenesis, must be carefully regulated to avoid premature death of an infected cell. This work reiterates ongoing struggle between the host and the virus that results in the generation and selection of viral variants with selective advantages.

**Methods**

**Ethics Statement**

The Institutional Human Ethical committee approved the study and written consent was obtained from all study participants.

**Study Participants and Sample Collection**

HIV-1-infected individuals from the Punjab/Haryana region of North India (immediately north of the Indian capital, Delhi) were selected on a random basis for our studies. They were obtained from the Immunodeficiency Clinic of the Post Graduate Institute of Medical Research and Education, Chandigarh, India after obtaining all the required ethical clearances. The clinical parameters of infected individuals (age, sex and CD4 counts) are shown in Table 1.

**Patient Population, Genomic DNA Isolation and Amplification of the vpu Gene**

Peripheral blood mononuclear cells were collected from peripheral blood and genomic DNA was isolated using qiaquick Genomic DNA isolation kit (Qiagen) as described previously for vpr gene by us and sequences spanning vpu gene was amplified by polymerase chain reaction (PCR) using gene specific primers [31]. The HIV-1 genomic fragment encoding full-length vpu was amplified using High fidelity Taq DNA polymerase (Qiagen, Germany) using one of two primer pairs as follows. The first set was used to amplify subtype B and the second set for vpu C.

**Provirial DNA Constructs and Virus Preparation**

Vpu from subtype B (pNL4-3, GenBankTM accession number AF324493) and C (Indian isolate 93IN905 GenBankTM accession number AF067158) HIV-1 (obtained from AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were PCR amplified and cloned in the mammalian expression vector pCMV-HA (Clontech). The cloning and sequencing was carried out at least twice on two separate occasion starting from genomic DNA to rule out the PCR generated mistakes in the sequence.

**Phylogenetic Analysis, Genetic Subtyping, dN/dS Ratio and Sequence Alignment**

These sequences were aligned with reference sequences of HIV-1 strains of all subtypes [http://www.hiv.lanl.gov)] using the Clustal W 1.83 program [32]. The phylogenetic analysis was performed using the Neighbor Joining (NJ) method based on the Kimura two-parameter distance matrix implemented in the MEGA 4.0 program [33–35]. The phylogenetic tree was constructed based on 576 nucleotide long vpu sequences (lies between the genomic region of 5041 to 5619 relative to HXB2) of 10 unique group of North Indian sequences and reference sequences which were retrieved from HIV Los Alamos database ([http://www.hiv.lanl.gov/]), includes M group (A,A1,A2,A3,B,C,D,F1,F2,G,H,J,K and U), N, O and P groups. The vpu reference sequences were from different part of the world (America, Japan, France, China, Thailand, Brazil, Kenya, Botswana, Zambia, South Africa and other countries). The GeneBank accession numbers for Indian vpu sequences are HQ239051 to HQ239066. To determine whether Vpu is under selection pressure in HIV-1 infected patients from North India, the SNAP analysis program ([http://www.hiv.lanl.gov/]) was used to compare the average ratio of normalized Non-synonymous to synonymous substitutions (dN/dS) [36]. The multiple sequence alignment and secondary structure prediction were done using CLC sequence server (CLC bio, Denmark).

**HIV-1 Subtype-Specific Adaptations**

5'GGCGAATTCTTATGCAACCTATAATAGTAGGCAAAG-3'

**Reverse 1.**

5'GGCGTCGACCTACAGATCATCAATATCCCAAGGAGG-3'

**Forward 2.**

5'GGCGGTCCACTAATCATTAACTCAGGAGG-3'

**Reverse 2.**

If the PCR using the first set of primers failed to amplify viral genomic fragment, the second set of primers was used. The PCR conditions were as follows: one cycle of 2 min at 94°C for denaturation; 30 cycles of 10 sec at 94°C for denaturation; 30 sec at 52°C for annealing and 30 sec at 72°C for extension and a final extension cycle of 5 min at 68°C were carried out. In order to examine the genomic fragment of the major viral population in a sample, PCR products amplified at the end-point dilution of DNA templates were subjected to sequence analysis. The gel purified PCR products were cloned in pGEM®-T Easy Vector System (Promega, USA) in between T7 and Sp6 promoters and also in the expression vector pCMV-HA (Clontech). The cloning and sequencing was carried out at least twice on two separate occasion starting from genomic DNA to rule out the PCR generated mistakes in the sequence.
two hours as described by us earlier [17]. The His-Ubiquitin (His-Ub) expression plasmid was kindly provided by Dimitris Xirodimas (University of Dundee, United Kingdom).

**Generation of HIV-1 pNL 4-3 Backbone for Cloning vpu**

A pNL4-3 based viral backbone was used for intracellular expression of different vpu constructs (Vpu B and C) upon infection in T-cell line by specifically substituting the wt vpu locus with that of natural vpu variants as described earlier [37]. Briefly, two unique restriction sites (Xma-1 and Xba-1) were precisely introduced; one just before start codon (Xma-1) and other after stop codon (Xba-1) of vpu in pNL 4-3 proviral DNA using a PCR based strategy. The sequences of primer used were:

Xma-1.

P1 5’- AT CCGGGG CTACAGATCATCAATATCC-GAAGG-3’.

P2 5’- ATT TCTAGA ATGCATCATCATCATCATCATCATCATCATCATCATGCAAC-3’.

Xba-1.

The HA tagged Vpu variants were then amplified from pCMV Vpu HA constructs with primers carrying Xma-1/Xba-1 restriction sites and then ligated into pNL 4-3 proviral backbone.

**Cell Culture, Transfections and Immunoblot Analysis**

HEK-293T (Human Embryonic Kidney 293 cells), HeLa (Human Cervical Cancer line) and Tzmbl cells (HIV indicator cells, acquired from NIH, AIDS research reagent) were maintained in DMEM (Gibco, Invitrogen, California) supplemented with glutamine, 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, California) at 37°C with 5% CO₂. MOLT-4 T cells (T-lymphoblastoid cell line of human origin) were maintained in RPMI (Gibco, Invitrogen) medium supplemented with glutamine, 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen) at 37°C in presence of 5% CO₂. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen). Relative levels of different proteins were compared by immunoblot analysis. Cells were lysed using RIPA Lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin). Protein estimation was done using BCA protein estimation Kit (Pierce Biotechnology, Inc., Rockford). Proteins were resolved by polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore, USA). The membranes were blocked with 5% non fat dry milk (Sigma Aldrich, St. Louis, Missouri) in PBS, washed with PBS containing 0.1% Tween 20 (MERCK, New Jersey) and incubated in the same buffer overnight at 4°C in the presence of primary antibody (1:2000 dilution). The primary antibodies used were anti-HA, anti-GAPDH (Santa Cruz Biotechnology, Inc., California). The membranes were washed with PBS containing 0.1% Tween 20 and then incubated with either anti-mouse or anti-rabbit antibody conjugated with horse radish peroxidase (1:10000 dilutions, Jackson Immuno Research, USA) in 5% non fat dry milk in PBS with 0.1% Tween 20 at room temperature as secondary antibody. The proteins of interest were detected with EZ western horse radish peroxidase substrate ( Biological Industries, Israel). GAPDH or tubulin levels were used as a loading control in all cases.

**Cycloheximide Chase Assay and in vivo Ubiquitination Assay**

Vpu constructs were transfected (2 µg of wt or mutant Vpu constructs) in HEK-293T cells (1×10⁶ cells) for thirty six hours. Subsequently, CHX (Sigma-Aldrich) was added to a final concentration of 100 µg/ml and cells were harvested at the indicated time points. The lysate was prepared and immunoblotting was performed. For detection of ubiquitinated Vpu protein, HEK-293T cells were grown in 100-mm dishes and transfected with 5 µg of His-ubiquitin expression plasmid along with equal amounts of various Vpu-expressing constructs [38]. To normalize the DNA amount in each well pCMV-HA empty vector was used. After thirty six hours of transfection, 25 µM MG-132 (Sigma Aldrich) was added and cells were further incubated for eight hours. Thereafter, cells were collected in PBS and were resuspended in 1 ml lysis buffer (6 M guanidinium-HCl, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0, 0.5 mM imidazole and 10 mM β-mercaptoethanol), sonicated and centrifuged. Ni-NTA beads (50 µL) were added to supernatant and the mixture was incubated at room temperature for four hours while rotating. Subsequently the beads were washed for 5 minutes at room temperature with 750 µL of each of the following buffers: 6 M guanidinium–HCl, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0, and 10 mM β-mercaptoethanol; 8 M urea, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0, 10 mM β-mercaptoethanol; 8 M urea, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 6.3, 10 mM β-mercaptoethanol (buffer A) plus 0.2% Triton X-100; buffer A and then buffer A plus 0.1% Triton X-100. After the last wash ubiquitinated proteins were eluted by incubating the beads in 75 µl of buffer containing 200 mM imidazole, 5% SDS, 0.15 M Tris/HCl, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol for 20 min at room temperature. The eluates were mixed in 1:1 ratio with 2 x Laemmli buffer and separated on 8% SDS-PAGE followed by immunoblotting with anti-HA antibody.

**Virus Release Assay, Infection by HIV-1 pNL4-3 Mutants and Cell Death Analysis**

For virus release assay, HeLa cells were transfected with equal amounts of pNL based proviral clones using Lipofectamine 2000. Thereafter, culture supernatants from transfected cells were collected forty eight hours post transfection. Infectious virus yields associated with culture supernatants were determined using Tzmbl HIV-indicator cells [39]. Briefly following forty eight hours of infection, Tzmbl cells were washed twice with ice cold 1XPBS buffer. Cells were then fixed with fixation buffer (0.25% Glutaraldehyde in 1X PBS) for 10 minutes at room temperature while gently rocking the plates. The cells were washed twice with ice cold 1X PBS and stained with freshly prepared staining buffer (5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 1 mg/ml X Gal solution, 2 mM MgCl₂ for six hours at 37°C. Total number of infected cells was counted as a measure of total virus release. Infection of various cell lines was accomplished by incubating the cells for four hours with equal amounts of infectious virus (1 MOI) as assessed previously by β-galactosidase staining using HIV-1 indicator Tzmbl cells [39]. For cell death analysis, infected cells were collected and washed in 1X PBS. Finally, the cells were resuspended in 1XPBS containing Propidium iodide (PI) at a final concentration of 10 µg/ml. The cells were analysed on the BD LSR flow cytometer for propidium iodide incorporation to measure cell death.

**Accession Numbers**

The GeneBank accession numbers for Indian vpu sequences reported in this study are HQ239051 to HQ239066.
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References

1. Cummins NW, Badley AD (2010) Mechanisms of HIV-associated lymphocyte apoptosis: 2010. Cell Death and Disease 1:e90.
2. Badley AD, Pilon AA, Landay A, Lynch DH (2000) Mechanisms of HIV-associated lymphocyte apoptosis. Blood 96: 2951–2964.
3. Emerman M, Malim MH (1998) HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. Science 280: 1880–1884.
4. Malim MH, Emerman M (2000) HIV-1 accessory proteins—ensuring viral survival in a hostile environment. Cell Host Microbe. 3: 388–398.
5. Bonhoeffer S, Holmes EC, Nowak MA (1995) Causes of HIV diversity. Nature. 15: 125.
6. Wain-Hobson S (1993) The fastest genome evolution ever described: HIV variation in situ.Curr. Opin. Genet. Dev. 3: 878–883.
7. Ji J, Loeb LA (1994) Fidelity of HIV-1 reverse transcriptase copying a hypervariable region of the HIV-1 env gene. Virology 199: 325–350.
8. Yasin K, Kronin C, Genchen B, Addo MM, Altfield M, et al. (2002) Clustering patterns of cytotoxic T-lymophocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. J Virol. 76: 857–868.
9. Strebel K, Klümkait T, Martin MA (1988) A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science 241: 1221–1223.
10. Cohen EA, Tewerilger EF, Sodroski JG, Haseltine WA (1988) Human immunodeficiency virus type 1 accessory gene a provides a signal for nuclear export of regulatory protein. Cell 56: 1111–1119.
11. Badley AD, Pilon AA, Landay A, Lynch DH (2000) Mechanisms of HIV-associated lymphocyte apoptosis. Blood 96: 2951–2964.
12. Yang SJ, Lopez LA, Exline CM, Haworth KG, Cannon PM (2011) Lack of Vpu inhibits efficient membrane protein trafficking in human immunodeficiency virus type 1 infected cells. J Virol. 85: 7901–7917.
13. Margottin F, Bour SP, Durand H, Solig L, Benichou S, et al. (1998) A novel human WD protein, h-betaTrCP, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. Mol Cell. 1: 565–574.
14. Schubert U, Heukin C, Bickford B, Wingender E, Strebel K, et al. (1994) The human immunodeficiency virus type 1 encoded Vpu protein is phosphorylated by casein kinase-2 (CK-2) at positions Ser52 and Ser56 within a predicted alpha-helix-turn-alpha-helix-motif. J Mol Biol. 236: 16–25.
15. Akari H, Bour S, Kao S, Adachi A, Struebl K, et al. (2001) The human Immunodeficiency Virus Type 1 Accessory Protein Vpu Induces Apoptosis by Suppressing the Nuclear Factor kappab-dependent Expression of Antia apoptotic Factors. J Exp Med. 194: 1299–1311.
16. Verma S, Ali A, Arowa S, Banerjea NC (2011) Inhibition of beta-TrCP-dependent ubiquitination of p53 by HIV-1 Vpu promotes p53-mediated apoptosis in human T cells. Blood. 117: 6600–6607.
17. Schuett U, Bour S, Ferrer-Montiel AV, Montal M, Maldarelli F, et al. (1996) The two biological activities of the human immunodeficiency virus type 1 Vpu protein involve two separate structural domains. J Virol. 70: 809–819.
18. Pacynaik E, Gomez ML, Gomez LM, Mullally ER, Jackson M, et al. (2003) Identification of a region within the cytoplasmic domain of the subtype B Vpu protein of human immunodeficiency virus type 1 (HIV-1) that is responsible for retention in the Golgi complex and its absence in the Vpu protein from a subtype C HIV-1. AIDS Res Hum Retroviruses. 21: 379–394.
19. Hussain A, Das SR, Tanwar C, Janneel S (2007) Olignomerization of the human immunodeficiency virus type 1 (HIV-1) Vpu protein – a genetic, biochemical and biophysical analysis. Virol J. 4: 81.
20. Lee CN, Wang WK, Fan WS, Tsu SJ, Chen SC, et al. (2000) Determination of Human Immunodeficiency Virus Type 1 Subtypes in Taiwan by vpu Gene Analysis. J Clin Microbiol. 38: 2468.
21. Lee CN, Wang WK, Fan WS, Tsu SJ, Chen SC, et al. (2000) Determination of Human Immunodeficiency Virus Type 1 Subtypes in Taiwan by vpu Gene Analysis. J Clin Microbiol. 38: 2468.
22. Jens T, Fioretto S, Treurnicht K, Zeier M, Engelbrecht S, et al. (2001) Characterization and Phylogenetic Analysis of South African HIV-1 Subtype C Accessory Gene. Aids Research and Human Retroviruses. 7: 773–781.
23. Hill MS, Ruiz A, Pacciniak E, Pinzon DM, Couley N, et al. (2008) Modulation of the severe CD4+ T-cell loss caused by a pathogenic simian-human immunodeficiency virus by replacement of the subtype B vpu with the vpu from a subtype C HIV-1 clinical isolate. Virology. 371: 86–97.
24. Adachi A, Grisofelden HE, Koenig S, Folk T, Willey R, et al. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol. 59: 294–291.
25. Estrabaud E, Roszynski E, Lopez-Vergés S, Morel M, Belaidouni N, et al. (2007) Regulated Degradation of the HIV-1 Vpu Protein through a βTrCP-Independent Pathway Limits the Release of Virus Particles. PLoS Pathogens 7: e1001140.
26. Magadán JG, Bonafacio JS (2012) Transmembrane domain determinants of CD4 Downregulation by HIV-1 Vpu. J Virol. 86: 757–762.
27. Skasko M, Wang Y, Tian Y, Tokarev A, Mungia J, et al. (2012) HIV-1 Vpu protein antagonizes innate restriction factor BST-2 via lipid-embedded helix-helix interactions. J Biol Chem. 287: 56–67.
28. Lu M, Wang J, Wang X, Zuo T, Zhu Y, et al. (2011) Polarity changes in the transmembrane domain core of HIV-1 Vpu inhibits its anti-tertherin activity. PLoS One 6:e20890.
29. Miyagi E, Andrew AJ, Kao S, Strebel K (2009) Vpu enhances HIV-1 virus release in the absence of BST-2 cell surface down-modulation and intracellular depletion. Proc. Natl. Acad. Sci. USA 106: 2568–2573.
30. Paul M, Mazumder S, Raju N, Jbabda MA (1998) Mutational Analysis of the Human Immunodeficiency Virus Type 1 Vpu Transmembrane Domain That Promotes the Enhanced Release of Virus-Like Particles from the Plasma Membrane of Mammalian Cells. J Virol. 72: 1270.
31. Bano AS, Sood V, Neogi U, Groel N, Kuttiat YS, et al. (2009) Genetic and functional characterization of human immunodeficiency virus type 1 VpuC variants from north India: presence of unique recombinants with mosaic genomes from B, C and D subtypes within the open reading frame of Vpu. J Gen Virol. 90: 2768–2776.
32. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.
33. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol. 4: 406–425.
34. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 16: 111–120.
35. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 24: 1596–1599.
36. Korber B (2000) HIV Signature and Sequence Variation Analysis. Computational Analysis of HIV Molecular Sequences 4: 55–72.
37. De Candia C, Espada C, Duette G, Ghiglione Y, Turk G, et al. (2010) Viral replication is enhanced by an HIV-1 intersubtype recombination-derived Vpu protein. Virol J. 7: 259.
38. Kubstaet MH, Ludvig RL, Ashcroft M, Voudoue KH (1998) Regulation of Mdm-2 directed degradation by the C terminus of p53. Mol Cell Biol. 18: 5690–5698.
39. Cornall A, Sharma L, Solomon A, Gorry PR, Crowe SM (2010) A novel, rapid method to detect infectious HIV-1 from plasma of persons infected with HIV-1. J Virol Methods. 159: 90–96.