Chapter 5

GENETIC AND FUNCTIONAL CHARACTERIZATION OF HIV-1 VPU GENE CIRCULATING IN HIV-1 INFECTED INDIVIDUALS FROM NORTH INDIA
Chapter 5 - Genetic and functional characterization of HIV-1 vpu gene circulating in HIV-1 infected individuals from North India

5.1 Background

Five years after its discovery in North America, HIV was first reported in 1986 in India among sex workers in Chennai (Simoes et al., 1987). The total estimate according to United Nations Programme on HIV/AIDS (UNAIDS) about people living with HIV in India was 0.36% that is 2.47 million people (UNAIDS, 2008). The understanding of the mechanisms of natural protection from or spontaneous resistance to viral infections may contribute to the development of effective preventive measures. Also studies towards understanding the evolutionary history of the virus are important. Together such studies can reveal valuable insights for the design of effective vaccines. Several national and international agencies have worked towards the collection of data regarding the incidence and prevalence of HIV in the different states of our country. NACO (National AIDS Control Organisation) a government body, NIHFW (National Institute of Health and Family Welfare), ICMR (Indian council of Medical Research) are few of the national organizations working in collaboration with several international bodies such as ILO (International Labour Organisation), WHO (World Health Organisation), UNAIDS to work out the future projections as well as control strategies. The combined effort of the above mentioned national organizations with international bodies like UNAIDS provides the direction in which various molecular virology studies can be carried out by various researchers. Research is essential to keep a track on the spread of the virus and on the variations in their sequences to design effective intervention strategies.

HIV diversity is associated with the error prone reverse transcriptase enzyme (Mansky et al., 1995). The major subtypes of HIV identified are A-K depending on
their geographical distribution. While subtype A is prevalent in Central Africa, subtype B is predominant in North and South American continent (Bobkov et al., 2004). The Indian subcontinent has subtype C as the major prevalent subtype along with China and South Africa, which is the major subtype contributing to the epidemic worldwide (Hemelaar et al., 2006; Neogi et al., 2009). Although, there is no direct correlation between the fitness of various subtypes to their respective spread, the prevalence of subtype C among majority of the HIV infected individual throughout the world would suggest that it has been naturally selected over other subtypes. The transmission rate of subtype C HIV-1 long terminal repeats (LTRs) from mother to infant has been shown to be a more likely event when compared to either subtype A or intersubtype recombinant LTR (Blackard et al., 2001). Also, in a recent review by scientists at NARI (National AIDS Research Institute), subtype C was stated to have fitness advantage over subtype A (Lakhashe et al., 2008). In lieu of these findings, it is important to carry out separate studies in relation to subtype C. Major Studies pertaining to HIV-1 pathogenesis have been carried out on Subtype B from Europe and America. In contrast little information is available for subtype C, responsible for majority of the global epidemic (Africa and Asia). As far as the Indian subcontinent is concerned there are numerous reports available for the presence of subtype C throughout India (Sahni et al., 2002; Mandal et al., 2002, Kurle et al., 2004). Less prevalent HIV-1 subtypes, other than subtype C have also been reported by several investigators in different parts of the country. These include subtype B and A from New Delhi and Punjab (Northern India), West Bengal and Manipur (eastern and north-eastern region), Maharashtra, Andhra Pradesh, Tamil Nadu and Gujarat (southern, western, and south western) (Gadkari et al., 1998; Kumar et al., 2006; Sidappa et al., 2004; Jameel et al., 1995, Cassol et al., 1996; Tripathy et al., 2005; Deshpande et al., 2009). Several recent reviews have been published by different groups describing the HIV epidemiology in India (Singh et al., 2008; Neogi et al., 2009). The pure HIV subtypes are gradually phasing out and are being replaced by mosaic genome viruses owing to the recombinogenic nature of the virus (Levy et
al., 2004; Shriner et al., 2004; Suryavanshi et al., 2007). Since Vpu is the most recent evolutionary adaptation of HIV-1 genome we expected it to show extreme genetic variation which may follow a subtype specific signature pattern. The purpose of this study was to find out the levels of genetic changes that are present in the Vpu gene from HIV-1 infected individuals from North-India (Punjab/Haryana) and determine the functional relevance with respect to the two known important functions of this protein, its ability to activate virus release and cause apoptosis.

Also, Vpu alone is able to contribute substantially towards HIV-1 mediated pathogenesis, selectively inhibiting the expression of this gene product may constitute an important antiviral approach. Several nucleic acid based approaches (antisense, catalytic Rzs, Dzs, aptamers and small interfering RNAs) (Akkina et al., 2003; Banerjea et al., 2005) have been successfully used to suppress a particular target gene in a sequence-specific manner. Dzs, as originally described by Santoro and Joyce in 1997, are short catalytic DNA molecules that possess the ability to cleave any target RNA in a sequence-specific manner (Joyce et al., in 2004; Silverman et al., 2005). Dzs containing 10-23 catalytic motif has been used extensively to interfere with the expression of HIV-1 genes with varying results. In the current study, we designed 10-23 catalytic motif containing Dz against the most conserved regions of Vpu gene.
5.2 Experimental Procedures

Patient Information

We obtained peripheral blood mononuclear cells from HIV-1 infected individuals from North India (Punjab/Haryana region). These patients were registered for our study after obtaining requisite clearances. The patient information along with clinical parameters is summarized in (Table 5.1). They were monitored at the Immunodeficiency Clinic of Post Graduate Institute of Medical Research and Education, Chandigarh, by our Clinician A. Wanchu.

Table 5.1. Patient information and clinical parameters of subjects involved in studies. ND: Not Done.

<table>
<thead>
<tr>
<th>Subject Code</th>
<th>Age</th>
<th>Sex</th>
<th>Mode of Transmission</th>
<th>CD4 Count</th>
<th>WHO clinical stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>NII-PGI-IND-1</td>
<td>32</td>
<td>M</td>
<td>Heterosexual</td>
<td>169</td>
<td>1</td>
</tr>
<tr>
<td>NII-PGI-IND-2</td>
<td>40</td>
<td>M</td>
<td>Heterosexual</td>
<td>62</td>
<td>2</td>
</tr>
<tr>
<td>NII-PGI-IND-4</td>
<td>35</td>
<td>M</td>
<td>Heterosexual</td>
<td>37</td>
<td>3</td>
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<tr>
<td>NII-PGI-IND-7</td>
<td>30</td>
<td>M</td>
<td>Heterosexual</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NII-PGI-IND-24</td>
<td>23</td>
<td>M</td>
<td>Heterosexual</td>
<td>458</td>
<td>1</td>
</tr>
<tr>
<td>NII-PGI-IND-41</td>
<td>25</td>
<td>M</td>
<td>Heterosexual</td>
<td>82</td>
<td>2</td>
</tr>
<tr>
<td>NII-PGI-IND-S2</td>
<td>29</td>
<td>M</td>
<td>Heterosexual</td>
<td>111</td>
<td>1</td>
</tr>
<tr>
<td>NII-PGI-IND-42</td>
<td>40</td>
<td>M</td>
<td>Heterosexual</td>
<td>69</td>
<td>3</td>
</tr>
</tbody>
</table>

Genomic DNA isolation and amplification of Vpu gene

PBMCs isolated from the patients were used to extract genomic DNA using Qiagen genomic DNA extraction kit. Isolated DNA was used to amplify sequence spanning the vpu gene by polymerase chain reaction. All PCRs were performed with high fidelity Taq polymerase. The sequence of primers used were:

\[
\text{Vpu B forward} \quad 5'-\text{ATTTCTAGAATGCATCATCATCATCATCATGCCAACCCTATAATAGTA-3'}
\]

\[
\text{Vpu C forward} \quad 5'-\text{ATTTCTAGA ATGCATCATCATCATCAC ATGGTAGATTTAGATTAAAA-3'}
\]
Chapter 5

Studies on natural isolates of HIV-1 Vpu

Figure 5.1 PCR amplification of vpu gene (in frame with HA tag) from genomic DNA samples of HIV-1 infected individuals.

\[ \text{Vpu B Reverse} \]
5’- ATTCTAGA ATG CATCATCATCATCAC ATGCAACCTATAATAGTA-3’

\[ \text{Vpu C Reverse} \]
5’- ATTCTAGA ATG CATCATCATCATCAC ATGTTAGATTATTATAA-3’

The vpu gene was amplified from genomic DNA samples of HIV-1 infected individuals using primers engineered to introduce in frame HA tag for cloning into; pNL-HIV-1 based viral backbone. The positive clones were identified by the appearance of ~300 bp insert after digesting with Xba-1 and Xma-1 restriction enzymes and then further confirmed by sequencing.

Sequencing, Computer alignment and analysis of vpu gene

Forward and reverse sequencing was performed with T7 and SP6 universal primers using pGEM-T easy vector. Sequence alignment was carried out by ClC workbench software and compared with pNL4-3 (AF324493) and 93IN905 (AF067158) prototype as well as subtype consensus sequences.

Cell line

HEK 293T (Human embryonic kidney 293T cells), HeLa (Human Cervical Cancer line) and Tzmbi cells (HIV indicator cells, acquired from NIH, AIDS research reagent) were
maintained in DMEM (Gibco, Invitrogen) supplemented with glutamine, 10% FCS, 100 U/mL penicillin, and 100 g/mL streptomycin (Invitrogen) at 37° C with 5% CO₂. MOLT-4 T cells (T-lymphoblastoid cell line) were maintained in RPMI (Gibco, Invitrogen) media supplemented with glutamine, 10% FCS, 100 U/mL penicillin, and 100 g/mL streptomycin (Invitrogen) at 37° C with 5% CO₂.

**Western blot analysis**

Relative levels of different proteins were compared by immunoblot analysis. After 48 hours of transfection of 293T cells with indicated amounts of Vpu gene variants from HIV-1 infected individuals, cell lysates were prepared and protein content was estimated. 12% SDS-PAGE was used to resolve the proteins and they were transferred to the nitrocellulose membrane (Millipore) at 300 mA for 2 hour. Monoclonal anti-HA antibody (Santa Cruz) was used as the primary antibody and HRP conjugated Rabbit IgG (Santa Cruz, CA, USA) was used as the secondary antibody. The proteins of interest were detected with EZ Western HRP substrate (Biologic Industries). GAPDH was used as a loading control in all cases.

**Preparation of viral stocks and Virus release assay**

HEK 293T cells were grown in 100-mm dishes and co-transfected with different proviral constructs (pNL4–3wt/ M-Vpu+ /Vpu-deficient) and plasmid encoding VSV-G (ratio of 9:1). The culture supernatant was collected at 4°C, 48 and 72 hours after transfection in 10mM HEPES buffer and stored at -70°C. For Virus release assay, HeLa cells were infected with VSV-G-pseudotyped PNL based HIV-1 variants. Thereafter, culture supernatants from infected cells were collected 48 hour post infection. Infectious virus yields were determined using TZMbl HIV-indicator cells.
Cell death Assay
Cells were removed by treating with cell dissociation buffer. Cells were centrifuged at 1200 rpm at 4°C, the supernatant decanted and the cells were washed in 1X PBS. Finally, the cells were resuspended in 1X PBS containing Propidium iodide (at a final concentration of 10μg/ml). The cells were analysed on the flow cytometer for propidium iodide incorporation to measure cell death.

Target site selection for the DNA-enzyme
To accomplish this objective we first identified the most conserved region in vpu gene. Vpu RNA sequences were then subjected to secondary structure generating software programme, m-FOLD (http://mfold.burnet.edu.au). The various secondary structures generated by this program were studied for most conserved loop regions and were screened for target sites, AU or GU to be targeted by Dzs. The most conserved (100%) target site chosen was an ‘AU’ at nucleotide positions 167 of the Vpu B RNA. The twenty nine nucleotide long oligonucleotides (Dzs) were synthesized (sigma Aldrich), containing in the middle, and the previously identified 10-23 catalytic motif with the following sequence:

5’-TCACCGTGCTAGCTACAAAGACTCTAC-3’

The hybridizing arms, seven residues each on either side of the purine/pyrimidine pair were made complementary to the target RNA (leaving the purine residue unpaired).
5.3 Results

Genetic variation in HIV-1 Vpu gene from North Indian HIV-1 infected individuals

To explore the genetic and functional implication of genetic variations displayed by Vpu alleles in HIV-1 infected patients, we recovered complete ORFs corresponding to Vpu locus of HIV-1 genome from genomic DNA sample of PBMCs from infected patients. The sequences were PCR amplified, sequenced and then subjected to extensive genetic and literature based characterization. When subjected to Phylogenetic analysis (Figure 5.2), all of the samples clustered into two distinct groups depending on resemblance with prototype subtype B Vpu or subtype C Vpu.

According to the neutral theory of evolution, the number of synonymous substitutions with synonymous site (dS) is proportional to the rate of nucleotide mutation of a gene. The non-synonymous/synonymous ratio of rate constants (dN/dS) is indicative of the selection pressure at the protein level: dN/dS < 1 is indicative of purifying selection and amino acid conservation because of structural and functional constraints, and dN/dS > 1 is indicative of diversifying, positive selection where amino acid substitutions confer an advantage (Ganeshan et al., 1997). Interestingly, dN/dS ratio analysis for most of variant group gave value greater than 1 (Table 5.2) indicating positive evolutionary selection.
Figure 5.2 Phylogenetic analyses for Vpu variants. The phylogenetic analyses were carried out using MEGA4.1 software using the neighbor joining method and maximum composite likelihood model with 500 bootstrap replicates. The accession numbers of all sequence submitted to NCBI nucleotide database are given in brackets.
Table 5.2 The rate of accumulation of non-synonymous and synonymous substitution, calculated by dN/dS ratio with consensus B and consensus C as references.

<table>
<thead>
<tr>
<th>Representative Unique sample</th>
<th>dN/dS ratio</th>
<th>Predicted subtypes</th>
<th>Evolutionary selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NII-PGI-Vpu A4</td>
<td>0.907</td>
<td>B</td>
<td>Purifying selection</td>
</tr>
<tr>
<td>NII-PGI-Vpu 42</td>
<td>2.570</td>
<td>B</td>
<td>Diversifying selection</td>
</tr>
<tr>
<td>NII-PGI-Vpu 7</td>
<td>2.078</td>
<td>B</td>
<td>Diversifying selection</td>
</tr>
<tr>
<td>NII-PGI-Vpu S2</td>
<td>1.094</td>
<td>B</td>
<td>Diversifying selection</td>
</tr>
<tr>
<td>NII-PGI-Vpu 41</td>
<td>1.499</td>
<td>C</td>
<td>Diversifying selection</td>
</tr>
<tr>
<td>NII-PGI-Vpu 24</td>
<td>0.944</td>
<td>C</td>
<td>Purifying selection</td>
</tr>
</tbody>
</table>

A site informative multiple sequence alignment of all the resultant alleles is shown with respect to consensus subtypes B and C Vpu (Figure 5.3). Group B variants display higher degree of variations in all topological domains with some novel mutations with high allelic frequency as compared with group C variants which display less variation (analysis based on unique representative samples). It is noteworthy that the group B variants showed maximum variation importantly in Cytoplasmic helix-2 region as compared to group C variants which display variation in transmembrane domain. We also noticed for the first time the genetic evidences for positive selection of group B Vpu variants which have substitution of a serine residue (serine-61 conversion to alanine; S61A), previously shown to be involved in Vpu B degradation (Estrabaud et al., 2007) in all of our group B variants. Interestingly, we also found one mutant (Vpu 24) in group C variants which had lost its functional β-TrCP binding motif. As observed by others (Lee et al., 2000, Jens et al., 2001), the length of all different Vpu variants was also quite variable in different Vpu isolates. Most of our 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 functions. Beside these interesting changes, we observed that the specific determinants for CD4 (V20, W22, S23) (Magadan et al., 2012), BST-2 (A11, A15, I17, I18 and A19) (Skasko et al., 2012,
Figure 5.3 Multiple sequence alignment of representative unique primary isolates of HIV-1 Vpu collected from HIV-1 infected individuals in North India. The color coding generated by software represents difference in color for amino acid with different physiochemical properties.
Lv et al., 2011) and β-TrcP (DS52GNES56) (Margottin et al., 1998) binding remained highly conserved (Figure 5.3) among all the variants.

**The S61A Mutation conferred enhanced intracellular stability**

Incorporating S61A mutation in HIV-1 pNL4-3 (Vpu B) was reported to increase stability of Vpu protein as well as boost viral replication rate (Estrabaud et al., 2007). We wanted to test the functional implication of S61A natural mutation with respect to intracellular stability as well as expression of Vpu alleles among the B group variants. HEK 293T cells were transfected with mammalian expression vectors encoding S61A mutants and wild type Vpu alleles (Figure 5.4B). Forty eight hours after transfection cell lysate were prepared and probed for relative expression of Vpu variants. As shown (Figure 5.4A) different Vpu alleles displayed differential migration pattern (owing to difference in overall length, phosphorylation and other post-translational modifications) upon electrophoresis on a high percentage acrylamide gel (15% PAGE). The variants also displayed substantial differences in relative abundance. Notably, S61A variants (Figure 5.4A, Lane 4 and 5) showed higher expression levels than S61+ Vpu alleles (Figure 5.4A, Lane 2 and 3).

![Figure 5.4 The S61A Mutation conferred enhanced intracellular expression. A) Equal amount of various Vpu expression constructs were transfected into HEK 293T cells by Lipofectamine 2000 for 48 hours. Thereafter Cell lysate were subjected to immunoblotting to measure relative abundance of various Vpu variants. B) Identical residues are presented as dot.](image)
Also, Cycloheximide (CHX) chase assay was performed to study the effect of S61A mutation on the intracellular turnover of Vpu protein alleles. After 8 hours of chase period the level of wild type Vpu B and C proteins were reduced to undetectable levels in transfected HEK 293T cells (Figure 5.5A and B). In contrast, a comparative analysis of two Vpu alleles with or without S61A mutation revealed that S61A mutant allele (Vpu A5) showed almost no reduction in protein levels than S61 variant (Vpu 41) which was comparable to wild type subtype B and C Vpu protein (Figure 5.5C and D). 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. HEK 293T cells were co-transfected with plasmids encoding His-ubiquitin (His-Ub) and various Vpu alleles.

![Image](image_url)

Figure 5.5 Cycloheximide (CHX)-chase to check kinetic stability of various Vpu variants. HEK 293T cells transfected with various Vpu constructs were treated with 50µg/ml of CHX and harvested at the indicated time points for Immunoblotting.
Then, 36 hours after transfection, cells were treated with MG132 for 8 hours. After cell lysis, ubiquitinated proteins were pulled down using Ni-NTA beads and Vpu ubiquitination was checked by anti-HA antibody. Interestingly, all the three S61A mutants tested (Vpu 1, 7 and S2) showed marked inhibition of Vpu ubiquitination (Figure 5.6, Lane 7, 8 and 9) as compared to S61 wild type alleles (Figure 5.6, Lane 4, 5 and 6). These results clearly show that acquisition of S61A mutation inhibits Vpu ubiquitination conferring increased intracellular stability as well as relative protein abundance.

Figure 5.6 Intracellular ubiquitination profile of various Vpu isolates. HEK 293T cells were co-transfected with His-ubiquitin (His-Ub), and various Vpu constructs. After 36 hours, cells were treated with MG132 for 8 hours followed by lysis in denaturation buffer and then total ubiquitinated proteins were pulled down using Ni-NTA beads and Vpu ubiquitination was checked by immunoblotting using anti-HA antibody.
Studying natural Vpu variations with respect to viral replication

Since Vpu is primarily involved with function of virus release (Klimkait et al., 1990) as well as cell death (Akari et al., 2001), we further analyzed the functional impact of natural variations among Vpu alleles on virus release and potential to induce cell death in infected cells. All the Vpu alleles were cloned in a pNL backbone to ascertain viral functions primarily associated with the above two Vpu biological activities. Following forty eight hour post infection cell culture supernatants from HeLa cells were collected and used to determine relative infectious viral yield associated with each Vpu allele by counting the number of blue cells in Tzmbl-indicator cells present in identical area using an inverted microscope. Vpu B (Figure 5.7A, panel 3) released twice the amount of virus as compared to Vpu C (Figure 5.7A, panel 4). Two of our natural S61A mutants were most potent in the ability to cause virus release (Figure 5.7A, panel 5, 6, 7B and C). However, one S61A mutant allele, Vpu 7 (containing a transmembrane deletion of eight amino acids, Figure 5.3), when compared with other S61A alleles, showed moderate enhancement of viral release (Figure 5.7A, panel 8 and Figure 5.7C). All the group C variants (Vpu 4, 24 and 41) showed virus release activity comparable to subtype C Vpu (Figure 5.7A, panel 7, 9, 10 and Figure 5.7B). Interestingly, one Vpu C variant possessing a non-functional β-TrCP binding motif, show little viral release activity as much as other S61 and β-TrCP motif wild type Vpu alleles (Figure 5.7A, panel 9 and Figure 7C).
Figure 5.7 Virus release activity of various isolates of Vpu. A) HeLa cells were infected with an equal MOI (0.5) of various pseudotyped viruses and total virus released in culture supernatant was quantitated using HIV indicator Tzmbl cells by β-galactosidase staining. B) Relative number of infected cells were counted for each sample and plotted C).
Next, we investigated whether the observed phenomenon of enhanced virus release and kinetic stability correlated the subtype-specific difference in apoptotic potential of HIV-1 during infection. To address this question, MOLT-4 T-cells were infected with an equal MOI of VSV-G pseudotyped viruses and following 48 hours infected cells were harvested for determination of total apoptotic population by PI staining as well as western blotting to confirm Vpu expression. As shown (Figure 8A), when compared with Vpu null (PNLΔVpu) HIV-1 (Figure 8A, panel 2), all Vpu expressing viruses were found to induce higher cell death (Figure 8A) in infected cells in agreement with the previous reports. When analyzed for their relative ability to induce cell death group B S61A mutants (Figure 8A, panel 5, 6 and 7) caused moderate cell death as compared to wild type subtype B Vpu (Figure 8A, panel 3). The group C variants, (Figure 8A, panel 8 and 9) on the other hand showed higher apoptotic potential than all group B variants tested. The mutant Vpu 24 expressing virus (Figure 8A, panel 10) was found to induce moderate cell death (less than subtype C Vpu and comparable to PNLΔVpu). Western blotting (Figure 8B) also confirmed higher expression levels associated with group B S61A variants (Figure 8B, lane 7, 8 and 9) as compared to S61+ variants (Figure 8B, lane 4, 5 and 6). From this data we conclude that sequence divergence exhibited by two Vpu groups can influence widely varying biological activities of Vpu.
Figure 5.8 Cytotoxic potential of various isolates of Vpu. Infected MOLT-4 T Cells were harvested, stained with Propidium iodide (10 µg /ml) and were then analyzed by flow Cytometry to determine the extent of cell death. A) The extent of cell death is indicated in upper right corner of each panel. The FACS data were analyzed by WinMDI software. B) Equal amount of lysate from infected cells was subjected to Immunoblotting to measure Vpu expression.
Identification of most conserved region in HIV-1 Vpu gene and its functional targeting using DNAzyme

HIV differs from many viruses in that it has a very high genetic variability. The error prone process of reverse transcription and rapid replication kinetics of HIV-1 genome make HIV a rapidly evolving virus difficult to be targeted by genetic therapies. We therefore used the information from genetic analysis to design DNAzyme effective against HIV-1 subtype B and C vpu gene variants targeting most conserved position (Figure 5.9).

![Dz target site](image)

Figure 5.9 Pictorial representation of predicted target site of 10-23 DNAenzyme against vpu gene. Identical residues are represented as dots.
As shown in figure 5.10 a region in vpu gene from position 144 to 184 (around 37 nucleotides) was identified as the most conserved region (~100%) in both groups of variants and therefore seems to be a hotspot for sequence specific genetic therapies. Interestingly this region of vpu transcript codes for the β-TrcP binding motif in Vpu protein. A DNAzyme was therefore designed targeting this common conserved region from evolving clinical isolates Vpu variants.

Since we choose overlapping target sequences, we speculated it to be effective against both subtype B and C vpu gene variants. In order to evaluate knockdown efficacy of the DNAzyme we used a direct flow cytometry based assay. HEK 293T cells were transfected with plasmids encoding either Vpu B-GFP or Vpu C-GFP fusion protein alone and in combination with the Dz. This coding sequence includes GFP fused with Vpu gene and therefore total cellular levels of Vpu can be directly measured by measuring GFP. Following expression period cells were suspended in cell dissociation buffer (Himedia) and GFP fluorescence was measured by flow cytometry using WinMdi program. As shown (Figure 5.11), the negative control, GFP alone expression (56%) was unaltered in the presence of Dz-94 (50% and 54% at two dose tested). However, the tested DNAzyme showed very impressive knockdown efficiency against both Vpu B (7.8 and 5.6% from 46% in control) and Vpu C (8.6 and 3.4% from 41% in control) GFP constructs.
Figure 5.10 Identification of most conserved region in vpu gene to be targeted by DNAzyme. A) and B) 100% conservation in nucleotide sequence of region identified. C) Secondary structure predictions of the target region as predicted by m-Fold software.
Figure 5.11 Knock down efficiency of Dz designed targeting against common conserved region of Vpu gene as measured by flow cytometry analysis using Vpu-GFP reporter system. A) HeLa cells transfected with plasmid encoding GFP (negative control) alone or in presence of indicated doses of Dz. B) HeLa cells transfected with plasmid encoding Vpu B-GFP fusion protein alone or in presence of indicated doses of Dz. C) HeLa cells transfected with plasmid encoding Vpu C-GFP fusion protein alone or in presence of indicated doses of Dz. Forty eight hour post transfection cells were harvested and analyzed for evaluation of relative proportion of GFP expressing cells. The percentage of GFP positive cells is indicated with histograms.
5.4 Discussion

Generation of variations is the driving evolutionary force which helps the virus to adapt itself to varying selection pressures such as antiretroviral therapies. In the course of genetic analysis we observed extreme heterogeneous nature of Vpu locus in HIV-1 genome displaying notable variations in sequence as well as length. The samples depending on relative resemblance to prototype Vpu from HIV sequence database (www.hiv.lanl.gov) were divided into two groups. Group B variants displayed higher degree of variations in all topological domains with some novel mutations with high allelic frequency as compared with group C variants which displayed less variation highlighting differences in rate and pattern of variation in group B and C HIV-1 Vpu. One remarkably noteworthy observation was the substitution of a phosphorylatable serine residue in cytoplasmic tail of Vpu from all of the group B variants. Incorporating such a mutation in HIV-1 pNL4-3 Vpu was reported earlier to boost viral replication rate as well as stability of Vpu protein (Estrabaud et al., 2007). We therefore verified whether natural S61A mutants possessing additional numerous variations (including transmembrane deletions) in cytoplasmic regions displayed greater kinetic stability. Results of Cycloheximide chase assay confirmed higher kinetic stability associated with S61 mutants. It is noteworthy that despite exhibiting additional numerous variations, S61A mutation in group B Vpu variants drastically enhanced intracellular expression and kinetic stability. Ubiquitination profile also suggested lesser intracellular poly-ubiquitinated species corresponding to Vpu S61 mutants than S61 wild type variants. Since, Vpu is known to act like a molecular motor that facilitates virus release as well as cause cell death it becomes 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 primary isolates in T cell line to assess the impact of Vpu variations on viral replication. Results of infection based studies in T cell line showed that despite exhibiting superior viral release activity as well as intracellular expression
levels, group B variants retained the moderate cell death potential associated with Vpu B. All group C variants as well as a β-TrCP motif mutant showed comparable viral release activity to group C. The viral release activity observed in Vpu variant possessing mutant β-Trcp binding motif (Vpu 24) may have resulted from sequestration of endogenous BST-2 function in agreement with previous reports of BST-2 degradation independent enhancement of virus release activity (Miyagi et al., 2009). The reduced viral release activity observed in S61mutant Vpu 7 on the other hand can be explained by transmembrane deletion that seriously affected viral release process via loss of two essential Alanine residues (A11 and A15) (Paul et al., 1998) in Vpu transmembrane domain. Furthermore, we were also able to show equal efficacy of designed Dzs against both Vpu B and C gene despite nucleotide differences in their primary sequences. It is important to develop antiviral approaches that work with equal efficiency against subtype C gene products, because this subtype is responsible for causing greater than 50% epidemic in the world (Rousseau et al., 2007).

In summary, apoptosis and viral release are two opposite and crucial determinants of viral fitness and pathogenesis and must be used 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. Selection of similar S61 mutation in group C variants may not have been feasible owing to much higher apoptotic function associated with C Vpu which might lead to early premature death of infected cells before viral release.