Chapter 5 Comparative functional study of Vpr gene derived from HIV-1 subtype B and C

HIV-1 Vpr is a 14 kDa viral regulatory protein implicated in several functions during HIV-1 infection. It is either expressed de novo as a late transcript (Schwartz et al., 1991) or it is found in the nucleus and packaged into the virions in association with Gag p6 (Tungaturthi et al., 2003). Various roles that this small protein has been attributed with include fidelity of the reverse transcription process, nuclear import of the PIC (pre integration complex), cell cycle progression and regulation of apoptosis and transactivation of the viral LTR promoter as well as those of host cell genes. The earliest studies involved strategies which used two synthetic peptides corresponding to the N and C terminal halves of Vpr (Morellet et al., 2003). While the N-terminal domain confers Vpr its cytopathic effect (Piller et al., 1999), the regulation of cell cycle, apoptosis has been attributed to its C-terminal domain (Roumier et al., 2002). Contradicting reports are also available (Srinivasan et al., 2008). One of the first functions attributed to Vpr was HIV-1 LTR transactivation (Cohen et al., 1990). Not only the HIV-1 LTR but Vpr is known to affect the expression of several cellular genes. Cis acting elements such as NF-kB, Sp1, C/EBP, GRE enhancer sequences have been implicated to play role in its transactivation abilities (Hogan et al., 2003; Wang et al., 1995; Vanitharani et al., 2001). The two leucine repeat regions in the first and third alpha helix have been reported to be associated with the transactivation properties of Vpr. Apart from its transcriptional activity, it has been conclusively reported to cause disruption of cell cycle, to up regulate virus replication and cause programmed cell death. There are several reports which suggest that there is increased transcriptional activity in cells arrested in G2 phase of the cell cycle. Several cellular binding partners of Vpr have been implicated in G2 arrest. These are extensively covered in a recent review by Andersen et al., in 2008). It causes
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G2 cell cycle arrest by inactivating the cdc25c phosphatase, thereby rendering cyclinB-cdc2 complex inactive resulting in G2 arrest (Roshal et al., 2003). Recently another mechanism has been proposed in which Vpr directs several of the proteins involved in cell cycle arrest and DNA repair for proteasomal degradation by ubiquitinylation of those proteins associated with Ubiquitin E3 ligase system (Angers S et al., 2006). G2 arrest and transactivation have been thought to be a related phenomenon. The level of HIV-1 LTR transcription increased by 4 folds when cells were arrested in the G2 phase of the cell cycle with HIV-1 Vpr (Goh et al., 1998). But contradictory reports are also available. It has also been reported that G2 arrest and transactivation properties are independent because Vpr mutants defective in inducing G2 arrest were able to activate the GR pathway (Sherman et al., 2000). The transactivation mediated by p300/CBP was shown to be independent of G2 arrest (Kino et al., 2002). Apoptosis is thought to be induced by Bax, which causes a release of Cytochrome C from the mitochondria. This activates the caspase 9 which in turn activates the effector caspase 3.

Most of these functions have been studied using VprB derived from HIV-1 subtype B but comparative functional data pertaining to VprC is lacking. HIV-1 subtype C is the major subtype prevalent in sub-saharan Africa, regions of Asia and the Indian subcontinent. It is the major subtype responsible for causing greater than 50% of the epidemic in the world (Rousseau et al., 2007 and references therein). Since there are considerable changes (ten amino acids changes, five of them being conservative and the remaining being non-conservative) in the amino acids sequence between prototype subtype B (pNL4-3) and Indian isolate of subtype C (clone 93IN905 and accession no. AF067158) Vpr protein (Figure 5-1), it is quite possible that the above mentioned functions could also show variations. In Figure 5-1, "*" represents identical amino acid, ":" represents that the amino acid has
changed from polar to non-polar or non-polar to polar group and ""."" represents that both the amino acid belong to the same group of amino acids (either polar or non-polar group).

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\begin{align*}
\text{Vpr-B} & \text{ MEQAPEDQGPQREPYNEWTLEELKEAVRHFPRIWHLHNLGQHYETYGDTWAGVEAI } 60 \\
\text{vpr-C} & \text{ MEQSPEDQGPQREPYNEWTLEELKQEAVRHFPRLHGLGQYIETYGDTWTGVEI } 60 \\
\text{***.**************.***********.***.**.*} \\
\text{Vpr-B} & \text{ IRLIQQLLFHFRIGCRHSRIGVTRRRARNGASRS } 96 \\
\text{Vpr-C} & \text{ IRLIQQLLFHFRIGCQHSRILRRARNGASRS } 96 \\
\text{*************.******.*************}
\end{align*}
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Figure 5-1: VprB and VprC protein sequence comparison

**Experimental Procedure**

**Plasmids and cells used**

HEK 293 and Hela-CCR5 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Plasmid DNA pNL4-3 and 93IN905 were obtained from the National Institutes of Health (Bethesda, Maryland, USA) AIDS Repository and grown according to standard procedures. Recombinant plasmid DNAs encoding the Vpr genes, were purified and transfected with Lipofectin reagent, as described in materials and methods.

**Cloning of Vpr gene of subtypes B and C of HIV-1**

Vpr gene was PCR amplified from pNL4-3 and 93In905 templates, for subtype B and subtype C, respectively using the primers described in materials and methods. It was cloned into pcDNA3 vector (Invitrogen) in Bam HI and Xho I restriction sites, under T7 and CMV promoter (Figure 4-2). This has been explained earlier in Chapter 4.
Transactivation of HIV-1 LTR by HIV-1 Vpr of both subtype B and C

HEK 293 cells were seeded in a 24 well plate, 24hrs prior to transfection. The cells were then co-transfected with 100 ng of pBS-LTR-B-Luciferase plasmid (henceforth referred to as pLTR-B – the Luciferase reporter gene is placed downstream of the HIV-1 LTR-B promoter) (obtained from AIDS Research and Reference Reagent Program of NIH, MD, USA) in the presence of either VprB or VprC encoding expression plasmid. Lipofectin (Invitrogen) was used as the transfection reagent to introduce the mixture of various DNA combinations and cell lysates were prepared 24 hours later using 1x reporter lysis buffer (Promega). The extent of Luciferase activity was determined according to the manufacturer’s instructions (Promega). Similar experiment was repeated to measure transactivation from LTR-C luciferase construct upon addition of either VprBpcDNA3 or VprCpcDNA3 constructs.

DNA laddering observed with VprB and VprC encoding plasmids

0.5 x 10^6 HEK 293 cells were seeded in each well of a six well plate and transfected with 1µg of either VprB or VprC encoding plasmid DNA in 1ml volume of DMEM. DNA was isolated 48 hrs after transfection, quantitated and equal quantity was loaded on a 1.5% agarose gel. Further, the gel was analysed by ethidium-bromide staining.

Vpr B or Vpr C mediated G2 Cell cycle arrest

To analyse cell cycle arrest induced by HIV-1 VprB and Vpr C genes, 1µg of pcDNA3 vectors encoding them were transfected for 48 hrs in a final volume of 1 ml DMEM media. For cell cycle analysis, transfected Hela CCR5 cells were harvested using Cell Dissociation Buffer (Himedia), incubated in 1% paraformaldehyde for 10 mins at room temperature, washed with 1X PBS and then fixed with chilled 70% ethanol. Subsequently, the fixed cells were washed and stained with DNA staining solution (10µg/ml propidium iodide
in 1X PBS). Stained cells were examined on a BD LSR flow cytometer and analysed by FLOWJO software. Propidium iodide stains the nucleic acid in the cell, so the cell cycle profile can be visualized as G1, S, and G2 phases. In Figure 5-4, the second peak represents the G2 phase and values at the upper left corner of the figure represent the ratio of G2 vs G1.

Results

Transactivation of HIV-1 LTR by HIV-1 Vpr of both subtype B and C
Cells transfected with HIV-1 LTRB alone as well as with plasmids encoding either Vpr gene of subtype B or C were lysed using the reporter lysis buffer and the lysate was analysed for the comparative luciferase activity. In the assay system, the extent of Luciferase activity directly correlated with the LTR promoter activity. It was observed that there is a dose-dependent increase in the luciferase activity of LTRB construct, when treated with a varying dose of VprB expressing plasmid (Figure 5-2, compare lanes 3, 4 and 5 with lane 2). Similarly a dose dependent increase in the luciferase activity was also observed with VprB-pcDNA3 with LTRC-luc plasmid (Figure 5-2 compare lanes 10, 11, 12 with lane 9). Transactivation of LTRB was observed with VprC-pcDNA3 (compare lanes 6, 7 and 8 with lane 2). Similarly, VprC-pcDNA3 resulted in enhanced transactivation from LTRC promoter as well (compare lanes 13, 14 and 15 with lane 9). The increase in transactivation due to VprC-pcDNA3 was modest in comparison with VprB-pcDNA3.
DNA laddering observed with VprB and VprC encoding plasmids

DNA was isolated from cells transfected with either VprB or VprC encoding plasmid and their ability to cause apoptosis was analyzed by observing the DNA laddering pattern by gel analyses. Figure 5-3 depicts the laddering pattern observed. It was found that 2μg of the plasmids showed DNA laddering and more pronounced laddering pattern was observed with VprC-pcDNA3 in comparison to VprB-pcDNA3 (Figure 5-3 compare lane 3 with lane 5). No laddering was observed with the same concentration of vector DNA (lane 1). Lanes 2 and 4 represent the DNA isolated from cells transfected with 1μg of the plasmids VprB-pcDNA3 and VprC-pcDNA3, respectively.
Reversal of cell cycle arrest by Dz-94

To compare VprB and VprC induced G2 cell cycle arrest, we performed cell cycle profiles of the Hela-CCR5 cells that were transfected with the same for 48 hrs. Thereafter, the cells were fixed and stained with propidium iodide and flow cytometry was used to assess the DNA content. The extent of G2 arrest was evaluated by the G2/G1 ratio (as indicated in top-left of each of the squares in Figure 5-4). The majority of untransfected cells or those transfected with the pcDNA vector only were at the G1 phase of the cell cycle (Figure 5-4, panels A and B). As expected, the DNA profile shifted from the G1 phase to the G2 phase in cells transfected with VprB and VprC encoding DNA, indicating cell cycle arrest (panels C and E). It is noteworthy that we found that VprC induced greater G2 cell cycle arrest which was evident from the G2/G1 ratio of 0.72 as compared to 0.45 with VprB. This G2 blockade was released upon transfection with Dz-94 (panels D and F).
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Discussion

From the above results it could be concluded that VprB is more powerful transactivator of LTR luciferase promoter than VprC from an Indian isolate in both homologous (LTRB luciferase + VprB) and heterologous system (LTRC luciferase + VprB). Enhanced LTRC promoter activity in presence of VprB could be of functional relevance in individuals who are coinfected with subtypes B and C. A potent dose-dependent response in the transactivation of HIV-1 LTR was observed with VprB, but not with the VprC-pcDNA3. This could imply that transactivation was probably a more prominent function for Vpr gene of subtype B but not for VprC. Although the functional implications of such a biased effect needs to be further investigated, it is evident that this would play a role in conferring subtype C with some fitness advantage over other subtypes.

DNA fragmentation causing abilities of Vpr from both the subtypes were also studied. DNA fragmentation is an indicator of apoptosis. VprC was more potent in promoting DNA damage as demonstrated by a more prominent
pattern of DNA ladder formation (Figure 5-3; compare lane 5 with 3). The 293 cells when transfected with same amount of empty vector pcDNA3 as expected failed to show any DNA fragmentation. Also, the capability of Vpr subtype B and C to arrest cells in G2 phase of the cell cycle was compared. It was found that VprC was slightly more effective in causing G2 cell cycle arrest. Although, we could not associate any reason for this phenomenon, this might confer greater fitness capabilities to the subtype C clade, as it is responsible for 50% of the epidemic worldwide. It has been earlier reported that cells arrested in G2 phase have enhanced transcription (Goh et al., 1998). Also, when co-transfected with the earlier identified Dz-94, the G2 peak was reduced to the same level as observed with either untreated control or with expression vector transfected controls.

In summary, we were able to show that VprB was more powerful in activating HIV-1 LTR(B and C) promoter but VprC (Indian isolate) was more potent in arresting cells in the G2 phase of the cell cycle and causing programmed cell death. These have important implications in modulating HIV-1 subtype specific pathogenesis. Also, the earlier identified Dz-94 was able to interfere with the ability of Vpr protein to cause cell cycle arrest.