Chapter Six
Mechanism of Vpx incorporation into virus particles
Vpx is a virion-associated protein, packaged into the virus particles in equimolar amounts to that of structural polyprotein \( \text{Pr55}^{\text{Gag}} \). In the virus particles Vpx seems to be localized within virus core and is incorporated efficiently in the progeny virions formed in the absence of \( \text{pol} \) and \( \text{env} \) gene products and is independent of viral RNA encapsidation. This indicates that expression of Gag precursor (Pr55Gag) is sufficient to mediate the incorporation of Vpx into virions. Studies have shown that Vpx is packaged into the virus particles by interacting with C-terminal p6 domain of Gag polyprotein. It was also shown that a leucine triplet (LXX)3 in p6 region is critical but not essential for Vpx incorporation as deletion of all the leucine repeats affects but does not abolish the incorporation of Vpx suggesting the involvement of other regions in Gag polyprotein. The exact mechanism involved in Vpx virion incorporation and also the amino acids that are required for virion incorporation of Vpx was not known. This part of the study was designed to understand the exact mechanism of Vpx virion incorporation by determining the following (a) the intracellular sites for Vpx/Gag interaction, (b) amino acids residues involved in Vpx/Gag interaction, and (c) the involvement of upstream regions in Gag polyprotein apart from C-terminal p6 domain.

### 6.1. Vpx is a virion associated protein

In order to understand the mechanisms (s) involved in virion incorporation of Vpx, we first checked for the presence of Vpx in purified virus particles. SIVsmPBj1.9 wild type and SIVsmPBj1.9 \( \Delta \text{vpx} \) (\( \Delta \text{Vpx} \), a mutant in which the initiating and the internal methionine codons were replaced with threonine and leucine residues and contained a stop codon at amino acid position 80) proviral DNAs were transfected into 293T cells as described in Materials and Methods. The culture supernatants were collected 48h after transfection, centrifuged over a 20% sucrose cushion, and the viral pellets were subjected to SDS-12% PAGE followed by Western blot analysis. Probing with anti-Vpx
Figure 6.1: Vpx is packaged into SIVsmPBj1.9 virions. A. 293 T cells were transfected with wild type and Δ Vpx proviral clones. Viral particles were concentrated from culture supernatants by ultracentrifugation through a 20% sucrose cushion. The viral pellets were solubilized in gel loading buffer and resolved on a SDS-12% PAGE followed by Western blot using anti-Vpx and anti-Gag monoclonal antibodies. (Δ Vpx is a control construct lacking functional vpx open reading frame). B. Mutations in helical domain affect Vpx virion incorporation. 293 T cells were transfected with wild type and mutant Vpx proviral clones. The culture supernatants were collected 48h after transfection and centrifuged over a 20% sucrose cushion to purify the virus particles. The viral pellets (normalized by p27 Gag content) were resolved on a SDS-12% PAGE followed by Western blot using anti-Vpx and anti-Gag monoclonal antibodies.
and anti-Gag monoclonal antibodies revealed the presence of Vpx-specific signal in the wild type SIVsmPBj1.9 (Fig. 6.1A, lane 1) whereas the absence of Vpx from SIVsmPBj1.9 ∆ vpx transfected samples (Fig. 6.1A, lane 2) suggests that Vpx is selectively packaged into virus particles.

**6.2. Mutations in the helical domains of Vpx affect its virion incorporation**

In order to identify the determinants that are required for Vpx virion incorporation, we introduced point mutations into the vpx gene of the infectious molecular clone SIVsmPBj1.9. All the conserved amino acid residues like serine, threonine and tyrosine, the targets for phosphorylation were selected for mutagenesis. SIVsm (PBj 1.9) proviruses containing the variants of vpx were transfected into 293-T cells, and the virion incorporation ability of Vpx mutant proteins was assessed. Forty-eight hours after transfection, the cell culture supernatants were collected and clarified to remove cell debris. The supernatants were layered on a 20% sucrose cushion and the virus particles were purified by ultracentrifugation. The viral pellets (normalized by p27 Gag content) were examined by Western blot analysis. Probing with anti-Vpx antibody revealed the absence of Vpx signal from mutants Y66,69,71A and L174,75S (Fig. 6.1B). Furthermore, probing with anti-Gag antibody indicated that mutations in Vpx did not alter the expression, maturation and virus particle release (Fig. 6.1B, p27 capsid).

Since Gag is known to form virus-like particles and is sufficient for packaging of Vpx, we have employed an assay where Gag and different mutants of Vpx were co-transfected into Cos-7 cells. Twelve hours after transfection the cells were labeled with $^{35}$S methionine and cysteine for 6h as described in Materials and Methods. The cells were harvested in ice cold PBS and the supernatants were collected separately. The supernatants were clarified to remove cell debris and then concentrated through a centricon 30. Vpx and Gag were immunoprecipitated from the cell lysates and supernatant
Figure 6.2: Mutations in helical domain II affect Vpx packaging into virus-like particles. Cos-7 cells were co-transfected with Gag and various Vpx mutant expression plasmids and 12h after transfection the cells were labeled for 6h with $^{35}$S methionine and cysteine. The cells were harvested and supernatant was collected separately. Cells were lysed in lysis buffer and the supernatants were concentrated in a centicon 30. Gag and Vpx were immunoprecipitated from cell lysates and supernatant by using anti-Gag and anti-Vpx monoclonal antibodies respectively. After immunoprecipitation the samples were resolved on a SDS-15% PAGE followed by autoradiography.
Figure 6.3: Substitutions in conserved tyrosines in helical domain III blocks Vpx packaging into virus like particles. Cos-7 cells were co-transfected with Gag and various Vpx mutant expression plasmids and 12h after transfection the cells were labeled for 6h with $^{35}$S methionine and cysteine. The cells were harvested and supernatant was collected separately. Cells were lysed in lysis buffer and the supernatants were concentrated in centricon 30. Gag and Vpx were immunoprecipitated from cell lysate and supernatant by using anti-Gag and anti-Vpx monoclonal antibodies respectively. After immunoprecipitation the samples were resolved on a SDS-15% PAGE followed by autoradiography.
by using respective monoclonal antibodies, and the samples were resolved on SDS-15% PAGE followed by autoradiography. The results from figures 6.2 and 6.3 indicate the absence of Vpx signal from W49, W49,53,56S, Y66,69,71A, Y66,69,71P and Y66,69,71S mutants (packaging panel) even though equal levels of expression was found in cell pellets (expression panel). Introduction of helical destabilizing proline residue in the helical domain I (E30P) did not abrogate Vpx incorporation into virions as well as virus-like particles (Fig. 6.1 and 6.2) suggesting that the N-terminal helix I may be dispensable for Vpx incorporation. This is also true for mutations in the C-terminal region (amino acids between 82-112) like H82S, GC86,87S, R100A and P103,106S were packaged efficiently like wild type both into virions and virus-like particles (Fig. 6.1 and 6.2). Interestingly, when we analyzed the individual tyrosine mutants such as Tyr-66 and Tyr-69, they retained wild type virion incorporation, whereas exchange of Tyr-71 alone or in combination with either Tyr-66 or Tyr-69 has severely reduced Vpx incorporation into virus particles (Fig. 6.1B and 6.3). Collectively, these results suggest that amino acid residues Trp-49, Tyr-71, Leu-74 and Ile-75 may be involved in interactions with Gag, thereby facilitating Vpx incorporation. Interestingly, all these amino acids are located within predicted alpha helix II and III, suggesting that integrity of helix regions is critical for Vpx virion incorporation.

6.3. Phosphorylation is not required for Vpx virion incorporation

We described in chapter 5 that cellular kinase MAPK/ERK-2 phosphorylates Vpx and regulates Vpx nuclear import and virus replication in non-dividing macrophages. In order to understand whether phosphorylation modulates Vpx virion incorporation, we tested the virion incorporation ability of non-phosphorylated Vpx proteins. Since Gag expression was shown to be to sufficient to mediate Vpx incorporation into virions, we co-transfected Vpx and Gag expression plasmids into Cos-7 cells. Transfected cells were treated with inhibitors of MAPK and tyrosine kinase pathway alone or in combination and
Figure 6.4: Phosphorylation of Vpx is not essential for incorporation into virus particles. Vpx and Gag expression plasmids were co-transfected into Cos-7 cells. Transfected cells were treated with inhibitors of MAPK pathways (hypericin and PD98059) and tyrosine kinases (PP2, genestein) alone or in combination and labeled with $^{32}$P orthophosphoric acid or $^{35}$S methionine and cysteine. Vpx efficiently incorporated into virus particles and is not altered by inhibitors of MAPK pathway or tyrosine kinases (panel A, lane 4 and 5). Whereas inhibitors of MAPK pathway blocked Vpx phosphorylation (panel B, lane 4) without altering its incorporation into virus-like particles (panel A, lane 4) indicating that phosphorylation is not required for Vpx incorporation into virus-like particles. Virus-like particle release (Pr55 Gag) is not affected by kinase inhibitors (panel C)
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labeled with $^{32}$P orthophosphoric acid and $^{35}$S methionine and cysteine for 6h. The supernatant was collected and concentrated in a centicon 30. Gag and Vpx proteins were immunoprecipitated with respective monoclonal antibodies and the samples were resolved on a SDS-15% PAGE followed by autoradiography. Results in figure 6.4 indicate that Vpx was efficiently incorporated into virus-like particles generated by Pr55$^{\text{Gag}}$ precursor and was not altered by either inhibitors of MAPK pathways or tyrosine kinase alone or in combination (Fig. 6.4; panel A, lanes 4, 5 and 6). Interestingly, inhibitors of MAPK pathway blocked Vpx phosphorylation (Fig. 6.4, panel B, lane 4) whereas tyrosine kinase inhibitor did not inhibit phosphorylation (Fig. 6.4, panel B, lane 5). Furthermore, we observed equal amount of virus-like particle release (Fig. 6.4, panel C) in the presence or absence of kinase inhibitors suggesting that inhibition of Vpx phosphorylation did not alter virus-like particle release. Together, these results suggest that phosphorylation is not required for Vpx incorporation into virus particles.

6.4 Vpx interacts with P55 Gag in the cytoplasm

Gag was shown to be a nucleo-cytoplasmic shuttling protein and is sufficient to mediate Vpx incorporation into virions, but the underlying mechanisms of Vpx and Gag interaction inside the cells remain elusive. We therefore analyzed the in vivo interaction between Vpx and Pr55 Gag by using indirect immunofluorescence assay. Results in figure 6.5 indicate that Vpx co-localizes with Gag precursor in the cytoplasm, this suggest that these two proteins interact in vivo. This interaction was specific, as no co-localization observed between green fluorescent protein and Gag (Fig. 6.7). Further, to study this issue in detail we analyzed the relationship between co-localization of Vpx with Gag and Vpx virion incorporation. Various Vpx mutants were co-expressed in combination with Flag-Pr55$^{\text{Gag}}$ in Vero cells and their localization was visualized by indirect immunofluorescence assay using anti-Flag polyclonal antibody (for Gag) and anti-Vpx monoclonal antibody. Mutants like
Figure 6.5: Vpx interacts with Pr55\textsuperscript{Gag} in the cytoplasm. vTF7-3 infected Vero cells were co-transfected with various Vpx mutant expression plasmids and Flag-Gag. Localization of Vpx and Gag proteins was detected by indirect immunofluorescence with an anti-Vpx monoclonal antibody and anti-Flag polyclonal antibody for Flag-Gag, followed by an goat anti-mouse Alexa fluor 488 conjugated secondary antibody (for Vpx) and goat anti-rabbit Alexa fluor 594 (for Flag-Gag). Green indicates localization of Vpx and red indicates localization of Gag. (Vpx S-A-serines at 2, 13, 52, 63, 65 changed to alanine and Vpx T-A threonines at 17, 28, 67 changed to alanine).
Figure 6.6: Conserved tyrosines and tryptophan residues are critical for Vpx co-localization with Gag in the cytoplasm. vTF7-3 infected Vero cells were co-transfected with various tyrosine and tryptophan Vpx mutant expression plasmids and Gag. Localization of Vpx and Gag proteins was detected by indirect immunofluorescence with an anti-Vpx monoclonal antibody and anti-Flag polyclonal antibody for Flag-Gag, followed by an goat anti-mouse Alexa fluor 488 conjugated secondary antibody (for Vpx) and goat anti-rabbit Alexa fluor 594 (for Flag-Gag). Green indicates localization of Vpx and red indicates localization of Gag.
Figure 6.7: Vpx interacts and co-localizes with Gag precursor p55 in the cytoplasm. A. Variants of Vpx expression plasmids were co-transfected with the Gag expression plasmid in vTF7-3 infected Vero cells. Localization of Vpx and Gag proteins was detected by indirect immunofluorescence with an anti-Vpx monoclonal antibody and anti-Flag polyclonal antibody for Flag-Gag, followed by an goat anti-mouse Alexa fluor 488 conjugated secondary antibody (for Vpx) and goat anti-rabbit Alexa fluor 594 (for Flag-Gag). Green indicates localization of Vpx and red indicates localization of Gag. GFP is used as a negative control to show the specificity of interaction. B. Vpx specifically interacts and co-localizes with SIV Gag in the cytoplasm (indicated by yellow, inset box). Vpx L74,I75S did not co-localize with Gag despite its localization in the cytoplasm (indicated by separate red and green, inset box).
W49S, W49,53,56S, Y71A, Y66,71A, Y6971A and Y66,69,71A did not co-localize with Gag despite retaining wild type nuclear localization (Fig. 6.6) and were not incorporated into virus like particles (Fig. 6.2 and 6.3). Interestingly, the Vpx mutants (H39L, H82S, G86C87S and P103106S) that are defective for nuclear import but co-localize with Gag in the cytoplasm (Fig. 6.5) and retained the ability to incorporate into virus-like particles like wild type protein (Fig. 6.2). Taken together, these results suggest that Vpx interacts with Gag in the cytoplasm and is translocated to the site of virus assembly for virion incorporation. On the other hand, substitution of Leu-74 and Ile-75 resulted in cytoplasmic localization of mutant protein but failed to co-localize with Gag (Fig. 6.7A and 6.7B) and subsequently did not incorporate into virus particles (Fig. 6.1B) suggesting that these residues are critical for Vpx and Gag interaction.

6.5. Vpx interacts with p27 capsid and p6 portion of Gag

Vpx is localized with core p27 inside the virus particles and previous studies suggest that C-terminus of Gag i.e. p6 portion of p15 play a critical role in Vpx incorporation into virus particles. Since Vpx localizes within viral core, we determined whether Vpx interacts with viral capsid protein. In order to understand this mechanism, we expressed and purified the full length, matrix, capsid and p15 proteins of Gag as fusion with GST (Fig. 6.8A and 6.8B) and analyzed Vpx interactions by GST-pull down assay, followed by Western Blotting anti-Vpx monoclonal antibody. Results in figure 6.9A indicate that Vpx interacts with p55 and p15 as expected. Interestingly, we observed an interaction between Vpx and capsid similar to full length Gag whereas no interaction was noticed with Gag matrix. These results suggest that Vpx interact with capsid p27 in addition to p15 and this interaction may play a critical role in Vpx packaging into virus particles and subsequent viral infectivity in macrophages. Further, to understand the relevance of Vpx interaction with p27 capsid, we have selected various Vpx mutants with
Figure 6.8: Construction and purification of full length, MA, CA and p15 of SIV Gag as GST fusion. A. Schematic representation of various GST-Gag constructs. B. Coomasie brilliant blue staining of purified GST, Gag p55, Gag-MA, CA, p15. GST fusion proteins were purified using GST-sepharose beads as described in Materials and Methods.
Figure 6.9: Vpx interacts with p27 CA and p15 portion of Gag. A. Cell lysate containing Vpx protein was mixed with sepharose beads bound with various GST-Gag proteins and incubated overnight. The bounded proteins were resolved on a SDS-12% PAGE followed by Western blot analysis using anti-Vpx monoclonal antibody. B. GST pull down assay using GST-Gag deletion constructs and various Vpx mutants followed by Western Blotting using anti-Vpx monoclonal antibody. Vpx mutants which did not package into virus-like particles or virus particles did not interact with p55, p27 and p15 in vitro.
different localization patterns and packaging abilities. We selected mutants with a) wild type localization pattern and incorporated into virus-like particles (E30P), b) mutants which are localized to the cytoplasm and packaged into the virus particles (H82S, P103,106S), c) mutants which localized to the nucleus and are not package into virus particles (W49S and Y66,69,71A) and d) mutant protein localized to the cytoplasm but not packaged into virus particles (L74,I75S). Interaction between Gag and Vpx mutants was analyzed by GST pull-down assay followed by Western blot using anti-Vpx monoclonal antibody. The results in figure 6.9 B indicate that mutants like E30P, Y66A, H82S, P103,106S that are packaged into the virus particles interact with both p27 and p15 (Fig. 6.9B lane 2, 5, 9 and 10). In contrast, mutants like W49S, W49,53,56S, Y71A, Y66,69,71A, L74I75S that are not packaged into virus particles did not show any interaction with either p27 or p15 (Fig. 6.9B lane 3, 4, 6 and 7). Together, these results suggest that Vpx interaction with p27 and p6 portion of p15 in Pr55Gag is critical for its efficient incorporation into virus particles.

6.6. Vpx is essential for efficient replication in macrophages

We next analyzed the ability of various vpx mutant proviruses to elicit a spreading infection in monocyte derived macaque macrophages. All vpx mutant viruses replicated efficiently and to high titers in CEMx174 cells (Fig. 6.10A). However, this was not the case in terminally differentiated macaque macrophage cultures. PBj1.9 mutants like Y66,69,71A and L74,I75S that failed to package Vpx proteins (Fig. 6.1B) were severely impaired in their ability to replicate in macrophages (Fig. 6.10B). Furthermore, failure to replicate in macrophages was also observed for mutants that were packaged into virus particles at near wild type quantities but showed an impairment in nuclear import (H82S, GC86,87S and P103,106S) (Fig. 6.1B and 6.10B). In three independent experiments PBj1.9 mutants with substitution in the carboxyl-terminal half of Vpx replicated poorly in macrophages, while the E30P
Figure 6.10: Vpx is critical for efficient infection in non-dividing macrophages. CEMx174 cells (A), and terminally differentiated macaque macrophages (B) are infected with indicated SIVsmPBj1.9 wild type and mutant proviruses equilibrated by p27 Gag (10ng of p27 Gag). Both wild type and mutant proviruses replicated well in dividing CEMx174 cells (A). In contrast Vpx mutant proviruses with defective Vpx nuclear transport (H82S, GC86,87S and P103,106S) and virion incorporation (Y66,69,71A, L74I75S) replicated poorly in non-dividing macrophages (B). Twenty one days after infection, macrophages were co-cultured with CEMx174 cells for 24 hours and the replication levels of Vpx mutant viruses were rescued indicating that Vpx is critical for virus replication in non-dividing macrophages.
mutation designed to disrupt the predicted N-terminal helix resulted in replication identical to that of wild type. Replication of all Vpx mutant viruses was rescued by CEMx174 co-cultivation with macrophages after 21 days (Fig. 6.10B). Collectively, these results suggest that presence of Vpx in the virus particles with ability to localize to the nucleus is critical for optimal virus replication in non-dividing target cells like macrophages.