Chapter Seven

Discussion
This is the first study to undertake the extensive mapping of the determinants that are required for Vpx nuclear localization and to define the interrelationship between phosphorylation, nuclear import and virion incorporation with relevance to viral replication. HIV-2/SIVsm Vpx has two distinct localization properties that direct it either to the nucleus (Pancio and Ratner, 1998; Mahalingam et al., 2001; Di marzio et al., 1995) or, in association with Gag, to budding virus particles at the plasma membrane (Henderson et al., 1988; Kappes et al., 1989; Accola et al., 1999; Wu et al., 1994). These distinct localization properties of Vpx may be mediated through different protein-protein interactions. In order to identify the determinants that are involved in nuclear import of Vpx, we introduced a number of point mutations into Vpx structural domains, which are highly conserved among divergent isolates of HIV-2 and SIV (Fig. 4.1D). Our results suggest that Vpx encompasses a novel determinant for nuclear localization and the nuclear import function of Vpx is critical for efficient virus replication in macrophage cultures. This was supported by a) exchange of serines and threonines impaired Vpx nuclear localization (Fig. 4.5B), b) a fragment of 20 amino acid residues (61-80) was found to be necessary and sufficient to mediate the import of Vpx as well as heterologous cytoplasmic proteins into the nucleus (Fig. 4.13) and c) mutations that are capable of affecting the ability of Vpx protein’s nuclear import did not support the virus replication in primary macaque macrophages (Fig. 4.14). Virion incorporation occurs late in the infection when de novo synthesized Vpx is re-routed to the plasma membrane for packaging into budding virions through its interaction with Gag precursor polyproteins. Hence, the nuclear localization of Vpx is likely relevant early in the infection when Vpx found in the virion helps to translocate the viral genome into the nuclear compartment.

In this study, we have used confocal microscopic analysis to first check the localization of wild type Vpx in situ. Such analysis demonstrated that wild
type Vpx protein is efficiently translocated to the nuclear membrane and to the nuclear interior (Fig. 4.3) even in the absence of other viral encoded proteins. Given the nuclear accumulation of the wild-type protein, we sought to characterize cellular localization of different Vpx variants and to map the domain responsible for the nuclear import of Vpx. A striking finding of these experiments was that mutations in several widely separated regions affected Vpx nuclear localization. Exchange of serines and threonines has resulted in cytoplasmic localization whereas tyrosine and tryptophan mutants retain wild type localization (Fig. 4.5, 4.7 and 4.8). One of the critical regions was the conserved C-terminal domain between amino acids 61-80 in which mutations resulted in complete loss of the nuclear localization of Vpx (Fig. 4.6). Furthermore, C-terminal domain containing amino acids 61-80 of Vpx targeted β-Gal, a cytoplasmic protein into the nucleus indicating that Vpx has a novel nuclear transferable localization signal. The 20 amino acid minimal nuclear targeting signal identified overlaps with the helical domain III (amino acids 64-82) and is conserved among divergent isolates of HIV-2/SIVsm, such conservation of this motif and its sequence is highly indicative of the domain being important for Vpx function. Nuclear import of proteins in general, is a multi-step process where proteins are first targeted to nuclear pore complex (NPC) and subsequently translocated to the nuclear interior. This appears to be the case for Vpx, which is targeted to the nuclear membrane and subsequently imported into nuclear interior as observed by its localization patterns in this study. Similar results were also reported for HIV-1 Vpr (Jenknis et al., 1998; de Noronha et al., 2001). Though the minimal targeting signal is located between amino acids 61-80, the substitution of histidine 82 with serine has impaired nuclear import of Vpx suggesting that the integrity of putative helix III (amino acids 64-82) is important for efficient transport of Vpx into the nucleus. Helical domains are known to be involved in protein-protein interactions, protein-nucleic acid interactions (Saier and McCaldon, 1988; Subbramanian et al., 1998, Tacke et al., 1993). Interestingly, alteration of
helical domain I in HIV-1 Vpr blocked its nuclear import (Vodicka et al., 1998; Mahalingam et al., 1997; Sherman et al., 2001) suggest the critical role for helical domains in protein nuclear transport.

Recently, Pancio and co-workers reported that deletion of the C-terminal proline-rich domain (amino acids 102-112) in Vpx resulted in a block of nuclear localization of HIV-2 DNA, thus implicating that this domain is essential in the nuclear import of the HIV-2 PIC (Pancio and Ratner, 1998). The 20 amino acid nuclear targeting domain identified in this study lies upstream of the proline-rich region raising the possibility that like HIV-1 Vpr, Vpx may contain two independent nuclear targeting domains (Jenkins et al., 1998; Kamata and Aida, 2000). Alternatively the deletion of proline-rich domain may have impaired Vpx function by changing structural conformation of the protein. To distinguish between these two possibilities, we fused the C-terminal proline-rich domain (PGPPPPPPPPGLA) of Vpx to β-Gal and tested whether this proline-rich domain can mediate the nuclear import of a heterologous protein. Our results clearly indicate that C-terminal proline-rich domain is not absolutely required for nuclear import of Vpx (Fig. 4.11). This was further supported by confocal microscopic analysis of chimeric protein β-Gal/Vpx 81-112, containing the proline-rich domain of Vpx localized only in the cytoplasm whereas β-Gal/Vpx 1-80 localized to the nucleus (Fig. 4.12). These results suggest that proline-rich domain is not required for Vpx nuclear import and the minimal nuclear localization signal (NLS) identified in this study is necessary and sufficient to translocate Vpx to the nucleus.

We next assessed the role of Vpx nuclear import on replication potential of SIVsmPBj1.9 proviruses in dividing and non-dividing cells. Viruses that encoded the nuclear import defective Vpx protein failed to replicate or grew poorly in macaque macrophage cultures. For example, H82S and P103, 106S mutations impaired Vpx nuclear import and abrogated mutant protein’s ability
to support virus replication in primary macaque macrophages. The reduced efficiency of Vpx mutant virus replication in macrophages even in the presence of other NLS containing viral proteins like Gag matrix and integrase supports the notion that nuclear import of Vpx is critical for the efficient replication of HIV-2/SIV in macrophages. This is in agreement with current consensus that suggests Vpx is the major nucleophilic determinant coded by HIV-2/SIV (Pancio et al., 2000; Fletcher et al., 1996).

Nuclear transport of NLS containing proteins is regulated by multiple mechanisms of which phosphorylation is the major regulatory mechanism. Phosphorylation plays a critical role in NLS mediated nuclear transport, cell cycle progression and gene expression (Fridell et al., 1997; Jans and Hubner, 1996; Peterson and Schreiber, 1999; Schakney and Shankley, 1999). Phosphorylation-regulated NLS were found to control nuclear transport in eukaryotic cells from yeast and plants to higher animals. Recent studies have demonstrated that serine-threonine kinases like MAPK/ERK-2 and cAMP dependent protein kinase A (PKA) of the host cell are incorporated within HIV-1 particles (Camaur et al., 1997; Gallay et al., 1995; Luo et al., 1997; Paul and Jabbar, 1997; Jacque et al., 1998) and regulate early steps in the viral life cycle. Having demonstrated that Vpx is the critical determinant for HIV-2/SIV replication in non-dividing cells, we studied whether nuclear transport of Vpx is regulated by phosphorylation. To this end, we first determined the presence of cellular MAPK/ERK-2 in the SIV particles and the phosphorylation status of Vpx. Our results suggest that Vpx is phosphorylated by virion associated MAPK/ERK-2 both in vitro and in vivo (Fig. 5.1 and 5.2). Recent reports suggested that hypericin efficiently inhibits MAPK activity (Jacque et al., 1998). We observed inhibition of Vpx phosphorylation when Jurkat cells were treated with hypericin suggesting that Vpx is a substrate for MAPK/ERK-2 (Fig. 5.3A). Furthermore, inhibition of MAPK/ERK-2 activity by hypericin was specific as there was no effect on the activity of JNK (serine-threonine kinase), in the
presence of hypericin (Fig. 5.3B). Exchange of potential phosphorylation residues like serines and threonines in Vpx has resulted in cytoplasmic localization of mutant proteins suggesting that phosphorylation may regulate Vpx nuclear transport. To study this issue in detail, we have analyzed the presence of Vpx protein in different cellular compartments in the presence or absence of MAPK inhibitor, hypericin. Interestingly, we observed accumulation of Vpx in the nuclear fraction in the absence of hypericin whereas more Vpx protein was found in cytoplasm when transfected cells were treated with hypericin (Fig. 5.4A). This result was further supported by cytoplasmic localization of Vpx in the presence of inhibitors, hypericin or PD98059 (MEK inhibitor) (Fig.5.B). Collectively, these results suggest that MAPK/ERK2 mediated phosphorylation regulates Vpx nuclear transport. We also found that impairment of host cell MAPK/ERK-2 activity by hypericin resulted in the production of virions with reduced infectivity as assessed by infection assays performed in macaque macrophages (Fig. 5.9). Western blotting analysis of virion protein contents from cells exposed to MAPK inhibitor, hypericin revealed that it had no apparent effect on virus production or particle release from the infected cells (Fig. 5.8). These results suggest that the defects in viral infectivity may be due to the inhibition of virus-associated MAPK/ERK-2 activity, which may be critical for Vpx-mediated nuclear translocation of viral genome.

Our studies indicate that host cell MAPK/ERK-2 is selectively incorporated into virus particles and regulates SIV infectivity by modulating Vpx phosphorylation. It is likely that the MAPK inhibitor blocks SIV infection by interfering with the nuclear import of Vpx, thereby restricting subsequent virus replication in macrophages. MAPK/ERK-2 is a proline directed kinase (Davis, 1993) but there are no consensus MAPK/ERK-2 sites within Vpx. Despite this, immunoprecipitates of MAPK/ERK-2 from cell lysates as well as recombinant kinase were able to phosphorylate Vpx. MAPK/ERK-2 phosphorylation of a
protein, which lacks MAPK consensus recognition sites, has been reported recently (Corbalan-garcia et al., 1996) for both viral and cellular proteins and suggested that conformation of the substrate protein was sufficient to allow recognition by MAPK. For example, Gag matrix protein of HIV-1, which lacks MAPK consensus recognition motifs was phosphorylated by MAPK/ERK-2 and regulates the transport of viral DNA to the target cell nucleus (Jacque et al., 1998). We also assessed the role of Vpx phosphorylation and nuclear transport on the replication potential of vpx mutant SIVsmPBj1.9 in non-dividing cells. Viruses that encoded phosphorylation and nuclear import defective Vpx mutant protein failed to replicate or grew poorly in macaque macrophage cultures despite wild type packaging ability suggesting that phosphorylation of Vpx is required for efficient nuclear import of viral genome and subsequent replication in non-dividing cells like macrophages. Together our data demonstrate that presence of Vpx in the virus particles with ability to transport into the nucleus is critical for efficient virus replication in macrophages.

Vpx is incorporated into the virus particles by interacting with p6 portion of Gag (Kappes et al., 1993; Wu et al., 1994; Pancio and Ratner, 1998) and is present in molar amounts equivalent to that structural protein Gag. A leucine triplet motif in the N-terminal region of p6 was shown to be critical for virion incorporation of Vpx (Selig et al., 1998). Our results indicate that the integrity of helical domains II and III is critical for efficient virion incorporation, as supported by the mutations within these domains (W49S, W49,53,56S, Y71A and Y66,69,71A) has resulted in complete abrogation of Vpx packaging into virus particles as well as virus-like particles (Fig. 6.1, 6.2 and 6.3). To further understand the mechanism of Vpx and Gag interaction, we analyzed the in vivo interaction between Gag and Vpx by using indirect immunofluorescence assay. Vpx when transfected alone localized to the nucleus but in combination with Gag it is localized in the cytoplasm (Fig. 6.6), this provides evidence that
these two proteins interact in vivo. In order to understand the relation between Vpx incorporation into virus particles and its localization with Gag inside the living cells, we co-expressed various mutants of Vpx with Gag and studied their localization. Comparison of results from immunofluorescence and packaging indicate that Vpx interaction with Gag in the cytoplasm is critical for the incorporation of Vpx into virions. Vpx mutants H82S, GC8687S and P103,106S which are co-localized with Gag in the cytoplasm are incorporated into virions (Fig. 6.2 and 6.5) whereas mutants like W49S, W49,53,56S and Y66,69,71A which retained nuclear localization but did not co-localize with Gag in the cytoplasm are not incorporated into virions. These results suggest that nuclear transport property of Vpx is not required for its incorporation into virus particles. Interestingly, W49S and Y66,69,71A mutants failed to package but retained wild type nuclear localization, suggesting that Vpx may be exported to the cytoplasm and export property of Vpx may be required for its availability in the cytoplasm for subsequent incorporation into virus particles like HIV-1 Vpr (Sherman et al., 2001; Jenkins et al., 2001; Subbramanian et al., 1998. Collectively, our data suggest that Vpx interaction with structural protein Gag in the cytoplasm is critical for its incorporation into budding virus particles to support efficient virus replication.

Recent reports suggest that efficient uncoating of viral core is essential for efficient viral genome nuclear transport and replication in non-dividing target cells (Yamashita and Emerman, 2004; Forshey et al., 2002). Since Vpx is found inside the virion core (Kewalramani and Emerman 1996), we asked a question whether Vpx interacts with core capsid p27 in addition to its interaction with p15 to support the uncoating process. In order to understand the Vpx interaction with Gag, we expressed and purified the full length, matrix, capsid and p15 proteins of Gag as fusion with GST and analyzed Vpx interactions by GST-pull down assay, followed by Western blot using Vpx monoclonal antibody. Our results suggest that Vpx interacts with p27 capsid
in addition to p15 portion of Pr55Gag. In contrast, no interaction was observed
with Gag matrix. These results are in accordance with previous studies where
it was shown that SIVmac Gag lacking matrix p17 can still interact with Vpx in
a yeast two hybrid assay (Selig et al., 1998). These data suggest that Vpx
interact with core capsid p27 in addition to p15 and this interaction may play a
important role in Vpx packaging into virus particles. It is reasonable to
hypothesize that the observed interaction of Vpx with p27 CA may be critical
for efficient uncoating of viral core in non-dividing cells like macrophages
which may be required for optimal replication of HIV-2/SIV in macrophages.