Structure and Function of HIV-1 Vpu

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Introduction

Aside from the typical retroviral *gag*, *pol*, and *env* genes, HIV encodes a series of accessory genes, *vif*, *vpr*, *vpx*, *vpu*, and *nef*. The *vpu* gene is found exclusively in HIV-1 (Strebel et al., 1988; Cohen et al, 1988; Matsuda, 1988). In tissue culture systems, defects in accessory genes are frequently not correlated with a detectable impairment of virus replication and, as a result, these genes are often referred to as "non-essential". However, as more and more information on the function of the accessory proteins becomes available it also becomes increasingly clear that *in vivo*, these proteins indeed exert important functions. With respect to Vpu, Li et al found that macaques infected with Vpu-negative simian-human immunodeficiency virus chimeras (SHIV) had lower virus loads than Vpu-positive virus (Li et al., 1995). Also, using a SCID-hu model, Aldrovandi & Zack demonstrated that deletion of Vpu significantly affects virus infectivity and, to a somewhat lesser extend, pathogenicity of HIV (Aldrovandi & Zack, 1996). Thus, while there is currently only limited information available on the importance of Vpu *in vivo*, such model systems might be useful in investigating the *in vivo* relevance of Vpu. This article attempts to provide a brief overview on our current understanding of this viral accessory factor. More exhaustive reviews on Vpu can be found elsewhere (Jabbar, 1995; Bour et al., 1995a; Trono, 1995).

Structural Considerations

Vpu is a small integral transmembrane protein which is cotranslationally inserted into membranes of infected cells (Strebel et al., 1989). The protein consists of an N-terminal hydrophobic domain, which functions both as signal peptide and membrane anchor, and a hydrophilic C-terminal domain which protrudes into the cytoplasm. Vpu contains two highly conserved seryl residues, located in the cytoplasmic domain, which are phosphorylated by the ubiquitous protein kinase CK-2 (Schubert, et al., 1992; Schubert et al., 1994; Friborg et al., 1995). Phosphorylation is essential for at least one of the biological functions of Vpu as discussed below. Vpu forms homo-oligomeric structures (Maldarelli et al., 1993), a feature that may be critical for a proposed ion channel function of Vpu (see below). Based on 2D 1H NMR spectroscopy of a peptide corresponding to the cytoplasmic domain of Vpu (Wray et al., 1995; Federau et al., 1996), it was proposed that the cytoplasmic domain of Vpu contains two α-helical domains, helix-1 and helix-2 (Figure 1), which are connected by an unstructured region containing the two conserved phosphoseryl residues. In addition, computer models predict a third α-helical domain in the transmembrane domain of Vpu, which could play an important role in the formation of ion channels (Schubert et al., 1996b).

The *vpu* gene overlaps at its $3'$-end with the *env* gene (Figure 2). Indeed, Vpu and Env are expressed from the same bicistronic mRNA in a Rev-dependent manner (Schwartz et al., 1990), presumably by leaky scanning of ribosomes through the *vpu* initiation codon. In tissue culture, this arrangement results in the synthesis of roughly equimolar levels of Vpu and Env proteins and it is possible that this unusual utilization of viral transcripts might reflect a requirement for the coordinate action of the two viral gene products. Several HIV-1 isolates were found to carry point mutations in the Vpu translation initiation codon but have otherwise intact *vpu* genes. Since removal of the Vpu initiation codon results in increased expression of the downstream *env* gene, it is possible that HIV-1 actually uses this mechanism as a molecular switch to regulate the relative expression of Vpu or Env in infected cells. The possible benefits of such a regulation are unclear; however, it is conceivable that, under certain circumstances, *vpu*-defective isolates expressing increased levels of Env protein have a selective advantage over "wild-type" viruses expressing Vpu.

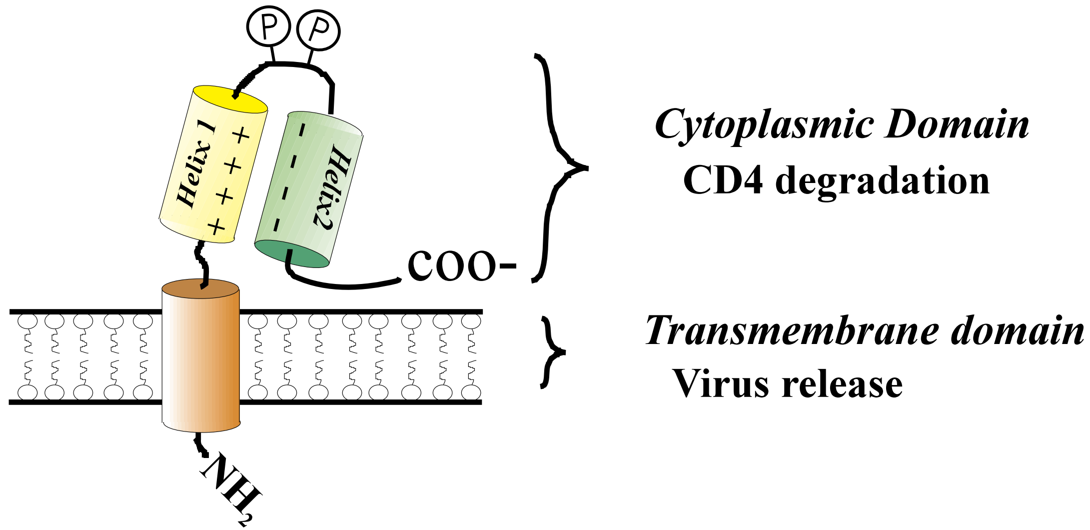

Figure 1. Structural domains of Vpu. Vpu consists of an N-terminal hydrophobic domain, that functions as membrane anchor, and a hydrophilic cytoplasmic domain. The cytoplasmic domain contains two amphipathic α-helical domains of opposite polarity. They are separated by an unstructured region containing two conserved seryl residues which are phosphorylated by protein kinase CK-2. The cytoplasmic domain contains seqences critical for CD4 degradation while the membrane anchor domain has a critical function in regulating virus release and plays an important role in the formation of cation selective ion channels. Vpu forms homo-oligomeric complexes. Only the monomeric form is shown. A putative interaction between helix 1 and helix-2 as shown in the cartoon suggests only one of many possible conformations of Vpu.

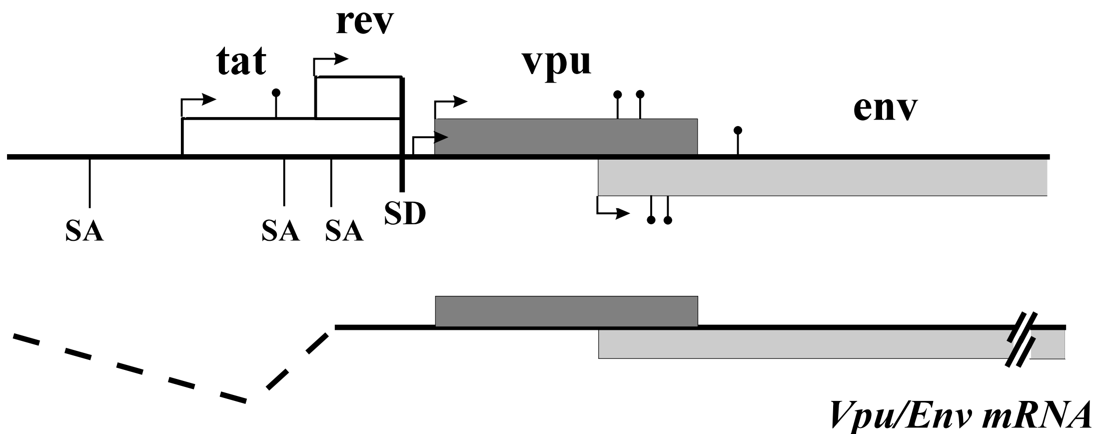

Figure 2. Structure of the *in vpu* gene. The *vpu* gene overlaps at its 3′-end with the *env* gene. Both Vpu and Env are expressed from the same bicistronic mRNA at roughly equimolar levels. The positions of AUG codons at the beginning of open reading frames are marked by arrows. Internal AUG codons are denoted by filled circles. SA = splice acceptor sites; SD = splice donor site.

Vpu has two primary biological activities which are discussed in detail below and are summarized in Figure 3. These include the degradation of CD4 in the endoplasmic reticulum and the augmentation of virus secretion from the plasma membrane. In addition, expression of Vpu has been associated with a reduction in syncytia formation of infected cells (Strebel et al., 1989; Terwilliger et al., 1989; Klimkait et al., 1990; Yao et al., 1993; Schubert et al., 1994). This latter phenomenon may be a consequence of the reduced presence of viral Env protein at the cell surface (Yao et al., 1993) due to the more efficient shedding of viral particles in the presence of Vpu. Aside from that, several other, less well defined functions have been associated with Vpu activity. These include the regulation of ER-to-Golgi transport of proteins (Vincent & Jabbar, 1995), or the modulation of MHC I antigen complexes (Kerkau, 1995).

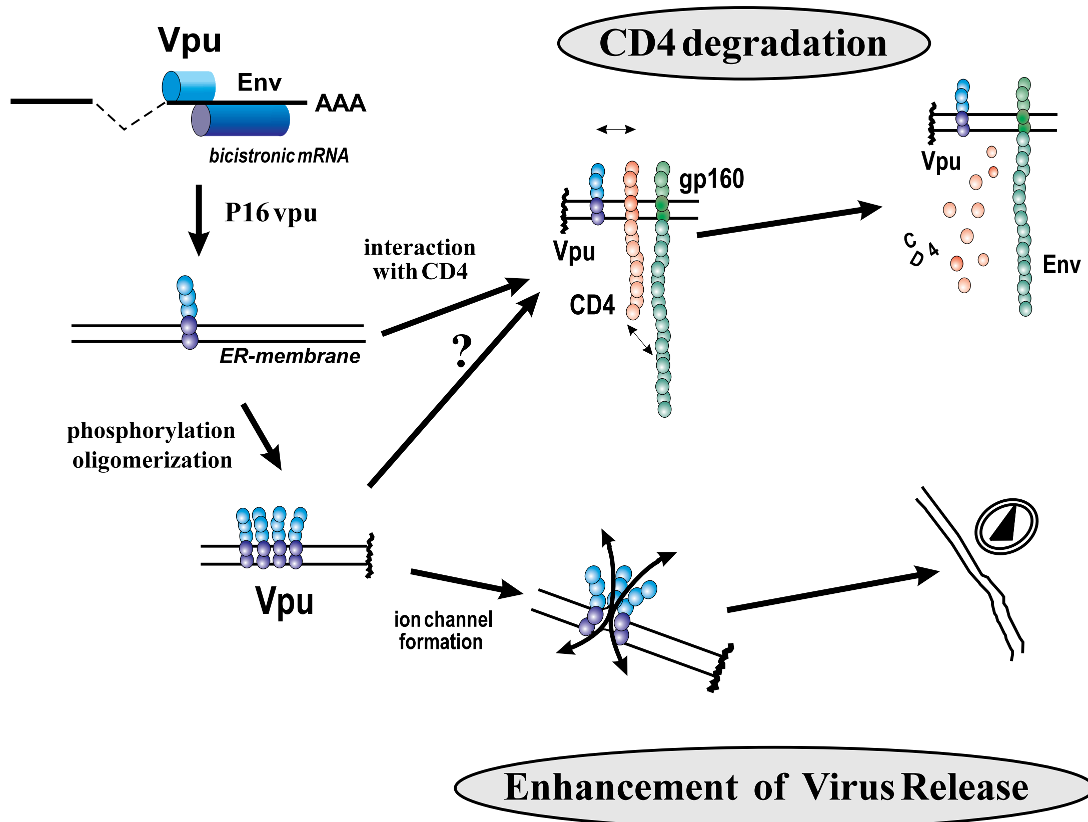

Figure 3. Vpu enhances virus particle secretion and induces degradation of CD4. The 81 amino acid Vpu protein (P16vpu) is synthesized from a bicistronic mRNA and cotranslationally inserted into membranes of the endoplasmic reticulum. Vpu is phosphorylated by CK-2 and forms homo- oligomeric structures. Newly synthesized CD4 is retained in the ER due to the formation of stable complexes with Env protein. Vpu physically associates with CD4 thereby triggering a mechanism that results in the destruction of CD4 and in the release of Env protein from the ER blockage. It is unclear whether this function of Vpu involves monomeric or oligomeric Vpu (? in the cartoon). Vpu exits the ER and accumulates at or near the Golgi. In cells overexpressing Vpu, small amounts of Vpu reach the plasma membrane. Oligomeric forms of Vpu have the capacity to form ion channels which presumably are involved in the regulation of virus particle release.

Vpu Facilitates the Release of Virus Particles

The original biological phenotype associated with viruses lacking a functional *vpu* gene was the impairment of virus particle secretion from infected cells (Strebel et al., 1988; Terwilliger et al.,

1989). This defect is manifested by the increased budding of viruses from internal membranes and the accumulation of budding particles at the cell surface where they remain loosely attached to the plasma membrane (Klimkait et al., 1990). Such particles can be released however, by vigorously shaking the cultures, and they are fully infectious (Klimkait et al., 1990). This suggests that Vpu regulates one of the final steps in virus production that is required for the efficient detachment of virions from the plasma membrane. Although it has been generally accepted that Vpu augments virus secretion from a variety of human cells, including PBMC, macrophages or CD4+ T cell lines, as well as non-T cell lines such as epithelial HeLa cells or SW480 cells (Strebel et al., 1988; Terwilliger et al., 1989; Klimkait et al., 1990; Westervelt et al., 1992; Yao et al., 1992; Göttlinger et al., 1993; Balliet et al., 1994; Kawamura et al., 1994; Schubert et al., 1994; Schubert et al., 1995; Sakai et al., 1995; Theodore et al., 1996), the enhancing effect of Vpu varies between different cell types and has been reported to range from a mere 2-to 3-fold in PBLs to up to 1000 fold in primary macrophages. Despite these cell type specific variations, the principal function of Vpu is not restricted to certain human cell types. In contrast, Geraghty et al. and Göttlinger et al. who analyzed the function of Vpu in Cos-1, Cos-7, or CV1 cells, were unable to detect any Vpu-mediated enhancement of virus secretion in simian cell lines (Geraghty et al., 1994; Göttlinger et al., 1991). It is possible that the efficient and Vpu-independent release of viruses from Cos cells is due to the expression of a cellular eqivalent to Vpu in simian cells.

The mechanistic details of Vpu activity are not well understood; however, several models can be proposed that are consistent with existing experimental data. First, Vpu could actively facilitate detachment of progeny virions from the cell surface, for example by altering the characteristics of the plasma membrane or by actively facilitating membrane fusion similar to the function exerted by annexin VI, which is required for budding of clathrin-coated pits during endocytosis (Lin et al., 1992). At least in the latter case, Vpu would be required in significant amounts at the cell surface. However, while Vpu was indeed observed at the plasma membrane of cells when overexpressed, the bulk of the protein is clearly sequestered on internal membranes (Maldarelli, personal communication; Schubert et al., 1996a). In addition, attempts to demonstrate the presence of Vpu in virions have thus far failed, also arguing against the presence of significant amounts of Vpu at the cell surface. Alternatively, Vpu could regulate virus release from internal membranes either by passively preventing budding on internal membranes, thus redirecting or restricting virus assembly to the cell surface, or by indirectly affecting particle release through the modulation of so far unidentified cellular factors.

Recent evidence suggests that Vpu has the ability to form cation selective ion channels (Ewart et al., 1996; Schubert et al., 1996b); there is some evidence that this activity of Vpu correlates with its ability to regulate virus release. The ability of Vpu to form ion channels requires the integrity of the TM domain, and alterations in the primary structure of the TM domain not only abolish the ability of Vpu to form ion conductive pores but also negate the capacity to regulate virus release (Schubert, 1996a/b). While these findings imply that regulation of virus release is mediated through an ion channel activity of Vpu, it remains to be shown how an ion channel activty of Vpu could affect the detachment of budding particles from the plasma membrane or how it could redirect virus budding from internal membranes to the plasma membrane.

Vpu Induces CD4 Degradation

A second function which has been extensively investigated is the ability of Vpu to induce degradation of CD4 at the endoplasmic reticulum (ER). One of the complications that HIV faces when replicating in CD4+ cells is the formation of stable complexes between cellular CD4 and the HIV Env protein. Such complexes are trapped in the ER (Bour et al., 1991; Crise et al., 1990; Kawamura et al., 1989; Jabbar & Nayak, 1990; Willey et al., 1992a/b) thereby preventing transport of both CD4 and Env to the cell surface. In the presence of Vpu, however, Env was found to be liberated from CD4/Env complexes (Willey et al., 1992a; Kimura et al., 1994) concomitant with a significant reduction in the detectable steady state levels of CD4. The reduction of CD4 was caused by Vpu-induced degradation which reduced the half-life of CD4 in Hela cells from normally 4–6 hours to approximately 10 minutes (Willey et al., 1992b). Efficient degradation of CD4 requires its retention in the ER. This is normally accomplished by the formation of stable complexes with HIV Env. However, Env protein per se is

not involved in CD4 degradation since it is not required when CD4 is artificially retained in the ER (Willey, 1992b). Also, CD4 degradation can be observed in an *in vitro* translation system where no viral proteins other than Vpu are required (Chen et al., 1993). Unlike Nef, Vpu is unable to target cell surface CD4, or, for that matter, CD4 that has exited the ER. Nevertheless, the rapid degradation of de novo synthesized CD4 in the presence of Vpu ultimately leads to a depletion of cell surface CD4 due to the reduction of the overall cellular CD4 pool.

Based on mutational analysis of CD4, it is known that deletion of the cytoplasmic domain of CD4 renders the protein insensitive to Vpu, suggesting that this domain of CD4 contains Vpu-responsive sequences (Chen et al., 1993; Lenburg & Landau, 1993; Vincent et al., 1993). This is supported by the fact that transfer of CD4 cytoplasmic sequences to CD8, a cellular receptor molecule that is not normally targeted by Vpu, resulted in Vpu-dependent degradation of the chimeric molecules (Willey et al., 1994). In fact, transfer of a 18 amino acid membrane proximal fragment of the CD4 cytoplasmic domain (amino acids 403 to 420) into the CD8 cytoplasmic tail was sufficient to confer Vpu sensitivity. While these results suggest that cytoplasmic sequences of CD4 are sufficient to confer Vpu sensitivity to heterologous membrane proteins, other studies suggest that sequences located in the TM domain of CD4 may also be required (Raja et al., 1994; Buonocore et al., 1994). It is possible that structural constraints imposed by the extracellular or TM domains of the model proteins employed in the different studies explain the apparently differing results. While the importance of the CD4 TM domain for this process thus remains unclear, the importance of the CD4 cytoplasmic domain is undisputed. It is now clear that this domain of CD4, which is predicted to form an α-helical structure (Yao et al., 1995), is involved in the physical interaction with the cytoplasmic domain of Vpu (Bour et al., 1995b; Schubert et al., 1996a; Margottin et al., 1996). Such interaction between CD4 and Vpu appears to be a prerequisite for CD4 degradation but is in itself not sufficient to trigger CD4 degradation. This is evidenced by the fact that mutants of Vpu that are unable to induce CD4 degradation are still able to bind to CD4 (Bour et al., 1995b).

Mutational analysis of Vpu demonstrated that phosphorylation of Vpu at two conserved seryl residues, Ser52 and Ser56, in its cytoplasmic domain is essential for its ability to induce CD4 degradation (Schubert, 1994; Friborg et al., 1995) but not for its ability to bind to CD4 (Bour et al., 1995b). The Vpu TM domain is not required for the interaction with CD4 (Margottin et al., 1996) and alterations in the Vpu TM domain have no apparent effect on CD4 degradation, provided the protein retains its ability to properly associate with membranes (Schubert et al., 1996a; Friborg et al., 1994). These results suggest that sequences critical for the induction of CD4 degradation are located in the Vpu cytoplasmic domain while the TM domain merely serves as a membrane anchor. How Vpu triggers degradation of CD4 is still unclear. It is conceivable that binding of Vpu to CD4 induces a conformational change in CD4 which in turn activates a cellular pathway designed to eliminate aberrantly folded proteins. However, the fact that CD4-Vpu interaction is necessary but not sufficient to induce CD4 degradation indicates that Vpu performs a catalytic function beyond the binding step.

Functional domains of Vpu and Vpu-like activities in HIV-2

In light of the multiple biological activities provided by the Vpu protein during the HIV-1 life cycle, it is intriguing that, except for the chimpanzee isolate SIVcpz (Huet, et al, 1990), no functional equivalent to Vpu is found in related viruses such as HIV-2 and SIV. This fact seems especially paradoxical since Vpu was shown to augment the release of chimeric viruses bearing the gag-pol regions of retroviruses that naturally lack a *vpu* ORF, such as HIV-2, visna virus and Moloney murine leukemia virus (Göttlinger et al., 1993). By examining the efficiency of particle release and Vpu-responsiveness of the ROD10 full-length molecular clone of HIV-2, we recently showed that mutations that disrupt the pROD10 env ORF, but not the *vif*, *vpr*, *vpx* or *nef* ORFs, have a profound negative effect on virus particle release (Bour et al., 1996a). Concomitantly, the pROD10 envelope glycoprotein provided in *trans* could rescue the envelope mutants and restore wild-type levels of particle release (Bour et al., 1996a). Similar results were independently reported by Ritter et al. who used a different HIV-2 isolate, HIV-2/ST (Ritter et al., 1996). The efficiency with which the ROD10 Env protein enhanced HIV-2 particle release was very similar to that of Vpu. Both activities could be provided in *trans* and were sensitive to treatment

of cells with Brefeldin A, suggesting that they both operate in a post-ER compartment (Bour et al., 1996a). Like Vpu, HIV-2 Env can regulate release not only of HIV-2 but of HIV-1 or SIV particles as well (Bour et al, 1996b). When compared in parallel, the effects of Vpu or ROD10 Env on HIV-1 or SIV virus release were identical. These findings suggest that the HIV-2 Env protein has a true Vpu-like activity with respect to virus particle release. However, in contrast to Vpu, there is no evidence that HIV-2 Env can induce degradation of CD4 (Bour et al., 1996b). This suggests that the activity of Vpu on CD4 is HIV-1-specific, a notion that may shed some light on the evolutionary relationship between the HIV-2 *env* and *vpu* genes. The selection pressure that promoted the appearance of the *vpu* gene in HIV-1 as well as the unique activity of Vpu on CD4 degradation may have been the increased affinity of the HIV-1 envelope for the CD4 receptor (Hoxie et al., 1991; Ivey-Hoyle et al., 1991; Mulligan et al., 1992), leading to more stable intracellular complexes and trapping of the envelope glycoprotein in the endoplasmic reticulum. The fact that the HIV-2 Env has a Vpu-like activity on particle release but not on CD4 degradation suggests that the ancestral activity of Vpu is particle release while the activity on CD4 was developed specifically by HIV-1 to counteract increased affinity of HIV-1 Env with the CD4 receptor.

The domain in HIV-2 Env which is responsible for the regulation of virus release has yet to be determined. Ritter et al. reported that an isolate containing a full-length 164 amino acid cytoplasmic tail is capable of performing this function while a variant, carrying a truncated, 17 amino acid cytoplasmic domain was inactive. It is tempting to speculate that the length of the cytoplasmic tail regulates this function of the HIV-2 Env protein. However, the ROD10 isolate used in our own studies carries only a short, 18 amino acid cytoplasmic tail yet efficiently activates virus release (Bour et al., 1996b). Thus it is likely that sequences other than the cytoplasmic domain of HIV-2 Env play a crucial role.

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