A single amino acid substitution within the transmembrane domain of the human immunodeficiency virus type 1 Vpu protein renders simian–human immunodeficiency virus (SHIVKU-1bMC33) susceptible to rimantadine

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Abstract

Previous studies from our laboratory have shown that the transmembrane domain (TM) of the Vpu protein of human immunodeficiency virus type 1 (HIV-1) contributes to the pathogenesis of SHIVKU-1bMC33 in macaques and that the TM domain of Vpu could be replaced with the M2 protein viroporin from influenza A virus. Recently, we showed that the replacement of the TM domain of Vpu with that of the M2 protein of influenza A virus resulted in a virus (SHIVM2) that was sensitive to rimantadine [Hout, D.R., Gomez, M.L., Pacyniak, E., Gomez, L.M., Inbody, S.H., Mulcahy, E.R., Culley, N., Pinson, D.M., Powers, M.F., Wong, S.W., Stephens, E.B., 2006. Substitution of the transmembrane domain of Vpu in simian human immunodeficiency virus (SHIVKU-1bMC33) with that of M2 of influenza A results in a virus that is sensitive to inhibitors of the M2 ion channel and is pathogenic for pig-tailed macaques. Virology 344, 541–558]. Based on previous studies of the M2 protein which have shown that the His–X–X–X–X–Trp motif within the M2 is essential to the function of the M2 proton channel, we have constructed a novel SHIV in which the alanine at position 19 of the TM domain was replaced with a histidine residue resulting in the motif His–Ile–Leu–Val–Trp. The SHIVVpuA19H replicated with similar kinetics as the parental SHIVKU-1bMC33 and pulse-chase analysis revealed that the processing of viral proteins was similar to SHIVKU-1bMC33. This SHIVVpuA19H virus was found to be more sensitive to the M2 ion channel blocker rimantadine than SHIVM2. Electron microscopic examination of SHIVVpuA19H-infected cells treated with rimantadine revealed an accumulation of viral particles at the cell surface and within intracellular vesicles, which was similar to that previously observed to SHIVM2-infected cells treated with rimantadine. These data indicate that the Vpu protein of HIV-1 can be converted into a rimantadine-sensitive ion channel with the alteration of one amino acid and provide additional evidence that drugs targeting the Vpu TM/ion channel can be effective anti-HIV-1 drugs.

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Introduction

The Vpu protein is the smallest membrane protein encoded by human immunodeficiency virus type 1 (HIV-1) and consists of a short amino terminal domain, an uncleaved signal sequence-transmembrane (TM) domain, and a cytoplasmic domain consisting of two α-helices and two highly conserved casein kinase sites (Strebel et al., 1988; Cohen et al., 1988; Schubert et al., 1994, McCormick-Davis et al., 2000a; Hout et al., 2004a). Previous studies have shown that the Vpu protein has at least two functions within HIV-1-infected cells. The first of these functions involves the interaction of Vpu with the CD4 molecule and subsequent targeting of CD4 to the proteasome for degradation (Fujita et al., 1997; Schubert et al., 1998). Both α-helices as well as the highly conserved casein kinase II sites (CK-II) in the cytoplasmic domain have been implicated in CD4 down-regulation (Schubert et al., 1996b; Paul and Jabbar, 1997; Tiganos et al., 1998). The second function associated with the Vpu protein is the ability to enhance virion release from infected cells (Klimkait et al., 1990). While the exact mechanism of enhanced virion release is unknown, some studies have associated this property with the TM of Vpu (Schubert et al., 1996a). The TM of Vpu has been shown to form ion channels...
Selective for some monovalent or divalent cations but not monovalent anions when expressed in frog oocytes (Ewart et al., 1996; Schubert et al., 1996a). This ion channel activity was localized to the TM domain and appears to be independent from the cytoplasmic domain in enhancing viral release from cells (Schubert et al., 1996b). More recently, drugs have been identified that interfere with the Vpu-mediated virion release from cells (Ewart et al., 2002, 2004).

Recently, we showed that the transmembrane domain of Vpu could be replaced with the TM domain of the M2 protein of influenza A virus (Hout et al., 2006). This virus, SHIVM2, was capable of causing a severe loss of CD4+ T cells and AIDS when inoculated into macaques. In addition, we found that unlike the parental SHIVKU-1bMC33, replication of the SHIVM2 virus was sensitive to an M2 ion channel blocker rimantadine. This study showed that drugs targeting the TM domain of Vpu (in this case, a chimeric Vpu with the TM of M2 protein) could reduce virus release from infected cells. Previous studies of the M2 protein have shown that the His–X–X–X–Trp motif within the TM domain was essential for ion channel activity and inhibition by amantadine, another M2 ion channel blocker (Okada et al., 2001; Tang et al., 2002; Takeuchi et al., 2003). Alteration of the histidine residue within the M2 TM domain to an alanine resulted in a constitutively open channel, indicating its importance to channel activation (Holsinger and Lamb, 1991; Holsinger et al., 1994; Pinto et al., 1992; Tang et al., 2002). Interestingly, the subtype B Vpu contains the sequence Ala–X–X–X–Trp in approximately the same position in the Vpu TM, with the tryptophan being invariant (McCormick-Davis et al., 2000a). In the present study, we have substituted the alanine in this motif with a histidine residue. We show that the replication of a SHIV (SHIVVpuA19H) expressing this mutant

**Fig. 1.** Sequence of the proteins analyzed in this study. Shown are the sequence of the Vpu, VpuA19H, and VpuM2 proteins.

**Fig. 2.** VpuEGFPA19H is expressed in the same intracellular compartments as the wild type Vpu. 293 cells were co-transfected with vectors expressing VpuEGFP or VpuA19H EGFP and DsRed2-ER or ECFP-Golgi. At 48 h, cells expressing EGFP and DsRed2 or ECFP were identified and images collected using laser scanning confocal microscopy as described in the Materials and methods section. (A–D) 293 cells co-transfected with VpuEGFP and DsRed2-ER. (A) Expression of VpuEGFP. (B) Expression of DsRed2-ER. (C) Merge of panels A and B. (D) Fluorescence micrograph of EGFP and DsRed2 fusion proteins from panels A and B. (E–H) 293 cells co-transfected with VpuEGFP and ECFP-Golgi. (E) Expression of VpuEGFP. (F) Expression of ECFP-Golgi. (G) Merge of panels E and F. (H) Fluorescence micrograph of EGFP and ECFP fusion proteins from panels E and F. (I–L) HeLa CD4+ cells co-transfected with VpuEGFPA19H and ECFP-Golgi. (I) Expression of VpuEGFPA19H. (J) Expression of ECFP-Golgi. (K) Merge of panels I and J. (L) Fluorescence micrograph of EGFP and ECFP fusion proteins from panels I and J.
Vpu became more sensitive to rimantadine than the SHIV<sub>M2</sub> virus. These results indicate that a single amino acid substitution in Vpu protein converts a rimantadine-resistant to a rimantadine-sensitive SHIV and provides additional in vivo evidence that the Vpu TM domain is a potential target for anti-HIV1 drugs.

**Results**

The Vpu<sub>A19H</sub> protein is transported to the same intracellular compartment as the unmodified Vpu protein

The sequences of the Vpu protein and the Vpu<sub>A19H</sub> mutant are shown in Fig. 1. We analyzed the intracellular transport of the Vpu<sub>A19H</sub> protein by fusion to the enhanced green fluorescent protein (EGFP) (Singh et al., 2003; Pacyniak et al., 2005). Co-transfection of 293 cells with the vectors expressing the unmodified subtype B Vpu protein (VpuEGFP) and DsRed2-ER resulted in this protein being partially co-localized with this intracellular marker (Figs. 2A–D). Co-transfection with the vectors expressing the unmodified subtype B protein (VpuEGFP) and ECFP-Golgi resulted in nearly complete co-localization (Figs. 2E–H). Co-transfection of 293 cells with the vectors expressing Vpu<sub>A19H</sub>EGFP and ECFP-Golgi resulted in the two proteins being almost completely co-localized (Figs. 2I–L). Similar to unmodified protein, co-transfection of 293 cells with a vector expressing the VpuEGFP<sub>A19H</sub> and either DsRed2-ER or ECFP-Mem markers revealed partial co-localization with the VpuEGFP-A19H but did not appear to be transported to the cell surface (data not shown). These results indicate that the Vpu<sub>A19H</sub> protein was transported to the same intracellular compartment as the unmodified Vpu protein and correlates well with our recent study that identified the cytoplasmic domain as having a Golgi retention signal (Pacyniak et al., 2005).

The VpuEGFPA19H is capable of down-regulating cell surface CD4

We determined if the VpuEGFPA19H was capable of preventing cell surface expression of CD4. HeLaCD4<sup>+</sup> cells were transfected with vectors expressing EGFP, VpuEGFP, and VpuEGFPA19H. At 48 h post-transfection, live cells were stained for cell surface CD4, fixed, and examined by confocal microscopy. As shown in Figs. 3A–D, expression of EGFP did not prevent CD4 expression while transfection of
HeLa CD4+ cells with a vector expressing VpuEGFP prevented cell surface expression (Figs. 3E–H), which was similar to what we previously reported (Pacyniak et al., 2005; Singh et al., 2003). Transfection of HeLaCD4+ cells with the vector expressing VpuEGFPA19H also prevented cell surface expression of CD4 (Figs. 3I–L) and was similar to the unmodified VpuEGFP. In addition, we performed co-transfection experiments with vectors expressing EGFP, VpuEGFP, or VpuEGFPA19H and human CD4 in the absence or presence of rimantadine. The results confirmed that the VpuEGFP and VpuEGFPA19H induced CD4 degradation while CD4 was stably expressed in the presence of EGFP (Fig. 4). Rimantadine treatment during the chase period did not appear to influence CD4 degradation in this assay. Together, these results indicate that replacement of the alanine in Vpu TM domain with a histidine residue did not affect the ability of Vpu to induce degradation of CD4.

**SHIVVpuA19H** expresses a Vpu protein with the same Mr as the unmodified protein and replicates with similar kinetics compared to parental SHIVKU-1bMC33

We determined if the SHIVVpuA19H expressed a protein with a similar Mr as the parental SHIVKU-1bMC33 virus. C8166 cells were inoculated with either SHIVVpuA19H or SHIVKU-1bMC33 for 6 days and then radiolabeled for 12 h with 35S-methionine/cysteine. Cell lysates were prepared and Vpu proteins immunoprecipitated using an anti-Vpu serum. As shown in Fig. 5A, Vpu proteins with a similar Mr were immunoprecipitated from C8166 cells inoculated with SHIVKU-1bMC33 or SHIVVpuA19H but not from unoinoculated C8166 cells. In addition to the pulse-labeling experiment in Fig. 5A, p27 growth curves were performed to quantify the amount of virus released. Cultures of C8166 cells were inoculated with an equivalent dose (10 ng) of infectious SHIVKU-1bMC33 or SHIVVpuA19H. Culture media were collected at 0, 1, 3, 5, 7, and 10 days post-inoculation and assayed for p27 release. As shown in Fig. 5B, growth curves revealed that the kinetics of viral p27 released into the culture medium were similar to the parental SHIVKU-1bMC33. In addition, we neither observed differences in the appearance of syncytial cytopathology nor in the extent of syncytia formation.

**Pulse-chase analysis reveals that SHIVVpuA19H and parental SHIVKU-1bMC33 process proteins with similar kinetics**

We examined the processing of the Env and Gag precursors in C8166 cells inoculated with SHIVKU-1bMC33 or SHIVVpuA19H at 7 days by pulse-chase analyses (Fig. 6). Fig. 6A shows normal synthesis and processing of precursor proteins from parental SHIVKU-1bMC33, and Fig. 6C shows the SHIVKU-1bMC33 proteins released into the culture medium. As shown in Fig. 6C, Gag proteins were released into the culture medium starting at the 1 h chase period, similar to what we
have previously reported (McCormick-Davis et al., 2000b; Stephens et al., 2002). The results of the pulse-chase analysis of SHIV\textsubscript{VpuA19H}-infected cells are shown in Figs. 6B–D. As shown in Fig. 6D, viral Gag proteins were detected in the culture medium beginning at the 1 h chase period. These results indicate that the release of viral proteins virus from SHIV\textsubscript{VpuA19H}-inoculated cultures was similar to the parental virus.

**The replication of SHIV\textsubscript{VpuA19H} is more sensitive to rimantadine than SHIV\textsubscript{M2}**

Previously, we showed that, when the TM domain of Vpu was replaced with that of the M2 protein of influenza A virus, the virus became sensitive to the drug rimantadine between 50 and 100 μM (Hout et al., 2006). We determined if SHIV\textsubscript{VpuA19H} was sensitive to rimantadine. C8166 cells were inoculated with equivalent doses of SHIV\textsubscript{VpuA19H} or parental SHIV\textsubscript{KU-1bMC33} for 4 h, the inoculum removed by washing cells and then incubated with various concentrations (0, 10, 25, 50, 75, or 100 μM) of rimantadine. At 7 days post-inoculation, culture media were retained for p27 measurement and the cells radiolabeled overnight with 35S-methionine/cysteine. Viral proteins were immunoprecipitated from the culture medium and from cell lysates. As shown in Figs. 7A–B, the addition of rimantadine to SHIV\textsubscript{KU-1bMC33}-inoculated cultures did not affect the release of p27 antigen in the culture supernatants or the synthesis of viral proteins. In contrast, the addition of rimantadine to SHIV\textsubscript{VpuA19H}-inoculated cultures reduced p27 release into the culture medium in a dose-dependent manner by an average of 20% at 25 μM and 87% at 50 μM to >99% for 75 and 100 μM (Fig. 7A). In addition, SHIV\textsubscript{VpuA19H} appeared to be more sensitive to rimantadine than SHIV\textsubscript{M2}. The sensitivity of
SHIVVpuA19H to rimantadine was also reflected in the ability to immunoprecipitate viral proteins from the culture medium (Fig. 7C).

Electron microscopy reveals that rimantadine-treated C8166 cells infected with SHIVVpuA19H accumulate viral particles at the cell surface and within intracellular membranes as our inhibitor studies indicated that rimantadine inhibited the replication of the SHIVVpuA19H, we examined the maturation of the SHIVVpuA19H and parental SHIVKU-1bMC33 in the presence of 25 μM rimantadine. We chose to examine infected cells treated with 25 μM rimantadine as this was a concentration that showed partial (~80%) inhibition of SHIVVpuA19H release as determined by p27 assays and that this concentration of rimantadine is not toxic to cells. Previously, we showed that parental SHIVKU-1bMC33 matured at the cell surface and that 50 μM rimantadine did not appear to affect virus maturation (Hout et al., 2006). The maturation of SHIVVpuA19H was similar to SHIVKU-1bMC33 in the absence of rimantadine (Fig. 8A). Electron microscopy revealed that the mean number of viral particles per cell was 47 and 45 for untreated and 50 μM treated SHIVKU-1bMC33-inoculated cultures, with the majority of the cells having less than 50 viral particles per cell (Figs. 9C–D). However, maturation of SHIVVpuA19H was considerably altered in the presence of 25 μM rimantadine. As shown in Figs. 8B–D, virus particles appeared to accumulate at the cell surface and within intracellular vesicles. The mean number of viral particles per cell was 39 for untreated SHIVVpuA19H-inoculated C8166 cells, while the mean number of particles per cell was 94 in 25 μM rimantadine-treated SHIVVpuA19H-inoculated cells, over twice that observed for the untreated cells (Figs. 9A–B). These results suggest that SHIVVpuA19H release from

![Fig. 7. Rimantadine interferes with the replication and spread of SHIVVpuA19H and SHIVM2 but not parental SHIVKU-1bMC33. (A) C8166 cells were inoculated with either SHIVVpuA19H, SHIVM2, or SHIVKU-1bMC33 in the presence of various concentrations of rimantadine or in the absence of rimantadine. At 7 days post-inoculation, culture supernatant was removed, clarified, and assayed for the presence of p27 antigen. The numbers above the bars represent the range and the mean percentage of p27 released compared to the control. (B and C) At 7 days post-inoculation, untreated or treated cells were starved for methionine and cysteine and radiolabeled overnight with 35S-methionine/cysteine for 12 h. Viral proteins were immunoprecipitated from the culture medium as described in the Materials and methods section from SHIVKU-1bMC33 (B) or SHIVVpuA19H (C).](image-url)
infected cells was abrogated in the presence of 25 μM rimantadine.

**Discussion**

The M2 protein of influenza A virus is the prototypic member of a class of viral proteins with ion channel activity known as viroporins, which also includes the BM2 protein of influenza B virus, the 6K protein of Sindbis virus (SV), and the 2B protein of poliovirus and the Vpu protein of HIV-1 (Gonzalez and Carrasco, 2003). The Vpu protein of HIV-1 has been modeled after the M2 protein of influenza A virus as they have a similar orientation within the membrane, similar length, and the ability to form an oligomeric structure (Klimkait et al., 1990; Grice et al., 1997; Lamb and Pinto, 1997; Sansom et al., 1998; Cordes et al., 2001, 2002; Fischer and Sansom, 2002; Hout et al., 2004a). The ion channel of M2 protein involves the formation of a homotetrameric structure. The amino acid residues that line the pore (Ala30, Gly34, His37, and Trp41) have been identified by cysteine-scanning mutagenesis and inhibition with transition elements (Gandhi et al., 1999; Shuck et al., 2000). Of these residues, the His37 residue is thought to act as both the pH sensor and selectivity filter, while the Trp41 serves as the activation gate (Tang et al., 2002). Thus, the His–X–X–X–Trp motif in the M2 ion channel is critical for proper functioning of the proton ion channel. The M2 ion channel is sensitive to the actions of the polycyclic amines amantadine and rimantadine. Both amantadine and rimantadine act as surrogate proton donors. However, treatment of patients with amantadine generally results in the selection of amantadine-resistant escape mutants, and these mutations generally occur at the pore-lining residues (Hay et al., 1986). Viral resistance to these drugs is thought to occur by two different mechanisms. First, mutations can occur in substitution of a larger amino acid that may reduce the diameter of the pore or change the properties of the pore such that the drug does not bind. Alternatively, mutations in the TM domain may actually increase the pore diameter such that, if the drug binds, it will not block the ion channel (Astrahan et al., 2004). The results presented here are based on our previous study in which we constructed a virus (SHIVM2) in which the TM domain of Vpu was replaced with the TM domain/ion channel of the M2 protein of influenza A virus (Hout et al., 2006). In that study, we showed that the replication and spread of SHIVM2 were inhibited by the drug rimantadine. Furthermore, we showed that SHIVM2 retained the ability to cause disease in macaques. This suggested that the M2 viroporin could substitute for the viroporin of Vpu and that anti-viral drugs targeting this domain could represent a new class of anti-HIV-1 drugs. Previously, we reported an invariant tryptophan residue at position 23 of the Vpu protein (McCormick-Davis et al., 2000a). This residue is approximately at the same position within the Vpu TM domain.
as the tryptophan residue (Trp41) of the M2 ion channel. Thus, instead of the His–X–X–X–Trp motif found in the M2 protein, the HIV-1 subtype B Vpu has the sequence Ala–X–X–X–Trp. Interestingly, replacement of M2 His37 with other amino acids (including alanine) impaired the selectivity of the ion channel resulting in constant current under neutral or acidic conditions (Pinto et al., 1992; Wang et al., 1995).

Replacement of the alanine at position 19 of Vpu with a histidine residue resulted in a protein that was transported to the same intracellular compartment as the unmodified Vpu and was still capable of CD4 down-regulation from the cell surface. Thus, it appeared that structural properties and biological functions were preserved in the VpuA19H protein. However, when we constructed an SHIV expressing the Vpu protein, the SHIVVpuA19H was phenotypically more similar to SHIVM2 than the parental SHIVKU-1bMC33. First, at concentrations of 25–50 μM rimantadine, SHIVVpuA19H replication was reduced from 80% to 13%, while a concentration of 75–100 μM rimantadine almost completely abolished the spread of SHIVVpuA19H in culture. Second, electron microscopic examination of infected cells treated with 25 μM rimantadine revealed particles accumulated at the surface of infected cells and within intracellular vesicles, similar to rimantadine-treated SHIVM2 (Hout et al., 2006).

The results of this study have implications for the structure of the Vpu transmembrane domain. Recently, the three-dimensional structure of the Vpu TM domain in micelles and bilayer samples was reported using NMR spectroscopy (Park et al., 2003). In this study, Vpu adopted a transmembrane α-helix spanning residues from the isoleucine at position 8 to the valine at position 25. The helix was found to be kinked slightly in the middle at the isoleucine at position 17, and there was a 13° average slant angle of the helix to the membrane normal. These investigators presented data that the Vpu TM formed oligomeric structures and presented both tetrameric and pentameric models of the Vpu TM structure based on their data. In their model, the invariant tryptophan produced unfavorable clashes unless its
side chain faced the surrounding lipids. They also suggested that the serine at position 23 faced the surrounding lipids while the isoleucine at position 17 faced the pore lumen. In the model of amantadine blockade of the M2 ion channel, the histidine and tryptophan residues at positions 37 and 41 face the pore of the channel, and their interactions are critical to the function of pore. Amantadine is thought to act as a surrogate H⁺ donor, and interactions with the histidine residues result in blocking of the pore. Our results provide additional data that the alanine at position 19 faces the lumen of the Vpu channel as alteration of this residue to a histidine results in a virus that is sensitive to rimantadine. It will be of interest to determine if the VpuA₁₉H protein is an ion channel for protons. If so, it may be a more simplistic channel than the M2 protein as the side chain of tryptophan in Vpu is thought to face the surrounding lipids and thus would not be in a position to form an activation gate like the M2 protein. The finding of increased rimantadine sensitivity when compared to SHIVM₂ suggests that the pore structure of Vpu may have a wider diameter than that of the M2 protein such that the pore structure formed by VpuA₁₉H is more accessible to rimantadine than VpuM₂. It may also be possible that the larger imidazolium side group of the histidine residue (as opposed to the methyl group of the alanine residue) may widen the pore structure formed by VpuA₁₉H and make it more accessible to the rimantadine.

The M2 protein of influenza virus has two important functions in the influenza A virus replication cycle (Gonzalez and Carrasco, 2003). During virus entry by receptor-mediated endocytosis, acidification of endosomes activates the M2 ion channel resulting in the influx of H⁺ into the virus resulting in a low pH conformational change in the hemagglutinin (HA). This results in exposure of the fusion peptide domain of the HA, fusion of the endosomal and viral membranes, and release of the viral nucleocapsids into the cytoplasm. In addition to its role in virus entry, the M2 protein is important for transport of the HA molecule to the cell surface. As there is a gradual drop in the pH from the rough endoplasmic reticulum (RER) to the trans-Golgi network (TGN), the M2 protein (based on its orientation) becomes activated and causes an efflux of H⁺ from the TGN, thereby preventing a low pH conformational change of the newly synthesized HA molecule as it transported to the cell surface (Sakaguchi et al., 1996; Henkel and Weisz, 1998; Henkel et al., 1999). It will be of interest to determine if blocking of the Vpu pore with rimantadine treatment of SHIVVpuA₁₉H-infected cells will affect the processing of the HIV-1 envelope glycoprotein in a way similar to that observed for influenza A virus hemagglutinin (HA).

The results presented here provide additional data to support a role for the Vpu protein in virion maturation and as a target for the design of novel anti-HIV-1 drugs. In our previous study using SHIVM₂, which contained the entire TM domain from the M2 virus, we showed that rimantadine was capable of inhibiting the replication and spread of this virus in culture. The results in this study expand on these findings and show that replacement of a single amino acid within the Vpu TM domain was sufficient to render the virus susceptible to rimantadine. Taken together, these studies indicate that rimantadine is capable of interacting with the pore structure of Vpu protein and that this class of compounds may serve as the basis for the discovery of novel rimantadine/amantadine derivatives that interact with the unmodified Vpu protein. Because we have incorporated this modified vpu gene in the genetic background of a pathogenic SHIV, we should be able to determine if rimantadine treatment of SHIVVpuA₁₉H-inoculated macaques will reduce virus burdens in macaques and/or this drug will drive the selection of the mutants that are resistant to rimantadine and result in accompanying increases in viral loads.

Materials and methods

Cells and viruses

The lymphocyte C8166 cell line was used for transfection of plasmids to generate viruses as well as indicator cells to measure infectivity and cytopathicity of the viruses used in this study. C8166 cells were maintained in RPMI-1640, supplemented with 10 mM HEPES buffer pH 7.3, 2 mM glutamine, 5 μg per ml gentamicin, and 10% fetal bovine serum (R10FBS). HeLa CD4⁺ cells were used to assess CD4 down-regulation, and 293 cells were used to assess the intracellular localization of the unmodified and mutant Vpu proteins and were used in CD4 degradation assays. The derivation of SHIVKU-1bMC₃₃ has been previously described (McCormick-Davis et al., 2000b; Stephens et al., 2002). The construction and characterization of the SHIVM₂ virus have been previously described (Hout et al., 2006).

Construction of VpuEGFPΔ₁₉H

In order to assess the intracellular transport of VpuA₁₉H and its ability to down-regulate CD4 expression, we constructed an expression vector in which the VpuA₁₉H was fused to the gene for EGFP using the same methodology as we used to express the subtype B and C proteins of HIV-1 as well as four SIVcpz Vpu proteins (Singh et al., 2003; Pacyniak et al., 2005; Gomez et al., 2005; Hout et al., 2006). For the site-directed mutagenesis, we used the plasmid ppuEGFP and the Quick-Change Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. Two rounds of mutagenesis were performed using the oligonucleotide 5'-GTAGCAATAATAATACAAATTACAAATAGTTGTGGTGCC-3' (first round) and 5'-GAATATAATACAAATACAGTTGTGGTGCC-3' (second round) to alter the alanine residue to a histidine within the Vpu TM sequence. Clones were isolated, plasmids isolated, and the entire insert sequenced to determine if the mutations were introduced as expected and to ensure that no additional changes were introduced during the mutagenesis. The resulting plasmid, ppuEGFPΔ₁₉H, was digested with KpnI and Stul, the vpuEGFPΔ₁₉H gene was isolated by gel electrophoresis and subcloned into the expression vector pcDNA3.1(+), which was digested with KpnI and EcoRV as we have previously described (Singh et al., 2003; Pacyniak et al., 2005; Gomez et al., 2005; Hout et al., 2006). The sequence of the vpuEGFPΔ₁₉H gene in the pcDNA3.1(+) vector was confirmed prior to any expression
Transfections and laser scanning confocal fluorescence microscopy analysis

Plasmids expressing VpuEGFP and other fusion proteins were transfected in human 293 cells to assess their subcellular localization using a cationic polymer (polyethyleneimine) transfection 5 reagent (ExGen™ 500, MBI Fermentas) using the manufacturer’s protocol. Briefly, 1–3 × 10⁵ cells were seeded onto cover slips in each well of a 6-well tissue culture plate 24 h prior to transfection. Transfection was carried out on cultures that were 50–60% confluent using 4.75 μg plasmid DNA and 15.5 μl of ExGen™ 500 corresponding to 6 equivalents. Each plasmid DNA sample was diluted in 300 μl of 150 mM sodium chloride solution separately. Samples were vortexed gently and immediately centrifuged at low revolution for a few seconds. Polyethyleneimine was then added to the plasmid DNA solution, mixed with a vortex and allowed to stand at room temperature for 10 min. The 293 cells were washed with serum-free media twice, and 3.0 ml of serum-free DMEM was added. Polyethyleneimine/DNA mixture was added to the cells, and the plate swirled by slow hand rotation for a couple of seconds. Culture plates were centrifuged at 280 × g for 5 min and incubated at 37 °C for 30 min. The medium from transfected cultures was replaced with fresh complete growth media, and cells were incubated at 37 °C in 5% CO₂ atmosphere.

Transfected cells were observed by confocal microscopy so that fluorescence could be detected from cells in different optical sections. Transfected cells were grown in 35 mm Petri dishes and were prepared for confocal microscopy as follows. Cells were rinsed briefly in phosphate-buffered saline (PBS, pH 7.2) at room temperature. The cells were fixed in freshly prepared, ice-cold, 1% paraformaldehyde in 0.13 M in sodium phosphate pH 7.2 for 2 min. The fixative was removed, and the cells briefly rinsed in phosphate-buffered saline. The saline was removed, and one drop of mounting media was placed on each dish. The mounting media is composed of 75% glycerol, 25% 0.13 M sodium phosphate buffer pH 8.0 plus 0.02% n-propyl gallate. A square number one glass cover slip was placed over each dish. The cells were imaged with a Zeiss LSM 510 confocal microscope equipped with an Argon/2 laser (25 mW) for the excitation (488 nm, 50% laser power) and detection (band pass 505–530 nm filter; BP505–530) of EGFP, for the excitation (458 nm, 100% laser power) and detection (band pass 475–525 nm filter; BP475–525) of ECFP, and for excitation (558 nm, 100% laser power) and detection (band pass 583 nm filter; LP560) of DsRed2. Images were acquired in Multitrack channel mode (sequential excitation/emission) with LSM510 (v 3.2) software and a Plan-Apochromat 100×/1.4 Oil DIC objective with frame size of 2048 × 2048 pixels. Detector gain was set initially to cover the full range of all the samples and background corrected by setting the amplifier gain, and all images were then collected under the same photomultiplier detector conditions and pinhole diameter.

Assays for detection of cell surface CD4 and degradation of CD4

To determine if the VpuEGFP-A19H construct was capable of promoting degradation of CD4 in transfected cells, we employed HeLa CD4⁺ cells. Cultures of HeLa CD4⁺, in which approximately 80–85% of the cells stained positive for surface CD4, were recloned to obtain a population of cells in which >99% of the cells stained positive for cell surface CD4. Briefly, HeLa CD4⁺ cells were grown on cover slips and transfected with pCMV-EGFP-A19H as described earlier. At 48 h post-transfection, cells were washed twice with wash buffer (PBS pH 7.2, containing 2% fetal calf serum and 0.01% NaN₃) and reacted with mouse anti-CD4 (clone SFC12T4D11CD4, Beckman-Coulter, 1:500 dilution) for 45 min. After the incubation, cover slips were washed three times and incubated with rhodamine-conjugated secondary antibody (goat anti-mouse, Chemicon, 1:50 dilution) for 35 min followed by washing five times in buffer without serum. Cells were then fixed in 1% formalin for 10 min, washed twice, equilibrated with anti-fade buffer, and mounted on microscope glass slides using Anti-fade (Molecular Probes, Oregon). Cells were immediately observed under a fluorescent microscope (Nikon...
TE300) with fluorescein filter (for visualization of EGFP) and rhodamine filters (for visualization of CD4 staining). All staining procedures were performed on ice and in the dark. HeLa CD4+ cells transfected with EGFP alone served as control for down-regulation of CD4 and cultures of HeLa CD4+ cells treated with wash buffer instead of primary antibody served as negative control for CD4 staining. A minimum of 400 EGFP expressing cells were counted.

In addition to detection of CD4 on the cell surface of transfected HeLa CD4+ cells, co-transfection experiments were performed using vectors expressing each VpuEGFP fusion protein and a vector expressing the human CD4 protein (pCMV4Neo; Goldsmith et al., 1995). 293 cells were transfected with Vpu fusion and pCMV4Neo vectors at a 3:1 ratio, respectively. This was to ensure that all cells transfected with the CD4 expressing vector were also transfected with the vector expressing the Vpu fusion proteins. At 48 h post-transfection, cells were starved for methionine and then radiolabeled with 500 μCi 35S-methionine/cysteine for 4 h. The radiolabel was removed and chased with 100× cold methionine/cysteine for 4 h. Cells were lysed in radioimmunoprecipitation assay (RIPA; 50 mM Tris–HCl, pH 7.5; 0.2% SDS; 0.5% sodium deoxycholate, 5 mM EDTA; 50 mM NaCl) buffer, lysates prepared and the CD4 immunoprecipitated using a rabbit anti-CD4 (sc-7219; Santa Cruz Biotechnology) and protein A Sepharose (PAS). Immunoprecipitates were collected, washed three times with 1× RIPA, and analyzed by SDS-PAGE (10% gel). Controls included co-transfection with pcEGFP (expressing on EGFP) as negative control for CD4 degradation and co-transfection with a pcypuEGFP (expressing the subtype B VpuEGFP fusion protein) as a positive control for CD4 degradation.

Construction of SHIV VpuA19H

A virus designated as SHIV VpuA19H was constructed in which the alanine residue at position 19 in parental virus SHIV-KU1bMC33 was changed to a histidine residue. Plasmid pUC19ΔSNvpu12, containing the SphI to KpnI fragment of SHIV-KU1bMC33 in pUC-19 (and coding for the tat, rev, vpu, and 5′ end of env), was used for the site-directed mutagenesis studies of the Vpu TM. The plasmid used was pUC19-ΔSNvpu12 and the Quick-Change Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. Two rounds of mutagenesis were performed using the oligonucleotide 5′-GTTAGCAATAATAAATAATGTTTGTTGGTCC-3′ (first round) and 5′-GTTAGCAATAATAAATAATGTTTGTTGGTCC-3′ (second round) to alter the alanine residue to a histidine within the Vpu TM sequence. Clones were isolated, plasmids isolated, and the entire insert sequenced to determine if the mutations were introduced as expected and to ensure that no additional changes were introduced during the mutagenesis. The plasmid, designated as pUC19ΔSNvpu, was digested to completion with SphI and KpnI, the 450 base pair insert isolated and ligated into p3′SHIV-KU1bMC33 using T4 DNA ligase, which was also digested with KpnI and SphI and gel purified. The resulting plasmid, p3′SHIVvpuA19Hb, was sequenced to ensure that no insertions or deletions had occurred during the cloning process. The SHIV VpuA19H virus was generated by digestion of p3′-SHIV-4 and p3′SHIVvpuA19H with SphI, ligation of the two plasmids together with T4 DNA ligase, and transfection into C8166 cells as previously described (McCormick-Davis et al., 2000b; Stephens et al., 2002; Hout et al., 2004b). Stocks of virus were prepared and stored at −86 °C.

Immunoprecipitations

To determine if Vpu proteins were expressed in SHIV VpuA19H-inoculated cultures, C8166 cells were inoculated with 10³ TCID of either SHIV VpuA19H or SHIV KU1bMC33. At 7 days post-infection, the medium was removed, and infected cells were incubated in methionine/cysteine-free Dulbecco’s modified Eagle’s medium (DMEM) for 2 h. The cells were then radiolabeled for 12 h with 500 μCi per ml of 35S-Translabel (methionine and cysteine, ICN Biomedical, Costa Mesa, CA). Vpu proteins were immunoprecipitated from the cell lysates using an anti-Vpu serum as previously described (McCormick-Davis et al., 2000b; Singh et al., 2003). Lysates were subjected to centrifugation to remove nuclei prior to the addition of antibody. Cell lysates were incubated with antibody for 16 h at 4 °C, and immunoprecipitates were collected on protein A Sepharose. The beads were washed three times with RIPA buffer, and the samples resuspended in sample reducing buffer and boiled. Proteins were separated by SDS-PAGE and visualized by standard autoradiographic techniques.

Pulse-chase analysis of viral proteins

To analyze the viral proteins synthesized and released from cells, C8166 cells were inoculated with 10⁴ TCID₅₀ of either SHIV VpuA19H or SHIV KU1bMC33. At 7 days post-inoculation, the medium was removed, and infected cells were incubated in methionine/cysteine-free Dulbecco’s modified Eagle’s medium (DMEM) for 2 h. The cells were then radiolabeled for 30 min with 1 mCi per ml of 35S-Translabel (methionine and cysteine, ICN Biomedical, Costa Mesa, CA) and the radiolabel chased for various periods of time in DMEM containing 100× unlabeled methionine/cysteine. SHIV proteins were immunoprecipitated from the cell culture medium and infected cell lysates using plasma pooled from several rhesus monkeys infected previously with non-pathogenic SHIV-4. Briefly, the cell culture medium was clarified (16,000 × g) for 2 min. The supernatant was transferred and made 1× with respect to cell lysis buffer (50 mM Tris–HCl, pH 7.5; 50 mM NaCl; 0.5% deoxycholate; 0.2% SDS; 10 mM EDTA), and SHIV proteins were immunoprecipitated with 10 μl of the serum described above. For immunoprecipitation of cell-associated SHIV proteins, cell lysates were prepared as described previously (Stephens et al., 1995, 1997, 2002; McCormick-Davis et al., 2000b; Hout et al., 2004b) prior to incubation with antiserum. Lysates were subjected to centrifugation to remove nuclei prior to the addition of
antibody. Cell lysates and culture medium were incubated with antibody for 16 h at 4 °C. All immunoprecipitates were collected on protein A Sepharose, the beads washed three times with RIPA buffer, and the samples resuspended in sample reducing buffer. Samples were boiled and the SHIV specific proteins analyzed by SDS-PAGE. Proteins were then visualized by standard autoradiographic techniques.

**p27 assays**

p27 growth curve assays were used to assess replication kinetics of the SHIVVpuA191I or parental SHIVKU-1bMC33. Cultures of 10⁶ C8166 cells were inoculated with equivalent amounts (10 ng) of cell-free virus stocks for 2 h. At the end of 2 h, the cells were centrifuged at 400 × g for 10 min, and the pellet washed with 10 ml of medium. Cells were washed two additional times. The cells were resuspended in RPMI-1640 supplemented with 10% FBS and antibiotics, and this was considered the 0 time point of the assay. Cultures were incubated at 37 °C, and aliquots of the culture were removed at 0, 1, 3, 5, 7, and 10 days. The culture medium was separated from the cells by centrifugation, and the culture supernatants assayed for p27 according to the manufacturer’s instructions (Coulter). For assays involving rimantadine, the drug was freshly prepared in RPMI-1640 medium as 10 mM stocks and filter sterilized prior to addition to cultures. Drug was added to cultures at the 0, 3, 6, and 9 days time points.

**Electron microscopy**

To determine the site of intracellular maturation, infected cells were examined by electron microscopy. Cultures of C8166 cells were inoculated with SHIVVpuA191I or SHIVKU-1bMC33 at a multiplicity of infection (M.O.I) of 0.01. Cells were incubated for 7 days at which time cells were pelleted at 400 × g for 10 min. Cells were washed three times with 10 ml of phosphate-buffered saline (pH 7.4) and then fixed in 2% glutaraldehyde overnight at 4 °C. Cells were postfixed in 1% osmium tetroxide (OsO₄) for 1 h. The cells were washed twice with water and dehydrated through a series of alcohols (30–100%) followed by embedding in Embed 812 resin. Thin sections were cut at 80 Å, stained with uranyl acetate and lead citrate, and examined under a JEOL 100CXII transmission electron microscope. The number of virus particles associated with infected cells (either at the surface or within the cell) was enumerated for 100 cells. Data were analyzed by planned comparisons using unpaired t test. Data from the untreated and 25 μM treated SHIVVpuA191I infected cultures was compared to 50 μM SHIVKU-1bMC33 infected cultures.

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**References**


