Effects of vpu start-codon mutations on human immunodeficiency virus type 1 replication in macrophages

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The human immunodeficiency virus type 1 (HIV-1) vpu protein increases the release of virus particles from infected cells. Mutations that abrogate vpu function have a profound effect on HIV-1 replication in primary macrophage cultures. About 1.24% of primary isolates in the HIV databases have vpu start-codon mutations. In addition, the envelope of the AD8 isolate was reported to compensate for the lack of vpu, whilst the YU-2 virus (cloned directly from the brain tissue of an infected individual) is macrophage-tropic, despite having a vpu start-codon mutation. These observations raise the possibility that envelopes evolve to compensate for the loss of vpu function in vivo. Chimeric vpu+ and vpu− replication-competent clones were constructed that contained the envelopes of SF162, AD8 or YU-2. Macrophages were infected with these chimeras and virus release was measured over time by a reverse transcriptase ELISA. It was found that vpu-deficient chimeras carrying AD8 and YU-2 envelopes were consistently released at lower levels than their wild-type (wt) vpu counterparts, indicating that these envelopes did not compensate for the lack of vpu. Non-chimeric vpu+ and vpu− AD8 and YU-2 followed similar patterns, although replication by vpu-deficient AD8 was variable, with virion release reaching 60% of that recorded for AD8 with a wt vpu. In summary, no evidence was found that the AD8 or YU-2 envelopes can compensate for the lack of vpu for replication in macrophages.

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) vpu gene encodes a 77–86 aa, approximately 16 kDa protein (Cohen et al., 1988). vpu is expressed predominantly in the endoplasmic reticulum and the Golgi (Pacyniak et al., 2005; Varthakavi et al., 2006), but also colocalizes with markers for recycling endosomes (Varthakavi et al., 2006). The vpu protein contains a transmembrane (TM) domain at the amino terminus and two cytoplasmic α-helices at the carboxyl terminus.

Over 80 nt at the 3′ end of vpu overlap the 5′ end of env in the HIV-1 genome and they are transcribed together on a bicistronic mRNA (Schwartz et al., 1990). Translation of the vpu protein and the env glycoprotein appears to occur via leaky scanning by ribosomes (Schwartz et al., 1992). Mutations that disrupt the vpu reading frame were reported to result in increased translation of env (Schubert et al., 1999; Stephens et al., 2002).

vpu has two distinct functions during viral replication, conferring (i) increased release of virus particles from plasma membranes and (ii) degradation of intracellular CD4. The TM domain is responsible for virus release from host-cell plasma membranes (Schubert et al., 1996a), overcoming a dominant host-cell block that is present in some cells, e.g. HeLa (Neil et al., 2006; Varthakavi et al., 2003) and macrophages (Balliet et al., 1994; Dejucq et al., 2000; Kawamura et al., 1994; Schubert et al., 1995), but absent in others, e.g. HOS (Neil et al., 2006) and 293T (Adachi et al., 2001; Sakai et al., 1995) cells. The vpu-mediated virus-release mechanism is unclear. The TM domain forms oligomers that act as ion channels (Ewart et al., 1996; Schubert et al., 1996b). A single amino acid substitution in the TM domain was shown to render vpu activity sensitive to the ion-channel blocker rimantadine, causing a decrease in the release of virus particles from infected cells (Hout et al., 2006). vpu was reported to interact with a host-cell ion channel, TASK-1, which may have antiviral activity disrupted by vpu (Hsu et al., 2004). vpu interacts with several cellular proteins besides TASK-1. vpu has been reported to associate with a member of the tetratricopeptide family called vpu-binding protein (UBP) (Callahan et al., 1998). UBP was shown to bind to vpu and p55 gag (Callahan et al., 1998). Overexpression of UBP diminished the release of virus particles, suggesting that vpu may remove UBP from gag in order to facilitate its transport to the cell surface (Callahan et al., 1998; Handley et al., 2001). In addition, Varthakavi et al. (2003) constructed heterokayons between cells permissive for virus release in the absence of vpu, and cells that were
not permissive. These experiments demonstrated the presence of an unidentified dominant inhibitory factor that can be overcome by vpu. More recently, Neil et al. (2006) showed that vpu prevents the endocytosis of nascent virions from the plasma membrane in restrictive cells. This suggests that vpu overcomes the entrapment of viruses at the cell surface by a putative tether factor. The relationship between this tether factor, the dominant inhibitory factor reported by Varthakavi et al. (2003), TASK-1 or UBP is unknown.

The cytoplasmic region of vpu downregulates CD4. This region recruits cellular proteins that ubiquitinate CD4 and induce its degradation in a multi-step process. First, the vpu α-helix interacts with the cytoplasmic tail of CD4 (Bour et al., 1995; Lenburg & Landau, 1993; Vincent et al., 1993; Yao et al., 1995). Second, serines at residues 52 and 56 are constitutively phosphorylated (Schubert et al., 1994) and recruit the cellular proteins β-TrCP, skp1 and the E3 ubiquitin ligase complex (Besnard-Guerin et al., 2004; Margottin et al., 1998). Finally, these proteins ubiquitinate CD4 (Schubert et al., 1998), triggering CD4 translocation from the endoplasmic reticulum to the proteasome for degradation (Fujita et al., 1997; Schubert et al., 1998). The removal of CD4 from the secretory pathway by vpu limits the formation of CD4–envelope complexes in the endoplasmic reticulum, allowing more efficient envelope trafficking through the secretory system (Kimura et al., 1994; Willey et al., 1992). The viral-release function of vpu, rather than CD4 degradation, was shown to be more important for replication in macrophages (Schubert et al., 1995), which typically express low levels of CD4 (Bannert et al., 2000; Lee et al., 1999; Mori et al., 1993).

HIV-2 and most simian immunodeficiency viruses (SIVs) lack a vpu gene, yet are fully functional in its absence. Determinants in the HIV-2/SIV envelope have been reported to confer virus release (Bour & Strebel, 1996; Iida et al., 1999). In the HIV-2 envelope, these determinants have been proposed to be an endocytosis signal (GYXX0) in the cytoplasmic tail and an uncharacterized region in the ectodomain of gp41 (Abada et al., 2005). The GYXX0 region in the gp41 cytoplasmic tail has been shown to recruit adaptor protein 2 (AP-2) complex and this activity was required to maintain the enhanced virus-release function (Noble et al., 2006).

It is possible that HIV-1 envelopes may also evolve to overcome a lack of functional vpu in a fashion similar to HIV-2/SIV envelopes, and that increased envelope expression in the absence of vpu may provide an advantage in vivo. The envelope of the AD8 isolate was reported to be vpu-independent because it conferred virus release from transfected HeLa cells and replication in macrophages in the absence of vpu (Schubert et al., 1999). YU-2, which possesses a mutated vpu start codon, was cloned directly from the brain tissue of an infected individual with neurological complications (Li et al., 1991) and was reported to infect macrophages efficiently, despite its vpu start-codon mutation (Li et al., 1991). Mutations in the vpu start codon also occur in 1.24% (12 of 967) of sequences derived from primary isolates in the HIV databases (Dejucq et al., 2000). We hypothesized that vpu start-codon mutations that occur in vivo, causing the loss of vpu function, can be compensated for by the evolution of vpu-independent envelopes.

Here, we compared the capacity of AD8 and YU-2 envelopes that lack functional vpu genes to replicate in macrophages. Our results show that the elimination of vpu function severely affects virion release and virus replication for AD8 and particularly for YU-2 in macrophages. Neither the AD8 nor the YU-2 envelope was able to rescue macrophage replication for vpu+ chimeric viruses. We also show that decreased virion release in vpu-defective infections of macrophages is due to a defect in viral release exacerbated by inefficient viral spread.

**METHODS**

**Construction of molecular clones.** Molecular clone pNL4.3 was used to construct all chimeric viruses used in the experiments described here. p3*SF162 (containing the 3’ half of the genome of the SF162 isolate) was used to provide the vpu gene for all chimeras (Cheng-Mayer et al., 1990). The construction of vpu+ p3*SF162 (ATG to ATA start-codon mutation) was described previously (Dejucq et al., 2000). Because the vpu and env genes overlap, we constructed two sets of chimeric clones. One set of clones was constructed to contain chimeric vpu genes and complete env genes; the second set was constructed to contain chimeric env genes and complete vpu genes. Premature stop codons were introduced into the nef genes of some of these latter constructs (Fig. 1).

The first set of chimeric clones was constructed by using unique BbsI and XhoI sites in p3*SF162. This allowed the introduction of different env genes into p3*SF162. vpu and env genes were then subcloned into pNL4.3 via the EcoRl and XhoI sites. Chimeric vpu+ and vpu− clones containing complete envelope genes from SF162, AD8 and YU-2 were constructed. This approach resulted in the NL4.3/AD8 and NL4.3/ YU-2 chimeric clones, containing chimeric env sequences (69 aa from SF162; 28 aa from AD8 or YU-2).

The second set of chimeric clones was constructed by using the KpnI site downstream of the vpu gene. These clones contain complete SF162 vpu genes and chimeric env genes. The leader sequences and first 12 aa of the env genes in these clones were derived from SF162. Premature stop codons were introduced by PCR mutagenesis of the nef gene at the XhoI site of the NL4.3/SF162, NL4.3/AD8 and NL4.3/ YU-2 chimeric clones described above.

The AD8 molecular clone with vpu+ or vpu− (ATA at the start codon) was described by Theodore et al. (1996). AD8 was cloned from circular DNA present in peripheral blood mononuclear cells (PBMCs) infected with AD87, a derivative of the ADA virus isolate (Theodore et al., 1996). vpu− YU-2 (CTG at the start codon) was described by Li et al. (1991). YU-2 was cloned directly from uncultured brain tissue (Li et al., 1991). The YU-2 vpu start codon was repaired by PCR mutagenesis.

**Production of virus stocks.** Virus stocks were prepared from infectious full-length HIV-1 clones and chimeric clones by calcium chloride transfection of HEK 293T cells as described previously (Peters et al., 2006). All virus stocks were prepared by co-transfection.
with a vector expressing the vesicular stomatitis virus (VSV) G protein. Supernatants containing virus were clarified by low-speed centrifugation and frozen at −152 °C.

**Cell culture and isolation of macrophages.** HEK 293T (DuBridge et al., 1987) and NP2 (Soda et al., 1999) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 10 μg gentamicin ml−1 (Invitrogen).

Macrophages prepared by elutriation (Gendelman et al., 1988; Sharova et al., 2005) were provided by the University of Massachusetts Medical School, Center for AIDS Research Cell Culture Core. Macrophages were also prepared from PBMCs by adherence (Simmons et al., 1995, 1996, 1998). Briefly, PBMCs were prepared from whole blood by Ficoll-Paque (Amersham Biosciences) density-gradient centrifugation. PBMCs (5 × 10^6–5 × 10^7) were placed in 150 cm^2 Petri dishes in DMEM containing 10% heat-inactivated human plasma (obtained from volunteer blood donors at the University of Massachusetts Medical School) and incubated at 37 °C for 3 h. Plates were washed gently with DMEM three times, and DMEM with 10% human plasma was added. Plates were incubated overnight, washed again the next day, then incubated at 37 °C. After 6 days, adherent macrophages were washed three times with EDTA and scraped gently off the plates with a cell scraper. Macrophages were resuspended at 2.5 × 10^5 cells ml⁻¹ in DMEM, 10% human plasma, seeded in 48-well trays (0.5 ml per well) and incubated overnight at 37 °C. Macrophages were infected the following day.

**Infectivity assays.** NP2 cells (4 × 10^5 cells ml⁻¹) were seeded in 48-well trays the day before infection. The cells were infected with serial tenfold dilutions of virus for 3 h at 37 °C. Virus was removed, fresh medium was added and cells were incubated for 72 h at 37 °C. Cells were fixed, stained in situ for p24 antigen and assessed for focus-forming units (f.f.u.) as described below. Macrophages in 48-well trays were infected with 100 μl viral stock, containing 25–50 pg reverse transcriptase (RT), of the VSV G-pseudotyped viruses or other doses as described. Macrophages were spinoculated by centrifugation at 1000 r.p.m. for 45 min (O’Doherty et al., 2000). After centrifugation, infected macrophages were incubated at 37 °C for 3 h. Virus inoculum was removed and cells were washed twice with fresh medium. Supernatants were harvested immediately following washing, then at approximately 3 day intervals for 2 weeks. Two weeks post-infection, the infected macrophages were fixed and stained for intracellular p24 antigen as described below. Harvested supernatants were assessed for RT activity by RT-ELISA (Cavidi Tech Inc.).

**Single-round infectivity assay.** Macrophages were infected with high doses of virus (1600 pg in 100 μl) by spinoculation as described above. After incubation at 37 °C for 4 h, cells were washed and 10 μM of the RT inhibitor indinavir sulfate (IVS; NIH AIDS Research and Reference Program) was added to cells to prevent subsequent rounds of replication. Supernatants were harvested at 24, 48, 72 and 96 h and infections were fixed after 96 h. RT activity of the supernatants was assessed by RT-ELISA.

**In situ immunostaining for p24 antigen and envelope.** Transfected 293T cells, infected NP2 cells or macrophages were fixed with cold (−20 °C) methanol:acetone (1:1), washed with PBS, then immunostained for p24 or envelope. For p24 staining, mAbs 38:96K and EF7 for p24 (UK Centre for AIDS Research), diluted 1:20, were used as described for p24 staining. The cells were washed twice in 1% fetal calf serum, 0.05% sodium azide in PBS, then incubated at room temperature for 1 h. For envelope staining, anti-gp41 mAb Chessie 8 (NIH AIDS Research and Reference Reagent Program) diluted 1:20 was used as described for p24 staining. The cells were incubated at 37 °C for 3 h. Infected cells stained blue and were regarded as foci of infection and counted by light microscopy.

**RT assays.** Virus stocks and supernatants from infections were assessed for RT activity by RT-ELISA (Cavidi Tech Inc.).
RESULTS

Construction of vpu⁺⁻ chimeric viruses
We previously described vpu⁺ and vpu⁻ chimeras of SF162 and NL4.3 (Dejucq et al., 2000). These constructs have complete vpu and env genes from SF162 in the background of NL4.3. The vpu⁻ construct contained a single mutation in the vpu start codon (ATG to ATA mutation) that eliminated vpu function. We constructed additional chimeric viruses carrying envelope sequences from HIV-1 AD8 and YU-2. Two strategies were used to prepare chimeras because the vpu and env genes overlap. The first set of chimeric clones was constructed with full-length AD8 or YU-2 env genes and chimeric vpu genes. These allowed us to evaluate the function of complete env genes, including the leader sequence, in the absence of functional vpu.

The second set of chimeric clones was constructed with a complete SF162 vpu gene and chimeric env genes. These viruses contain the env leader sequence from SF162, which allowed us to test a potential role for this region in vpu independence. This second set of chimeric clones included some constructs that carried a premature stop codon in nef, which enabled us to examine the possible effect of nef on virus release. Fig. 1 shows the structure of the genomes for both sets of chimeric clones. We also used virus derived from AD8 and YU-2 molecular clones with and without functional vpu genes to evaluate the role of vpu for viral replication in macrophages.

We used SF162 vpu in all chimeric viruses in this study because its amino acid sequence is related most closely to the consensus sequences of primary isolates compared with the vpu sequence from NL4.3. The SF162 envelope was used as a vpu-dependent control because it has consistently been shown to be dependent upon functional vpu (Dejucq et al., 2000; Kawamura et al., 1994; Schubert et al., 1995). Immunostaining of transfected 293T cells for envelope showed that all constructs produced envelope (not shown).

Infectedivity of VSV G⁺ viruses
Virus stocks for each of the viral clones described above were prepared by calcium chloride transfection of plasmid DNA into 293T cells. Plasmid DNA encoding viral or chimeric clones was co-transfected with a VSV G expression vector to create VSV G⁺ virions (see Methods). Incorporation of VSV G onto emerging virus particles subsequently conferred more efficient infection of primary macrophages.

HIV-1 virion release from 293T cells has been reported to be independent of vpu (Adachi et al., 2001; Sakai et al., 1995). However, to confirm that the infectedivity of our vpu⁺ and vpu⁻ viruses was not influenced by production from 293T cells, we compared the number of infectious and physical virus particles in each virus stock. Physical virus particles were measured as RT activity by RT-ELISA; infectious virus particles were measured by titrating virus stocks onto NP2 parental cells (CD4⁻ CCR5⁻) (Soda et al., 1999). Infectivity:RT ratios were calculated as an estimate of the infectivity per virus particle.

Table 1 shows the infectivity:RT ratios for each pair of vpu⁺ and vpu⁻ viruses. The ratios for each pair are generally very close; for six of the ten pairs, the difference was less than twofold (NL4.3/SF162 vpu⁺/⁻ nef⁺, NL4.3/AD8 vpu⁺/⁻, NL4.3/AD8 vpu⁺/⁻ nef⁺, NL4.3/2 AD8 vpu⁺/⁻ nef⁺, AD8 vpu⁺/⁻ and YU-2 vpu⁺/⁻). For vpu⁺/⁻ pairs with a greater than twofold difference, three of the four have a higher vpu⁻ ratio (NL4.3 vpu⁺/⁻ nef⁺, NL4.3/YU-2 vpu⁺/⁻ and NL4.3/YU-2 vpu⁺/⁻ nef⁺). One pair (NL4.3/YU-2 vpu⁺/⁻ nef⁻) had a nearly tenfold difference between the vpu⁺ and vpu⁻ virus.

Replication of vpu⁺ and vpu⁻ HIV-1 in macrophages

Virus stocks were equalized for RT activity, and primary macrophages were infected with equal amounts of VSV G⁺ replication-competent viruses as described in Methods. Virus release resulting from multiple rounds of infection and replication was measured over 2 weeks by RT-ELISA.

We first tested vpu⁺ and vpu⁻ AD8 viral clones for replication in macrophages (Fig. 2a). The envelope of AD8 was reported to compensate for the lack of functional vpu by enhancing virus release in HeLa cells (Schubert et al., 1999). We found that virus release by the vpu⁻ AD8 virus was variable depending on the experiment (not shown), but was consistently released at lower levels than AD8 vpu⁺.

Fig. 2(a) shows the maximum amount of released virions detected in one of several experiments, reaching 60% of vpu⁺ AD8 release (Fig. 2a, left panel).

YU-2, cloned directly from brain tissue without culture (Westervelt et al., 1992), also contains a vpu start-codon mutation (Li et al., 1991), yet retains the ability to infect macrophages. This raised the possibility that the YU-2 envelope was vpu-independent. However, YU-2 vpu⁺ replicated considerably more efficiently than vpu⁻ YU-2 in macrophages (Fig. 2a, right panel). We also confirmed that NL4.3/SF162 vpu⁺ replicated at lower levels than vpu⁻ NL4.3/SF162 (Fig. 2b), as reported previously (Dejucq et al., 2000; Schubert et al., 1995).

We next tested the vpu⁺ and vpu⁻ NL4.3/AD8 chimeras containing either full-length AD8 envelopes or full-length SF162 vpu genes as described above. In both cases, vpu⁺ NL4.3/AD8 chimeras were consistently released at higher levels than the corresponding NL4.3/AD8 vpu⁻ chimeras (Fig. 3a, b), regardless of the strategy used to construct the chimeric viruses. Similarly, we found that NL4.3/YU-2 vpu⁻ was consistently released from macrophages at higher levels than NL4.3/YU-2 vpu⁺, again regardless of whether
Table 1. Infectivity of VSV G+ virus stocks used for macrophage infections

The infectivity of virus particles produced in 293T cells was evaluated by comparing infectivity titres measured on NP2 cells with RT activity assessed by RT-ELISA. Infectivity:RT ratios provide a measure of virion infectiousness and these were similar for vpu+ and vpu− virus preparations.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Infectivity (f.f.u. ml−1)</th>
<th>RT (pg ml−1)</th>
<th>Ratio infectivity:RT (f.f.u. pg−1)</th>
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<tr>
<td>NL4.3/SF162 vpu+ nef+</td>
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the chimeric viruses carried full-length or chimeric vpu or envelope sequences (Fig. 3a, b).

Fig. 4 shows in situ immunostaining for p24 at different times after infection. Low virus release by vpu− viruses is not due to the lack of infected cells, but results partly from decreased spread. Thus, increased virus release for vpu+ viruses shown in Figs 2 and 3 is partly due to the spread to, and infection of, more macrophages.

The experiments described in Figs 2 and 3 were carried out multiple times on different batches of macrophages. We noted considerable variation from macrophage batch to batch, which presumably reflected macrophage donor variation. However, the macrophages used here were provided without donor-identification tags and we cannot be certain of this. Some batches of macrophages failed to yield sufficient virus particles to provide meaningful results for our experiments. Immunostaining in situ for p24 at the end of these assays showed that low levels of infected cells were the cause. Whether low infectivity was due to poor infection conferred by VSV G or to post-entry restrictions is unclear.

In summary, we found that vpu− AD8 replicated with variable efficiency in macrophages compared with vpu+ AD8. Using chimeric viruses, we failed to show that the AD8 envelope could compensate for the lack of a functional vpu. In addition, neither YU-2 nor its envelope compensated for the lack of a functional vpu.

Short-term virus release from infected macrophages

The infectivity assays described above followed virion release from macrophages during several rounds of replication over 2 weeks. Fig. 4 shows that the increased virion release by vpu+ viruses is at least partly due to more efficient spread of infection. It was possible that the vpu-independent phenotype reported for AD8 (Schubert et al., 1999) is more pronounced in early rounds of replication. We therefore examined whether the AD8 envelope conferred a vpu-independent phenotype when viral replication was limited to a single round. Macrophages were infected with high doses of virus and treated with IVS (a protease inhibitor) 3 h after infection to prevent subsequent rounds of infection. Virus release was measured over 4 days by RT-ELISA.

In the presence of 10 μM IVS, vpu+ viruses were released to higher levels than vpu− viruses (Fig. 5). We observed this phenotype for the SF162 and AD8 chimeras as well as for AD8.

These results failed to show that the AD8 envelope conferred a vpu-independent phenotype in the early rounds of viral replication. However, these results, along with our p24 immunostaining results in Fig. 4, confirm that the vpu− phenotype observed in macrophages results primarily from a defect in viral release, which subsequently impacts on viral spread.
The role of Nef

The strategy used to create the chimeric clones used in these studies resulted in the formation of chimeric nef genes (34 aa from AD8 or YU-2 and 162 aa from NL4.3; Fig. 1). Like vpu, nef has been reported to downregulate CD4. However, whilst vpu removes CD4 from the endoplasmic reticulum, nef downregulates CD4 from the plasma membranes of infected cells via clathrin-coated pits (Greenberg et al., 1997; Piguet et al., 1998, 1999). The requirement of nef for HIV-1 replication in macrophages is controversial (Brown et al., 2004; Swingler et al., 2003). To determine whether nef influenced the phenotypes described above, we introduced a premature stop codon in nef at the XhoI site into the vpu+ and vpu− chimeric viral clones (Fig. 1b). Viruses derived from these clones were then used to infect macrophages, and virus release into the supernatant was measured over 2 weeks by RT-ELISA as described above.

NL4.3/SF162 and NL4.3/YU-2 chimeras carrying mutated nef genes (nef−) were released from macrophages at low levels, similar to the vpu− chimeras, whereas the vpu+ nef− counterparts replicated efficiently (Fig. 6a, b). Curiously, the vpu+ nef− NL4.3/AD8 chimera varied in virus release depending on the experiment. In one experiment, the vpu+ nef− NL4.3/AD8 chimera replicated as efficiently as the vpu+ nef+ chimera (Fig. 6c, right panel), whilst in a second experiment, virus release was low and comparable to that of the vpu− nef− chimera (Fig. 6c, left panel). These fluctuating results with the NL4.3/AD8 vpu+ nef− virus may be due to variation between batches of macrophages. These results indicate that a functional nef is usually required for the replication of vpu+ viruses in macrophages.

DISCUSSION

In this study, we determined that neither the AD8 nor the YU-2 envelope was able to compensate for the lack of functional vpu in terms of virus release from macrophages. Release of vpu− viruses from macrophages was consistently lower than that of their vpu+ counterparts (Figs 2, 3 and 6). However, release of vpu− AD8 was variable, reaching 60% of vpu+ AD8 in one experiment. Short-term virus-release experiments showed that lower viral release by vpu− viruses is primarily due to a defect in release, but compounded by reduced viral spread (Fig. 5).
The ability of vpu to enhance the release of virus particles is usually critical for HIV-1 replication in macrophages. Mutation of the vpu start codon occurs at low frequency during PBMC culture of HIV-1 isolates in vitro (Dejucq et al., 2000). Thomas et al. (2007) described defects in the vpu genes present in several vpu–envelope sequences amplified from brain tissue, where macrophage-lineage cells are the main targets for infection. YU-2, a highly macrophage-tropic strain cloned directly from brain tissue of an AIDS patient, also carries a vpu start-codon mutation (Li et al., 1991). Finally, Schubert et al. (1999) reported that the envelope of the HIV-1 AD8 isolate carried determinants that could compensate for the lack of vpu. These observations led to the hypothesis that loss of vpu function in vivo may be compensated for by adaptive mutation in envelope. Loss of vpu function could be advantageous in some environments, as it has been reported to result in an increase in envelope synthesis (Schubert et al., 1999; Stephens et al., 2002). Here, we investigated whether the envelopes of AD8 and YU-2 could compensate for the loss of vpu function for virus release and replication in primary macrophage cultures. Our results do not lend support to a role for envelope in compensating for a loss of vpu function in macrophages.

Fig. 3. Effect of vpu mutations on virion release from macrophages using chimeric constructs. Macrophages were spinoculated with equal amounts of virus and incubated for 2 weeks. (a) NL4.3/AD8 and NL4.3/YU-2 constructs carrying full-length env and chimeric vpu genes. (b) NL4.3/AD8 and NL4.3/YU-2 constructs carrying full-length SF162 vpu genes and chimeric env genes. In all cases, vpu+ viruses (●) were released from macrophages at higher levels than vpu− viruses (■).
Our data appear to conflict with the study of Schubert et al. (1999), which showed that AD8 viruses with and without a mutated vpu start codon replicate efficiently and to equivalent levels in primary macrophages. In the same study, the authors used a pseudovirion system to show that the AD8 envelope could enhance virion release from HeLa cells. Interestingly, several groups have also reported that the HIV-2ROD envelope enhanced virion release (Abada et al., 2005; Bour et al., 1996; Noble et al., 2006). Therefore, it seems reasonable that some HIV-1 envelopes, e.g. AD8, may have evolved to perform the same virion-release function. It is unclear why we did not observe rescue of vpu

**Fig. 4.** Immunostaining of macrophages infected with vpu+ and vpu− HIV-1. Macrophages were infected with vpu+ and vpu− NL4.3/SF162, NL4.3/AD8 and AD8. Infected cells were fixed and immunostained for p24 at 24 h intervals. Blue-stained cells indicate infection. The micrographs show that vpu+ viruses confer more efficient spread and formation of small syncytia. Magnification, ×60.
start-codon mutations at least for the AD8 envelope. Using non-chimeric AD8 infectious clones, vpu− AD8 did confer significant (although variable) levels of virion release in primary macrophages, although always less efficiently than vpu+ AD8. However, whilst Schubert et al. (1999) implicated the AD8 envelope as the determinant for vpu-independent virion release by using NL4.3/AD8 chimeras similar to those described here, we did not. In our study, we used primary macrophages prepared from blood monocytes by elutriation (Gendelman et al., 1988; Sharova et al., 2005), similar to those used by Schubert et al. (1999). Both studies also monitored multiple rounds of viral replication in macrophages. We produced viruses from transfected 293T cells for macrophage infections, whereas Schubert et al. (1999) produced theirs in HeLa cells. HeLa cells carry the host-cell restriction overcome by vpu (Neil et al., 2006; Varthakavi et al., 2003), whereas 293T cells do not (Adachi et al., 2001; Sakai et al., 1995). vpu− viruses produced from HeLa cells may have altered envelope content and infectivity compared with virus particles from 293T cells. However, such a difference would only affect the initial infection stage and cannot explain the enhanced virion release that we observed for vpu+ viruses compared with the equivalent virion release of Schubert et al. (1999) for vpu+ and vpu− viruses over multiple rounds of replication in macrophages.

The apparent differences between our data and the previous report of Schubert et al. (1999) led us to undertake several control experiments. First, we assessed infectivity: particle ratios of the VSV G+ virions that were produced from 293T cells to ensure that vpu− virions conferred similar levels of infectivity to vpu+ virions. We confirmed that virus production in 293T cells is not affected by vpu. Next, we evaluated virus release in a single replication cycle and in a spreading infection to confirm that rescue of virion release by the AD8 envelope did not occur early before being overwhelmed by cell-to-cell spread. We also discounted an effect by nef, which, like vpu, downregulates CD4 (Lindwasser et al., 2007). These control experiments failed to alter our conclusion that the envelopes studied here did not affect vpu− virion release. Finally, we tested vpu+ and vpu− chimeric viruses constructed in two different ways, resulting in either full-length SF162 vpu and chimeric env, or chimeric vpu. However, the different AD8 or YU-2 chimeric constructs consistently failed to show that the loss of vpu function could be rescued by either AD8 or YU-2 envelopes for macrophage replication.

In our study, we focused entirely on the effects of vpu on HIV-1 replication in primary macrophages and have avoided studying HeLa cells. HeLa cells are used frequently to examine the effects of vpu defects on viral replication (Abada et al., 2005; Schubert et al., 1999; Varthakavi et al., 2003). However, whilst HeLa cells are valuable tools for studying events in vitro, it is unclear whether they are representative of any cell type targeted by HIV in vivo. A rapidly dividing culture of HeLa cells may not model a culture of terminally differentiated macrophages accurately. Recently, Neil et al. (2006) reported that vpu prevents the internalization of nascent virions from the cell surface in HeLa cells, conferring more efficient release of virions. In contrast, newly budded virions from primary macrophages are found predominantly in intracellular vesicles, even when HIV-1 carries vpu (Pelchen-Matthews et al., 2003). These observations suggest that the mechanisms that lead to virion endocytosis in the absence of vpu may be more potent in macrophages than in HeLa cells. Therefore, the
requirement for vpu for virus release in macrophages may be significantly more robust than the vpu requirement in HeLa cells. This possibility could explain the inefficient replication in macrophages by vpu−AD8 observed here, if the putative vpu-independent AD8 envelope could not overcome the macrophage-imparted block on virion release.

In summary, by using chimeric viruses based on NL4.3, we have failed to find evidence that the AD8 envelope can significantly rescue loss of vpu for macrophage replication. Moreover, the envelope from the YU-2 clone that carries a vpu start-codon mutation and is highly macrophage-tropic also failed to confer macrophage replication. The variable

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**Fig. 6.** Effect of nef on vpu phenotypes. Macrophages were spinoculated with equal amounts of vpu+/vpu−, nef+/nef− viruses and incubated for 2 weeks. (a) NL4.3/SF162; (b) NL4.3/YU-2; (c) NL4.3/AD8. Results from two separate but representative experiments are shown in the left and right panels. For NL4.3/SF162 (a) and NL4.3/YU-2 (b), the presence of non-functional vpu, nef or both resulted in severely reduced virus release from macrophages. For NL4.3/AD8 (c), the presence of non-functional vpu, nef or both resulted in reduced viral release for some experiments (left panel). However, virus release for vpu+ was observed even in the context of nef− in other experiments (right panel). ◆, vpu+ nef+; □, vpu− nef+; ▲, vpu+ nef−; ●, vpu− nef−.
replication in macrophages observed for vpu- AD8 suggests the presence of a viral determinant (presumably not envelope) that partially compensates for vpu loss. However, our results do not yet support the presence of fully vpu-independent HIV-1 variants that could preclude the development of vpu inhibitors for therapy.

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