JOURNAL OF VIROLOGY, Dec. 1995, p. 7699–7711 Vol. 69, No. 12
0022-538X/95/$04.00+0

Augmentation of Virus Secretion by the Human Immunodeficiency Virus Type 1 Vpu Protein Is Cell Type Independent and Occurs in Cultured Human Primary Macrophages and Lymphocytes

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Received 23 June 1995/Accepted 31 August 1995

The human immunodeficiency virus type 1-specific Vpu protein is a small integral membrane phosphoprotein that induces degradation of the virus receptor CD4 in the endoplasmic reticulum and, independently, increases the release of progeny virions from infected cells. To address the importance of Vpu for virus replication in primary human cells such as peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM), we used three different sets of monocyte-tropic molecular clones of human immunodeficiency virus type 1: a primary isolate, AD8$^+$, and two chimeric variants of the T-cell-tropic isolate NL4-3 carrying the *env* determinants of either AD8$^+$ or SF162 monocyte-tropic primary isolates. Isogenic variants of these chimeric viruses were constructed to express either wild-type Vpu or various mutants of Vpu. The effects of these mutations in the *vpu* gene on virus particle secretion from infected MDM or PBMC were assessed by determination of the release of virion-associated reverse transcriptase into culture supernatants, Western blot (immunoblot) analysis of pelleted virions, and steady-state or pulse-chase metabolic labeling. Wild-type Vpu increased virus release four- to sixfold in MDM and two- to threefold in PBMC, while nonphosphorylated Vpu and a C-terminal truncation mutant of Vpu were partially active on virus release in primary cells. These results demonstrate that Vpu regulates virus release in primary lymphocyte and macrophage cultures in a similar manner and to a similar extent to those previously observed in HeLa cells or CD4$^+$ T-cell lines. Thus, our findings provide evidence that Vpu functions in a variety of human cells, both primary cells and continuous cell lines, and mutations in Vpu affect its biological activity independent of the cell type and virus isolate used.

Peripheral blood mononuclear cells (PBMC) and macrophages have been widely described as the main target for human immunodeficiency virus (HIV). Because of the lack of an appropriate animal model for HIV-1, these primary cells represent the most relevant system available to study replication of HIV and to decipher the function of factors involved in the regulation of the virus life cycle. Monocytes are circulating phagocytic cells with the capacity to migrate and infiltrate most tissues in the body and to differentiate into mature macrophages. Persistent infection of macrophages plays an important role in pathogenesis of HIV-1 (13, 18–21, 33, 40, 58, 59) as well as other lentiviruses, including equine infectious anemia virus, visna-maedi virus, and caprine arthritis-encephalitis virus (41). While oncoretroviruses are able to infect only actively dividing cells, lentiviruses, including HIV-1, are able to establish productive infection in nondividing, terminally differentiated cells of the monocyte/macrophage lineage (18, 19). HIV-1 infection of monocytes/macrophages has been demonstrated for several organs, such as the brain, spinal cord, lungs, lymph nodes, and skin (3, 6, 18, 20, 33, 56, 60). The susceptibility of monocyte-derived macrophages (MDM) to HIV infection has been documented both in vivo and in vitro (4, 18, 19, 21, 30, 43, 58, 59). The observation that cytopathic changes in MDM are less severe than in PBMC or CD4$^+$ T-cell lines after infection with HIV-1 has led to the hypothesis that monocytes/macrophages may be an important reservoir for continuous virus replication even in the presence of an effective host immune response (13, 18–21, 33, 41, 43). HIV-1 replication within immature peripheral blood monocytes occurs at low levels, while significantly increased levels of virus replication can be observed in tissue macrophages found in several organs (13, 18, 20, 21, 33). Therefore, it has been widely speculated that HIV infection of macrophages plays a critical role in the establishment of chronic infections and in the progression to clinical manifestations of the disease (18, 20, 21, 33).

HIV isolates exhibit a host cell tropism and are categorized as T-cell-tropic or monocyte-tropic isolates (42, 49). T-cell-tropic isolates are generally capable of replicating in PBMC and established CD4$^+$ T cell lines, whereas monocyte-tropic isolates are capable of replicating in primary macrophages as well as PBMC but, in general, not in CD4$^+$ T-cell lines (11). The ability of macrophage-derived virus isolates to replicate not only in macrophages but also in primary human PBMC underlines the potential importance of macrophages as a main reservoir for virus replication and for spread into other host cells.

Vpu is encoded only by HIV-1 (9, 53), with no functional homologs known in the closely related primate lentiviruses HIV-2 and simian immunodeficiency virus (SIV) except for the SIV_{CPZ} chimpanzee isolate, which has the capacity to encode a Vpu-like protein (29). Vpu is an 81-amino-acid integral membrane phosphoprotein with two distinct biological functions, i.e., enhancement of virus release from the plasma membrane (32, 52–54) and degradation of the virus receptor CD4 in the endoplasmic reticulum (7, 22, 37, 57, 63, 64). The two biological functions of Vpu are independent, occur at different

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sites within the cell, and exhibit different sensitivity to Vpu phosphorylation (45, 47, 48). They are further regulated by two distinct molecular mechanisms involving two different structural domains of the Vpu protein: an ion channel activity of the Vpu transmembrane (TM) domain (46) is directly correlated with the ability of Vpu to enhance virus release, while the cytoplasmic domain is crucial for the Vpu-induced degradation of CD4 (5, 44, 62).

It has been reported by several authors that Vpu augments the release of progeny virions from various established human cell lines (23, 26, 32, 48, 52–54, 68). However, there are conflicting reports regarding the activity of Vpu in primary cells (2, 4, 30, 59): while Westervelt et al. (59) found only a moderate support of Vpu on virus replication in MDM, which was functionally complemented by Vpr, an up to 1,000-fold Vpu-mediated enhancement of virus replication was reported by Balliet et al. (4). In contrast, no Vpu effect was detected for monocyte-tropic viruses in PBMC by Balliet et al. (4) or Kawamura et al. (30). These findings could indicate a cell type-dependent function of Vpu. However, conclusions regarding the function of Vpu in primary cell systems, either macrophages or PBMC, are hampered because they rely exclusively on the analysis of extracellular virus, by either reverse transcriptase (RT) or p24 antigen capture assays (2, 4, 30, 59), and do not provide a comparison with the corresponding intracellular levels of viral proteins. The goal of our present study was therefore to analyze the function of Vpu in primary cells by a variety of biological and biochemical means, including Western blot (immunoblot) analyses, steady-state metabolic labeling, and pulse-chase analyses. For construction of chimeric monocyte-tropic viruses, we made use of the fact that T-cell-tropic isolates can be made competent for replication in macrophages by introduction of a portion of the *env* gene, including the V3 loop from primary monocyte-tropic isolates (16, 17, 42, 49, 59). This feature allowed us to analyze the activity of the well-characterized *vpu* gene from the T-cell-tropic isolate NL4-3 (1) in cultured PBMC and MDM and to compare its activity with the function of a *vpu* gene derived from the primary monocyte-tropic isolate $AD8^+$ (55). Two types of chimeric viruses carrying the monocyte-tropic *env* determinant from either the primary isolates $AD8^+$ (55) or SF162 (8), inserted into the backbone of the molecular T-cell-tropic HIV-1 clone NL4-3 (1), were constructed. In addition, we used a full-length molecular clone of the monocyte-tropic HIV isolate $AD8^+$ (55) to study the function of Vpu in PBMC and macrophages. All three virus isolates were analyzed in the context of wild-type Vpu or defined Vpu mutants. We show that wild-type Vpu augments virus release four- to sixfold in MDM and two- to threefold in PBMC. Furthermore, expression of nonphosphorylated Vpu or a C-terminal truncation mutant of Vpu resulted in reduced virus secretion. All results obtained are consistent with the effects observed previously in HeLa cells and in the $CD4^+$ T-cell line A3.01 (14, 32, 48, 52, 53). We conclude that the Vpu-mediated enhancement of virus secretion is independent of the cell type analyzed, is not restricted to a particular virus isolate, and thus is a general characteristic of this viral protein.

MATERIALS AND METHODS

Molecular HIV-1 clones and plasmid constructions. All plasmids containing HIV-1 sequences are derivatives of the infectious molecular clones pNL4-3 (1), SF162 (8), or $AD8^+$ (55). For construction of the mutant chimeric viruses, different *Eco*RI-*Kpn*I fragments were introduced into NL4-3(AD8) (16, 17), which carries the *Kpn*I-*Bsm*I *env* determinant of $AD8^+$ (55) inserted into pNL4-3 (1) (Fig. 1): (i) NL(AD8)-$U_{2/6}$ contains a 610-bp *Eco*RI-*Kpn*I fragment from pSP6-$U_{2/6}$ and encodes a Vpu phosphorylation mutant with serine-to-asparagine transitions at positions 52 and 56 of Vpu (45, 48); (ii) NL(AD8)-U_{35} contains a 618-bp *Eco*RI-*Kpn*I fragment from pAR-U_{35} and carries a linker insertion resulting in a translational frameshift after amino acid 32 of Vpu followed by three nonspecific amino acids and therefore has the potential to express the entire N-terminal TM domain of Vpu (53); (iii) NL(AD8)-U_{DEL1} contains a 562-bp *Eco*RI-*Kpn*I fragment from the molecular HIV-1 clone vpuDEL-1 (32) and carries a 48-bp deletion within the Vpu TM domain and is therefore unable to express any Vpu-specific sequences. For construction of the *vpu*-deficient chimeric virus NL(SF)-U_{DEL1}, a 562-bp *Eco*RI-*Kpn*I fragment from vpuDEL-1 (32) was introduced into chimeric virus NL(SF) (15) carrying a *Kpn*I-*Bsm*I *env* fragment of the monocyte-tropic HIV-1 isolate SF162 (8). For construction of the *vpu*-deficient virus AD8-U_{DEL2}, an 845-bp *Eco*RI-*Dra*III fragment (nucleotides 5972 to 6817) from the molecular clone $pAD8.2^+$, which carries an ATA-to-ATG restoration of the *vpu* translation initiation codon of the primary isolate pAD8.2 (55), was subcloned into pSP8ΔSspl (51), resulting in $pSP8EDAD8^+$. An 81-bp *Ssp*I-*Ssp*I deletion within the TM domain of Vpu_{AD8} (nucleotides 6301 to 6382 of $AD8^+$ [55]) followed by an 8-bp *Xho*I linker insertion was introduced. A 772-bp *Eco*RI-*Dra*III fragment was recloned into $pAD8.2^+$, resulting in the *vpu*-deficient clone pAD8-U_{DEL2}.

Cell culture, transfection, and infection. HeLa cells (ATCC CCL2) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Stocks of PBMC were prepared from gradient-isolated lymphocytes of a healthy, HIV-seronegative individual by using lymphocyte separation medium (Organon Teknika-Cappel, Durham, N.C.) and stored in liquid nitrogen. For each infection, 5×10^6 cells were stimulated for 2 days with 1 μg of phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, Mo.) per ml in the presence of 20 U of purified human interleukin-2 (IL-2, Boehringer, Mannheim, Germany) per ml. PBMC cultures were maintained in RPMI 1640 plus FBS supplemented with human IL-2.

For transfection, HeLa cells were grown to near confluency in 25-cm^2 flasks (5×10^6 cells per flask). At 2 h prior to transfection, the medium was replaced with 5 ml of fresh DMEM-FBS. Calcium phosphate-precipitated (28) plasmid DNA (25 to 30 μg) was added to the cells. After 6 h, the medium was removed and the cells were subjected to a glycerol shock for 2.5 min as described elsewhere (25). The cultures were then washed once with phosphate-buffered saline (PBS) and maintained in 5 ml of DMEM-FBS. Virus stocks were prepared in HeLa cells transfected with plasmid DNAs of individual molecular clones. Virus-containing supernatants were clarified by centrifugation ($1,000 \times g$ for 5 min) and filtered through a 0.45-μm-pore-size filter to remove residual cells and debris. Virions were pelleted by ultracentrifugation for 1 h at 35,000 rpm in a Beckman SW55 rotor, resuspended in 1 ml of RPMI 1640 plus FBS, and sterilized by filtration. The virus stocks were assayed for RT activity by an assay involving [^{32}P]TTP incorporation with an oligo(dT)-poly(A) template as described previously (66).

Routinely, 10^6 RT units was used to infect 10^6 PBMC or MDM. Following 15 h of adsorption, the medium was changed completely to remove residual input virus. PBMC were maintained in RPMI 1640 plus FBS at a density of approximately 10^6 cells per ml and were fed by replacing 80% of the medium at 2-day intervals. Infection of the cultures was monitored by determining the RT activity in the supernatant fluid as described above. The cultures were also examined by light microscopy for syncytium formation and scored by counting the number of syncytia per field.

Antisera and antibodies. Serum from an asymptomatic HIV-1-seropositive patient was used to detect HIV-1-specific proteins, including Vpu, by immunoprecipitation and Western blotting. In addition, a Vpu-specific polyclonal antiserum (from sheep) raised against a synthetic peptide comprising residues 41 to 58 of Vpu (45, 47), which was a gift of T. Porstmann, and a polyclonal Vpu antiserum (from rabbits) directed against the hydrophilic C-terminal domain of Vpu expressed in *Escherichia coli* (39) were used for detection of Vpu. Polyclonal rabbit antisera recognizing gp160 and gp120, which were a gift of R. Willey, were described recently (63, 64).

Monocyte isolation and culture. PBMC were isolated from the leukopheresed blood of HIV-seronegative donors after gradient separation in lymphocyte separation medium. Monocytes were isolated, as described in detail previously (12, 24), by countercurrent centrifugal elutriation in a Beckman system (Beckman Instruments, Fullerton, Calif.). Elutriated monocytes were >99% viable as determined by trypan blue exclusion and were >95% pure as determined by morphological analysis with Giemsa staining of representative cytocentrifuge preparations. All reagents used in the preparation and culture of monocytes were free of detectable endotoxin as determined by the *Limulus* amebocyte assay (38). Monocytes were precultured as adherent cell monolayers by a modification of the procedure of Lazdins et al. (36). Briefly, 3×10^6 cells were suspended in 2 ml of high-glucose (4.5 g/liter) DMEM (Gibco, Grand Island, N.Y.) supplemented with 50 U of penicillin per ml, 50 μg of streptomycin (Gibco) per ml, 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), and 10% pooled human serum. Cultures were then incubated at 37°C in a humidified incubator under an atmosphere of 5% CO_2 for 7 days; they were then supplemented with an additional 2 ml of DMEM, and incubation was continued. After 10 to 14 days, MDM were obtained by harvesting culture supernatants, adding 2 ml of cold Ca^{2+}/Mg^{2+}-free PBS to adherent cell monolayers, incubating the cells for 45 min at 4°C, and removing them with a plastic cell scraper. All cells were pooled, pelleted by centrifugation (10 min at $1,000 \times g$), and resuspended in fresh DMEM at a concentration of 0.5×10^6 cells per ml. Harvested cells (1.5 ml) were

finally replated into 24-well tissue culture plates (Nunc), allowed to adhere, and infected within 3 days.

Metabolic labeling and immunoprecipitation. For steady-state metabolic labeling of macrophages, 7.5×10^5 precultured cells per well were seeded in 24-well plates (Costar, Cambridge, Mass.) and infected with 7.5×10^5 RT units of purified virus stocks, and the spread of infection was measured as RT activity released into the culture supernatants. Medium was changed at 3-day intervals. At the time of maximal virus production, metabolic labeling of adherent cells was performed as follows. Cells were preincubated for 10 min in methionine-free DMEM (Specialty Media, Lavallette, N.J.) supplemented with 5% heat-inactivated pooled human serum to deplete the intracellular pool of methionine. The cells were then washed with 1 ml of methionine-free DMEM and incubated in 1 ml of methionine-free DMEM containing 450 μCi of [^{35}S]methionine (DuPont, Boston, Mass.). After 8 h, 450 μCi of [^{35}S]methionine in 0.3 ml of methionine-free DMEM was added, and the labeling was continued for an additional 16 h. The supernatants (1.3 ml) were harvested and then filtered through 0.45-μm-pore-size Spin-X filter tubes (Costar). Cell debris was removed by centrifugation for 2 min at 16,000 × *g*, and virions were pelleted by centrifugation in a refrigerated Eppendorf microcentrifuge for 100 min at 16,000 × *g* and 4°C. Immunoglobulins in the clarified supernatants were removed by two rounds of preabsorption with a 1:1 mixture of protein A-Sepharose and GammaBind-G–Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, N.J.). Pelleted virions were washed with 500 ml of ice-cold PBS and pelleted again for 100 min at 16,000 × *g* and 4°C. The pelleted virions were lysed in a buffer containing 300 mM NaCl, 50 mM Tris-hydrochloride (pH 7.4), and 0.1% (vol/vol) Triton X-100 by shaking at room temperature for 20 min. Cells attached to the tissue culture plates were lysed in 210 μl of a buffer containing 50 mM Tris-hydrochloride (pH 8.0), 5 mM EDTA, 100 mM NaCl, 0.5% (wt/vol) [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate] (CHAPS), and 0.2% (wt/vol) deoxycholate; incubated for 5 min at 37°C; and transferred into 1.5-ml Eppendorf tubes. Wells were washed with 0.5 μl of PBS, and cell debris from the pooled cell lysate was removed by centrifugation (16,000 × *g* at 4°C for 15 min) followed by filtration through 0.45-μm-pore-size Spin-X filter tubes. Lysates of cells and virions were further clarified by two rounds of incubation with protein A-Sepharose and protein G-Sepharose preadsorbed with normal rabbit serum. Viral proteins from 50% of the clarified lysates of cells and virions, as well as the clarified supernatants, were immunoprecipitated with a 1:1 mixture of HIV-1-positive human serum and a polyclonal rabbit serum specific for HIV-1 gp160/gp120 proteins, preadsorbed to protein G-Sepharose, as described elsewhere (48, 63, 64). As a control, the remaining half of the cell lysate was used for immunoprecipitation of the transferrin receptor molecule with a monoclonal anti-TFR antibody (Becton Dickinson, San Jose, Calif.). For pulse-chase metabolic labeling of MDM, a similar procedure was followed, except that labeling with [^{35}S]methionine (1.5 mCi/ml) was performed for 30 min. Cells remained attached to the tissue culture flasks during the pulse and chase periods. Different wells of infected cells were used for individual time points. Parallel cultures of 7.5×10^5 MDM were seeded in each well of a 24-well tissue culture plate, and one well was used for each time point during the chase periods. Cell lysates were prepared as described above, and viral proteins immunoprecipitated from the cell, virion, or supernatant fractions collected after steady-state or pulse-chase labeling were solubilized by boiling in sample buffer containing 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 1% glycerol, and 65 mM Tris-hydrochloride (pH 6.8) and separated in 10% polyacrylamide or 8% acrylamide-Acryl-Aide (FMC Corp., Rockland, Maine) SDS-containing gels by the method of Laemmli (34). Gels were fixed for 30 min by incubation in 40% methanol–10% acetic acid, rinsed with water, soaked in 1 M sodium salicylic acid for 30 min, and dried. Radioactive bands were visualized by fluorography. The radioactivity of the respective bands was quantitated in a Fuji BAS 2000 bio-image analyzer.

Immunoblotting. Infected MDM (7.5×10^5) or PBMC (10^6) were lysed in 210 μl of lysis buffer containing CHAPS and deoxycholate, adjusted with 200 μl of sample buffer, boiled for 5 min, separated in a 10% polyacrylamide–SDS gel, and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.). The membranes were incubated with the appropriate combination of antibodies as described below, and binding of the antibodies was identified by using ^{125}I-protein A (0.1 μCi/ml; New England Nuclear, Du Pont, Wilmington, Del.) followed by autoradiography. For analysis of virus associated proteins, viral particles from 1 ml of culture supernatants were pelleted (at 16,000 × *g* and 4°C for 100 min), washed with 0.5 ml of PBS, and lysed in 20 μl of sample buffer.

RESULTS

Vpu supports virus release from HIV-infected macrophages. To study the importance of Vpu for viral replication in primary cells, we used a chimeric molecular clone of HIV-1, pNL4-3(AD8) (16, 17), which contains a portion of the *env* gene from the monocyte-tropic clone AD8$^+$ (55) inserted into the T-cell-tropic molecular HIV-1 clone pNL4-3 (2) (Fig. 1, panel Ia). Replication of this isolate was compared with that of two isogenic variants: NL(AD8)-U_{DEL1}, which is unable to express Vpu-specific sequences, and NL(AD8)-$U_{2/6}$, encoding a nonphosphorylated form of Vpu, $Vpu_{2/6}$ (Fig. 1, panels Ib and Id). $Vpu_{2/6}$ exhibits approximately 50% of wild-type activity on augmentation of virus particle secretion when analyzed in HeLa cells or CD4$^+$ T-cell lines (48).

Parallel cultures of MDM were infected with equal RT units of virus stocks generated by harvesting culture supernatants from HeLa cells transfected with plasmid DNAs of the NL(AD8) chimeras. As a control for potential contamination of cultured macrophages with CD4$^+$ T lymphocytes, the T-cell-tropic NL4-3 virus, which fails to replicate at detectable levels in this macrophage system as estimated by RT assay, was included in all experiments. Aliquots of culture supernatants were collected at 3-day intervals over a period of 1 month, and secretion of virus particles into culture supernatants was subsequently determined by measuring the virus-associated RT activity. The resulting replication profiles (Fig. 2A) demonstrate that both wild-type NL4-3(AD8) (+Vpu) and the phosphorylation mutant NL(AD8)-$U_{2/6}$ (+$Vpu_{2/6}$) were able to productively infect MDM, with nonphosphorylated $Vpu_{2/6}$ producing about 50% of the RT activity observed with wild-type virus. Surprisingly, the ability of the Vpu-deficient NL(AD8)-U_{DEL1} variant (−Vpu) to replicate in MDM cultures was drastically reduced. While declining input virus measured as RT activity in the culture supernatants was readily detectable for approximately 9 days postinfection (p.i.), levels of de novo synthesized progeny viruses remained very low throughout the experiment. No replication of NL4-3 could be detected by RT analysis, attesting to the absence of CD4$^+$ T lymphocytes in the MDM preparation.

The inability of the Vpu-deficient isolate NL(AD8)-U_{DEL1} to efficiently replicate in macrophages is consistent with a report describing an up-to-1,000-fold effect of Vpu on virus release in macrophages (4). However, this result is inconsistent with the comparatively modest effect observed with the corresponding *vpu* mutant in the context of the T-cell-tropic isolate NL4-3 in A3.01 cells (32). Also, Westervelt et al. (59) and Kawamura et al. (30) reported only a modest effect of Vpu in macrophage systems. To rule out a potential experimental artifact, virus pellets collected at individual time points were subjected to Western blot analysis to look for the presence of cell-free virus (Fig. 2B). The relative amounts of the HIV-1 capsid protein, $p24^{gag}$, detected with an HIV-reactive human serum were calculated by using an image analyzer and plotted as a function of time (Fig. 2C). The results are consistent with the RT activity profiles shown in Fig. 2A and suggest a very low level of virus replication in the NL(AD8)-U_{DEL1}-infected culture. In all cases, input virus was detectable up to 6 days p.i. at comparable levels (Fig. 2B, panels I to III).

It has been previously reported that reduction of virus release in Vpu-deficient cultures correlates with an intracellular accumulation of viral proteins (32, 52–54). To assess the effects of Vpu on the intracellular accumulation of viral proteins in MDM, cells were harvested at the end of the experiment shown in Fig. 2 (day 30 p.i.) and the cell lysates were subjected to Western blot analysis (Fig. 2D) with the same antibody mixture as in the experiment in Fig. 2B. As expected, the intracellular levels of HIV-1 proteins in cells producing wild-type Vpu and nonphosphorylated $Vpu_{2/6}$ were comparable, with slightly larger amounts present in the $Vpu_{2/6}$ lysate, consistent with the partly reduced activity of nonphosphorylated $Vpu_{2/6}$ (Fig. 2D, lanes c and e). Despite the very low levels of extracellular virus detected in the Vpu-deficient culture [NL(AD8)-U_{DEL1}], significant amounts of viral proteins were detectable in the day 30 cell lysate (Fig. 2D, lane d), suggesting that the *vpu*-deficient chimeric virus replicated in MDM. This

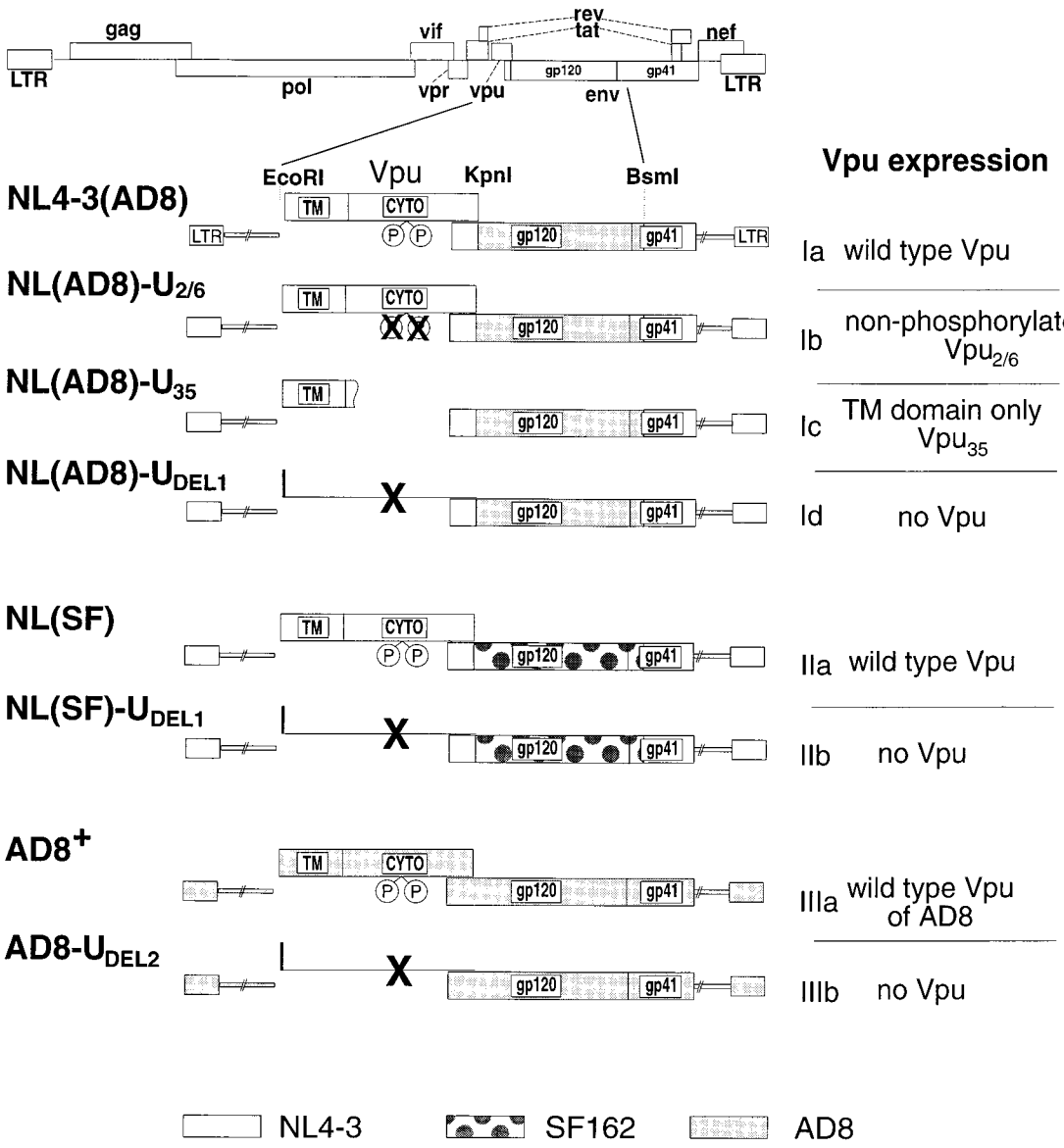

FIG. 1. Schematic outline of the monocyte-tropic chimeric or primary HIV-1 proviral genomes. (Panel I) Chimeric molecular clones carrying the *env* determinant (positions 6343 to 9878 in NL4-3) of the monocyte-tropic AD8$^+$ isolate (55) in the backbone of the T-cell-tropic NL4-3 isolate (1). NL4-3(AD8) (16, 17) expresses wild-type Vpu; NL(AD8)-$U_{2/6}$ expresses nonphosphorylated $Vpu_{2/6}$ (48); NL(AD8)-U_{35} expresses a truncated form of Vpu (53); NL(AD8)-U_{DEL1} does not express any Vpu-specific sequences. (Panel II) Chimeric variants of NL4-3 carrying the monocyte-tropic *env* determinant of the primary isolate SF162 (8). NL4-3(SF) expresses wild-type Vpu (15); NL(SF)-U_{DEL1} does not express Vpu-specific sequences. (Panel III) Molecular clones of the primary isolate AD8 (54). AD8$^+$ expresses wild-type Vpu; AD8-U_{DEL2} does not express Vpu. CYTO, cytoplasmic domain; P, phosphoseryl residues in Vpu.

phenomenon could be due to cell-to-cell transmission of *vpu*-deficient chimeric viruses. It is possible that the accumulation of budding structures at the surface of cells infected with Vpu-deficient viruses (32) facilitates such a mode of virus spread in MDM. No viral proteins were detected in lysates from mock-infected cells or cultures infected with the T-cell-tropic isolate NL4-3 (Fig. 2D, lanes a and b).

It is interesting that the Env glycoprotein produced in MDM exhibits altered mobility compared with the Env proteins produced in HeLa cells: while the HeLa cell-derived input virus exhibits two distinct species, representing mature gp160 and gp120 Env proteins (Fig. 2B, days 3 and 6), the mobility of de novo synthesized Env protein in macrophages is heterogeneous and migrates as a broad band in the SDS-polyacrylamide gel electrophoresis (PAGE) system used (Fig. 2B, panels I and III, days 9 and thereafter; Fig. 2D). Infection of PBMC with macrophage-derived virus results in the reappearance of two distinct Env species (results not shown). These changes in the migration behavior of MDM-derived Env result from differences in the posttranslational modification of Env in macrophages with respect to HeLa cells (61). This feature allows us to easily distinguish between HeLa cell-derived input virus and progeny virus produced in macrophages.

Purified virus stocks exhibit increased efficiency of replication in MDM. Virus stocks derived from transiently transfected HeLa cells contain considerable amounts of soluble gp120 (48, 65), which could interfere with infection of MDM by saturating potential virus-binding sites on cell surface CD4. In addition, it is possible that HeLa supernatants contain other cellular factors which could interfere with HIV infection of macrophages.

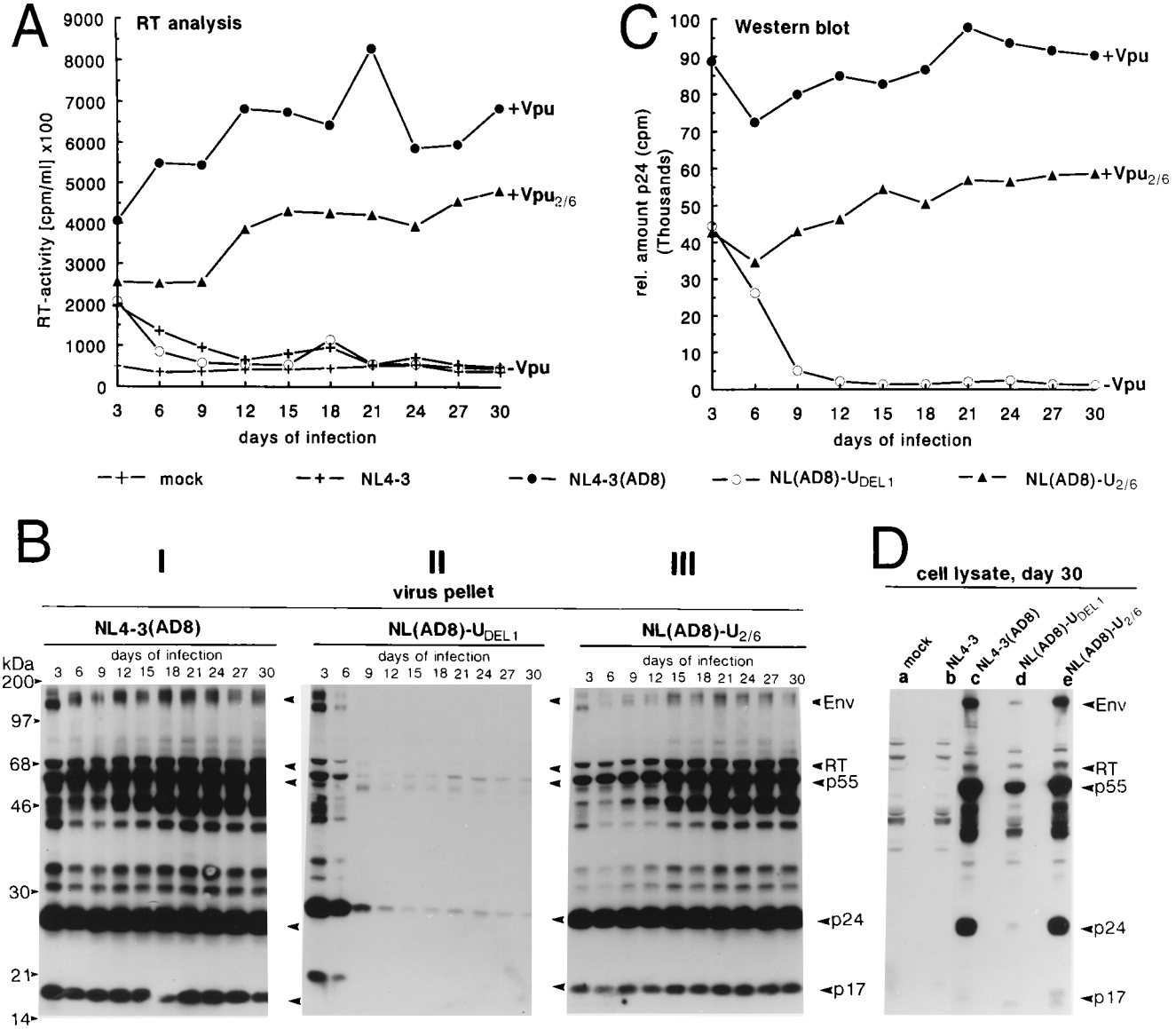

FIG. 2. Replication of NL4-3(AD8), NL(AD8)-U_{DEL1}, and NL(AD8)-$U_{2/6}$ in macrophage cultures. (A) Parallel cultures of 7.5×10^5 MDM were infected with equal RT units of NL4-3(AD8), NL(AD8)-$U_{2/6}$, and NL(AD8)-U_{DEL1}. As negative controls, cells were infected with T-cell-tropic virus NL4-3 or mock infected. Virus release was measured as the secretion of RT activity into the culture supernatants at 3-day-intervals. (B) Virions from 1 ml of culture supernatants collected during the course of infection were pelleted, separated by SDS-PAGE (10.5% polyacrylamide) and analyzed by Western blotting with a 1:1 mixture of an HIV-reactive human serum and a polyclonal rabbit serum against the HIV-1 gp160/gp120 Env products. Bound antibodies were detected with ^{125}I-protein A. (C) $p24^{gag}$ protein detected in panel B was quantitated with an image analyzer and plotted as a function of time. (D) Upon termination of the experiment (day 30), cells were harvested and lysates were prepared. Aliquots of each cell lysate were separated by SDS-PAGE (10.5% polyacrylamide) and analyzed by Western blotting with an HIV-reactive patient serum sample. Cells lysates of the control cultures (mock and NL4-3) were included.

To assess the effect of such potential inhibitory contaminants on virus replication, stocks of NL4-3(AD8) (+Vpu) or NL (AD8)-U_{DEL1} (−Vpu) viruses were purified by ultracentrifugation and used for the infection of MDM (Fig. 3). As an additional control, NL(AD8)-U_{35} (Fig. 1, panel Ic), which has the potential to express the TM anchor of Vpu and has residual biological activity for virus release in PBMC and T-cell lines (44), was included in this experiment.

As above, virus replication was analyzed by comparing RT profiles (Fig. 3A) as well as by Western blot analysis of pelleted virions (Fig. 3B and C) or intracellular viral proteins (day 30 p.i.; Fig. 3D). Interestingly, all three virus stocks including NL(AD8)-U_{DEL1} established productive infection of MDM. Virus replication was generally more efficient than in the infection described in Fig. 2. For example, production of wild-type virus reached its peak 12 days earlier than following infection with unpurified virus, even though similar RT units were used to initiate the infection. In addition, the levels of extracellular virus were significantly increased in cultures infected with purified virus stocks (compare Fig. 2A and 3A). Similarly, the results from the immunoblotting of pelleted virions are consistent with an increased replication potential of purified virus stocks (Fig. 3B). The level of wild-type virus (+Vpu) was approximately six times higher than in the *vpu*-deficient culture (−Vpu) or about four times higher than in the culture expressing the truncated mutant Vpu_{35} (+Vpu_{35}).

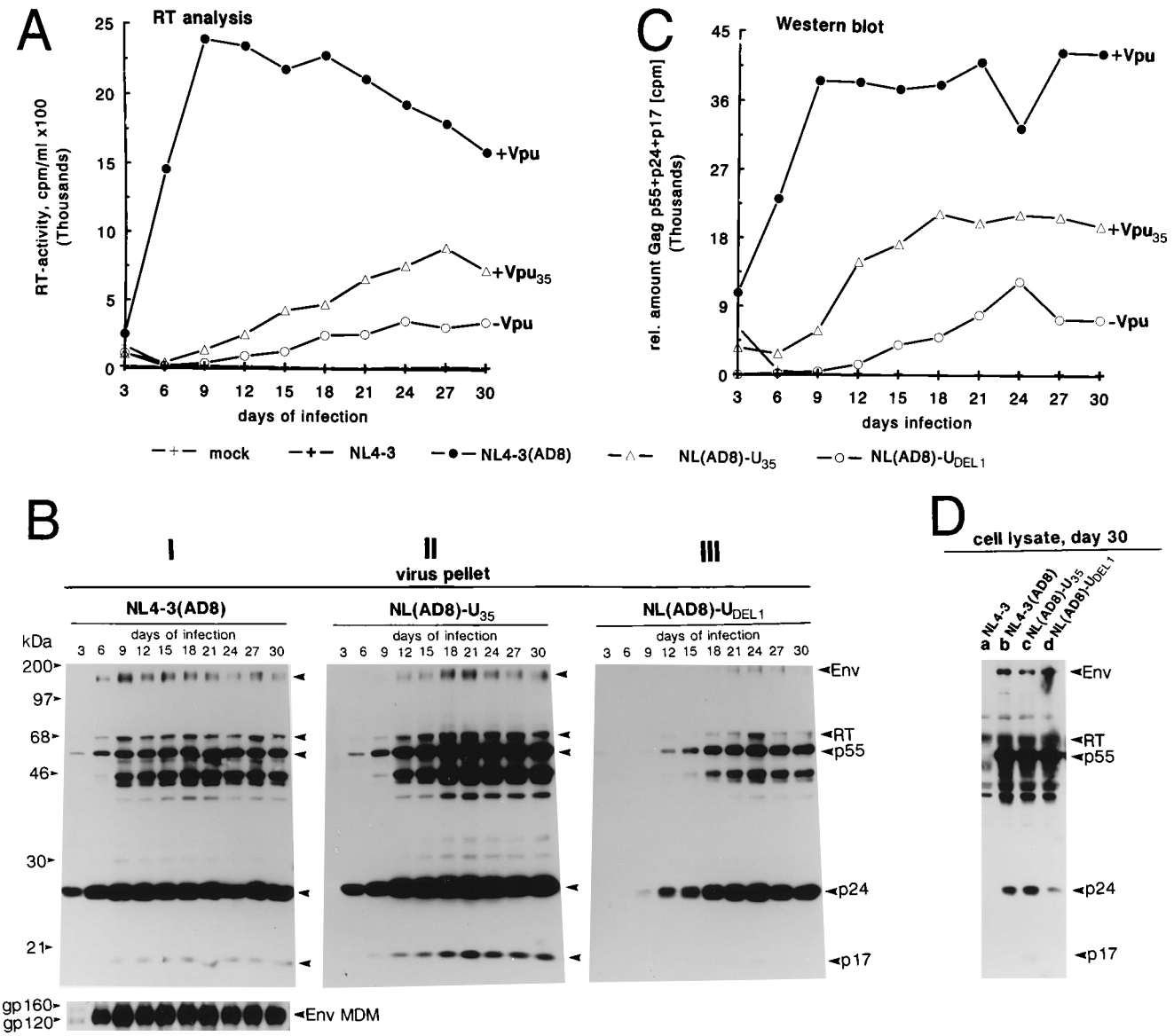

FIG. 3. Infection with purified virus stocks improves replication of *vpu*$^+$ and *vpu* viruses in MDM. (A) Parallel cultures of 7.5×10^5 MDM were infected with equal RT units (1 cpm per cell) of purified virus stocks of NL4-3(AD8), NL(AD8)-U_{35}, and NL(AD8)-U_{DEL1}. As a control, parallel cultures were infected with T-cell-tropic virus NL4-3 or were mock infected. RT profiles were acquired over a 1-month period. (B) Pelleted virions from individual time points were analyzed by Western blot analysis as described for Fig. 2B. Autoradiograms: panel I, NL4-3(AD8); panel II, NL(AD8)-U_{35}; panel III, NL(AD8)-U_{DEL1}. Note that the autoradiograms shown in panels II and III were exposed six times longer than the autoradiogram shown in panel I. At the bottom of panel I, a longer exposure of the region depicting the Env proteins of NL4-3(AD8) is shown. (C) Gag proteins (p17, p24, and p55) present in the virus pellets in panel B were quantitated with an image analyzer and are plotted as a function of time. (D) Lysates of cells harvested at day 30 of infection were analyzed by Western blotting as in Fig. 2D.

Variations in the amounts of extracellular virus observed for the three infected cultures (Fig. 3A to C) are contrasted by the relatively constant levels of intracellular proteins in these cultures (Fig. 3D, lanes b to d), indicating that defects in the *vpu* gene result in the relative accumulation of viral proteins in macrophages.

Taken together, these results demonstrate that the use of purified virus stocks significantly increases the capacity of wild-type and *vpu* mutant viruses to replicate in macrophages. Consistent with the results from other cell systems, *vpu*-deficient mutants nevertheless produced significantly reduced levels of extracellular virus, paralleled by the relative accumulation of intracellular viral proteins. This suggests that Vpu regulates the release of progeny virions from infected macrophages to a similar extent and through a similar mechanism to that observed previously with CD4$^+$ T-cell lines or HeLa cells (26, 32, 44, 46, 48, 52–54). As a result of this experiment, all subsequent studies were done with purified virus stocks.

Effects of Vpu on long-term infection of macrophages. The results of the experiments in Fig. 2 and 3 indicate that Vpu has the capacity to enhance virus release from macrophage cultures. However, because of the absence of an apparent peak in the replication profile of *vpu* mutant virus, it cannot be ruled out that replication of Vpu-deficient virus is merely delayed and the peak virus production was not observed in Fig. 3 because of premature termination of the experiment. In addition, it is possible that some of the differences observed in the previous experiments are attributable to donor variability.

Finally, it is conceivable that different virus isolates exhibit different responses to Vpu. To address these issues, we performed a comprehensive comparison of three different HIV-1 isolates and their corresponding Vpu mutants and analyzed their response to Vpu in MDM from different donors. The three isolates analyzed include AD8$^+$ (Fig. 1, panel IIIa), which is a Vpu-positive molecular clone of an original macrophage-tropic isolate (55); NL4-3(AD8) (Fig. 1, panel Ia); and NL4-3(SF) (Fig. 1, panel IIa) (15), which is an isogenic variant of NL4-3(AD8) with an *env* determinant derived from the monocyte-tropic HIV-1 isolate, SF162 (8). AD8-U_{DEL2}, NL(AD8)-U_{DEL1}, and NL(SF)-U_{DEL1} (Fig. 1, panels Id, IIb, and IIIb, respectively) are the corresponding *vpu*-deficient variants which were analyzed in parallel. It is worth noting that NL4-3(AD8) and NL(SF) encode the NL4-3 *vpu* gene while the *vpu* gene in AD8$^+$ is part of an original macrophage-tropic isolate (55) and has 79% amino acid homology with the NL4-3 *vpu* gene.

Purified virus stocks were prepared from HeLa cells, and parallel MDM cultures were infected with RT units equal to those used in the previous experiments. Culture supernatants were harvested at 3-day intervals over a period of 2 months, and the RT profiles were determined (Fig. 4). The overall replication efficiency of chimeric viruses in macrophages was dependent on the *env* gene: NL4-3(AD8), carrying the *env* determinant of the AD8$^+$ isolate, replicated more efficiently than did NL4-3(SF), carrying the SF162 *env* gene (Fig. 4A and B). However, independent of the particular virus isolate used, release of virus particles was consistently increased four- to sixfold in the presence of Vpu throughout the course of infection and was donor independent. Vpu-deficient cultures did not show a delayed peak in virus production even when virus replication was monitored over a period of 2 months. Thus, Vpu stimulates virus production from infected macrophage cultures independently of the host cell donor and of the virus isolate used.

Vpu supports virus release from PBMC. We next analyzed the effect of Vpu on replication of macrophage-tropic virus isolates in PBMC, another major target for HIV infection in vivo. Published reports describing the role of Vpu in replication of monocyte-tropic viruses in PBMC are inconsistent and range from almost no effect on virus production (2, 4, 30) to an approximately threefold increase in the level of extracellular virus (59). None of these studies included a direct comparison of extracellular and intracellular viral proteins, which would allow us to assess the efficiency of virus release in the presence or absence of Vpu.

We analyzed the role of Vpu in PHA- plus IL-2-stimulated PBMC from a healthy donor following infection with purified virus stocks of the primary isolate AD8$^+$ or AD8-U_{DEL2} (Fig. 5B), as well as the chimeric viruses NL4-3(AD8) and NL (AD8)-U_{DEL1} (Fig. 5A). The multiplicity of infection was identical to that used for infection of macrophages (1 RT unit per cell). Aliquots of the culture supernatants were collected every other day, and the replication profiles (RT activity) were determined. As shown in Fig. 5A and B, both *vpu*$^+$ and *vpu*-deficient viruses replicated efficiently in PBMC, with peak virus production on day 6 p.i. for the primary isolate AD8$^+$ and on day 12 p.i. for the NL4-3(AD8) chimera. The presence of Vpu resulted in an approximately twofold augmentation of virus production during the time course of infection for both primary and chimeric monocyte-tropic viruses. AD8$^+$-infected cultures (Fig. 5B) produced approximately fourfold-higher levels of cell-free virus than did NL4-3(AD8)-infected cultures (Fig. 5A), suggesting a better adaptation of the primary isolate than the chimeric monocyte-tropic isolate for replication in PBMC. Despite the more efficient replication of AD8$^+$ in PBMC, a comparable effect of Vpu was observed for both primary and chimeric viruses (Fig. 5A and B).

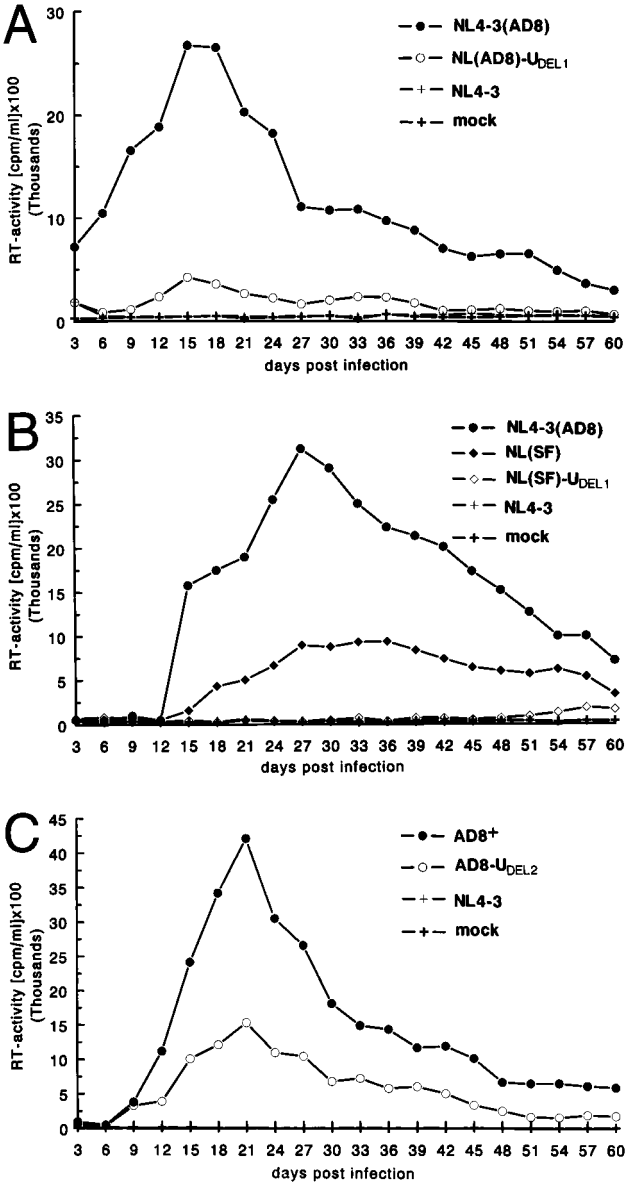

FIG. 4. Vpu-mediated enhancement of virus replication is donor independent and is a characteristic of multiple virus isolates. Parallel cultures of 7.5×10^5 MDM were infected with purified virus stocks of NL4-3(AD8) or NL(AD8)-U_{DEL1} (A); NL4-3(SF), NL4-3(AD8), or NL(SF)-U_{DEL1} (B); or AD8$^+$ or AD8-U_{DEL2} (C). As negative controls, cultures were infected with NL4-3 and were mock infected. Virus production was monitored over a 2-month period and quantitated by determining the RT activity secreted into the culture supernatants. MDM from different donors were used in panels A, B, and C.

To correlate the long-term effect of Vpu on replication of monocyte-tropic viruses in PBMC, as described in Fig. 5A and B, with its effect on short-term virus release kinetics, we performed pulse-chase analyses with infected PBMC (Fig. 5C and D). Parallel cultures of PHA- plus IL-2-stimulated PBMC were infected with AD8$^+$ (+Vpu) and AD8-U_{DEL2} (−Vpu) virus stocks. At peak virus production, cells were metabolically labeled with [^{35}S]methionine for 30 min and chased for the indicated periods as detailed in Materials and Methods. Viral

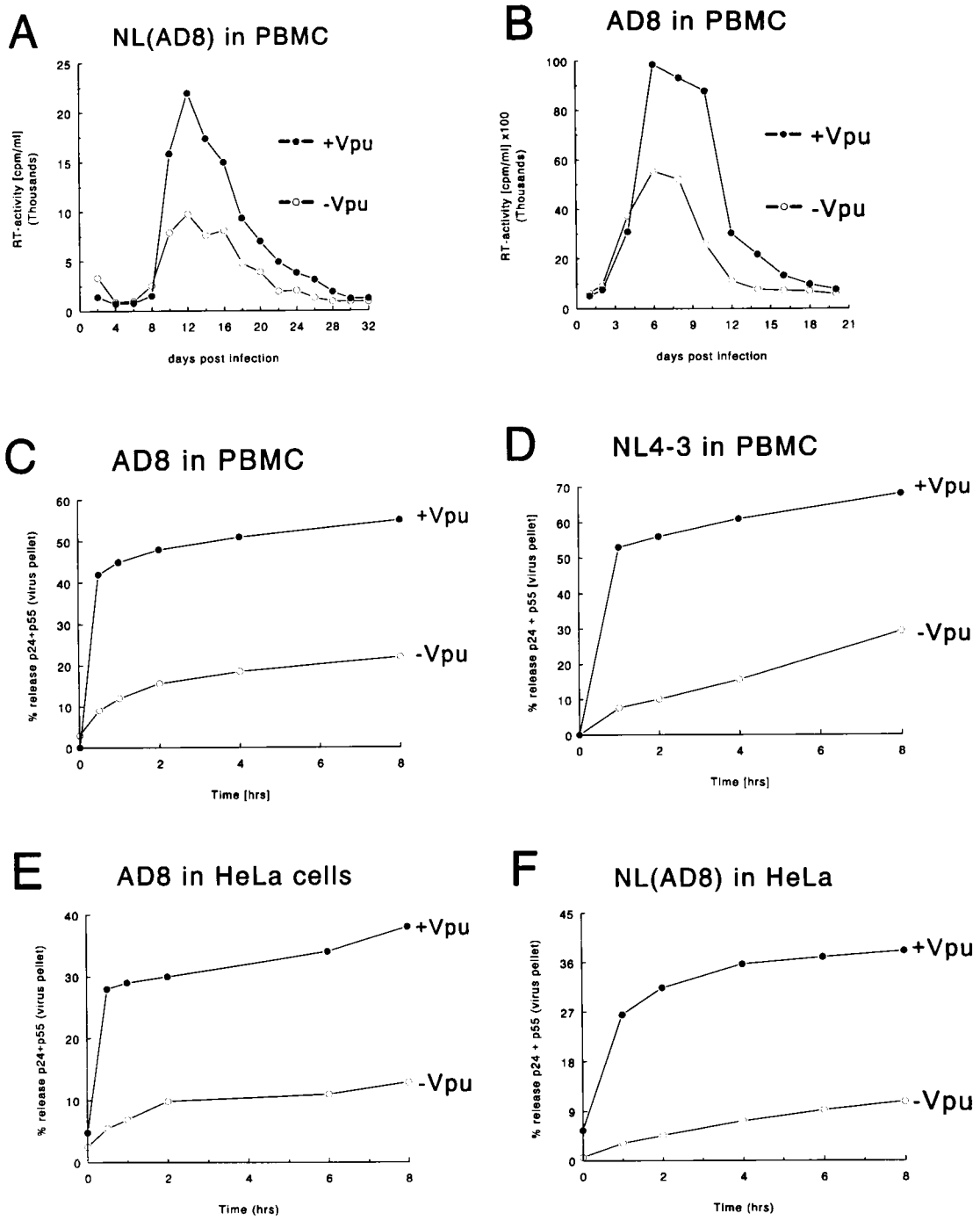

FIG. 5. Effect of Vpu on virus release from PBMC. PHA- plus IL-2-stimulated PBMCs were infected with equal RT units of purified viruses NL4-3(AD8) (+Vpu) or NL(AD8)-U_{DEL1} (−Vpu) (A) or $AD8^+$ (+Vpu) and AD8-U_{DEL2} (−Vpu) (B). Culture supernatants were collected every other day and assayed for RT activity. PHA- plus IL-2-activated PBMC were infected with *vpu*$^+$ and *vpu*-deficient variants of the AD8-type monocyte-tropic primary isolate (C) or the T-cell-tropic virus NL4-3 (D). Alternatively, HeLa cells were transfected with *vpu*$^+$ and *vpu*-deficient variants of the AD8-type (E) or the NL(AD8)-type chimera (F). At the peak of virus production, cells were metabolically labeled with [^{35}S]methionine for 30 min and chased for the indicated times as detailed in Materials and Methods. Viral proteins present in the cell lysates, the viral pellet fractions, and the clarified supernatants were immunoprecipitated with HIV-1-reactive human serum, separated by SDS-PAGE (data not shown) and quantitated with an image analyzer. Secretion of viral particles was calculated as the percentage of Gag proteins present in the viral pellet relative to the sum of Gag proteins detected intra- and extracellularly.

proteins present in the cell lysates, the viral pellet fractions, and the clarified supernatants were immunoprecipitated with HIV-1-reactive human serum, separated by SDS-PAGE (results not shown), and quantitated with an image analyzer. Secretion of viral particles was calculated as the percentage of Gag proteins present in the viral pellet relative to the sum of Gag proteins detected intra- and extracellularly (Fig. 5C). Virus release kinetics increased approximately two- to threefold

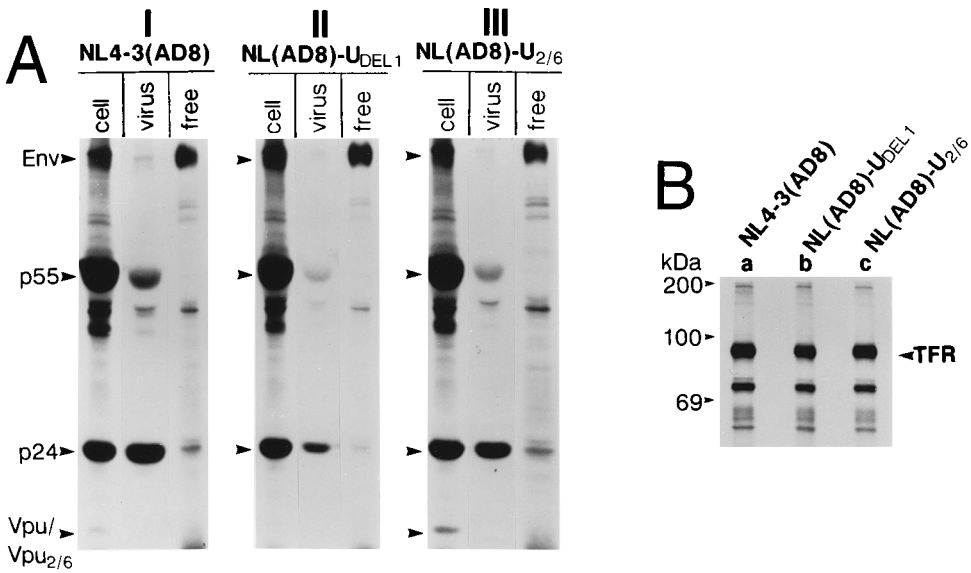

FIG. 6. Effect of Vpu on virus release evaluated by steady-state metabolic labeling of infected MDM cultures. (A) Parallel cultures of 7.5×10^5 MDM were infected with purified virus stocks of NL4-3(AD8) (*vpu*$^+$), NL(AD8)-$U_{2/6}$ (*vpu*$_{2/6}^+$), and NL(AD8)-U_{DEL1} (*vpu*). At peak virus production, cells were metabolically labeled with [^{35}S]methionine for 24 h. Viral proteins from cell lysates (cell), the pelleted virions (virus), or the clarified supernatants (free) were immunoprecipitated with a 1:1 mixture of polyclonal HIV-1 Env antisera (rabbit) and an HIV-1-reactive human serum sample, separated by SDS-PAGE (10.5% polyacrylamide), and analyzed by fluorography. (B) As a control, 50% of the cell lysates were immunoprecipitated with a monoclonal antibody specific for the transferrin receptor (TFR) and analyzed as described for panel A.

in the presence of Vpu in comparison with the *vpu*-deficient culture. These results are comparable to the release kinetics observed for the T-cell-tropic isolate NL4-3 in PBMC (Fig. 5D). Similar effects of Vpu on virus release kinetics were also observed when HeLa cells were transfected with plasmid DNAs of the *vpu*$^+$/*vpu*-deficient molecular clones of AD8$^+$ (Fig. 5E) or the NL4-3(AD8) chimera (Fig. 5F) and subjected to similar pulse-chase analysis.

Steady-state and pulse-chase metabolic labeling in macrophages. The analysis of the replication profiles of *vpu*$^+$ or *vpu*-deficient monocyte-tropic isolates by means of an RT assay or Western blot analysis, as demonstrated in Fig. 2 through 4, suggests that Vpu augments virus release from infected macrophages. To directly demonstrate the effects of Vpu on the release kinetics of HIV-1 from infected macrophages, we performed a series of biochemical studies including steady-state metabolic labeling and pulse-chase analysis. For steady-state labeling, parallel cultures of MDM were infected with purified NL4-3(AD8), NL(AD8)-$U_{2/6}$, or NL(AD8)-U_{DEL1} virus stocks. At peak virus production, cells were metabolically labeled with [^{35}S]methionine for 24 h as described in Materials and Methods. Metabolically labeled viral proteins from cell lysates, pelleted virions, and clarified supernatants were immunoprecipitated and separated by SDS-PAGE (10.5% polyacrylamide) followed by fluorography (Fig. 6A). In parallel, transferrin receptor molecules were immunoprecipitated from cell lysates to control for experimental variations (Fig. 6B). Efficient particle release was observed for wild-type NL4-3 (AD8) and the Vpu phosphorylation mutant NL(AD8)-$U_{2/6}$. Approximately 60 or 40% of p24^{gag}, respectively, was associated with cell-free virus particles (compare Fig. 6A, panels I and III). In contrast, virus secretion from the *vpu*-deficient culture was less efficient and the majority of viral proteins remained cell associated (Fig. 6A, panel II). Considerable amounts of soluble proteins, in particular Env, which migrated as a broad band, were detected in all cultures. However, the relative amounts of soluble proteins in the three cultures were similar and were not affected by Vpu. The amounts of transferrin receptor detected in the three lysates were indistinguishable, indicating that the experimental conditions were comparable.

To investigate the contribution of Vpu on virus release kinetics from macrophages by pulse-chase analysis, cells were infected with equal RT units of purified AD8$^+$ (+Vpu) and AD8-U_{DEL2} (−Vpu) virus stocks. Pulse-chase experiments were conducted at the time of maximum virus production, as detailed in Materials and Methods. Briefly, cells were pulse labeled with [^{35}S]methionine for 30 min and chased for up to 8 h as indicated in Fig. 7. Replicate wells of MDM cultures were used for each time point of the chase period, and cells were left attached during the pulse-labeling and chase periods. Cells were then harvested and lysed, and radiolabeled viral proteins from lysates of cells, pelleted virions, and the clarified supernatants were immunoprecipitated and separated in an 8% Acryl-Aide gel. Only fluorograms from cell lysates and virus pellets are shown in Fig. 7A and B. Gels were quantitated with an image analyzer, and the relative amounts of Pr55^{gag} and p24^{gag} present in the viral pellet were calculated from the total amount of cell-associated, soluble, and virion-associated proteins (Fig. 7C). The results of this experiment demonstrate that Vpu increases the kinetics of virus release from infected macrophages. In the presence of Vpu, approximately 40% of metabolically labeled proteins were associated with virus particles within 8 h after synthesis compared with only 7% of viral proteins in the absence of Vpu. Vpu had no apparent effect on protein synthesis or maturation of the structural HIV proteins (Fig. 7A and B, panels "cell") or the nonspecific secretion of non-particle-associated viral proteins (results not shown).

In summary, we conclude that Vpu affects the kinetics of virus particle release from infected macrophages to a similar extent to that observed in PBMC (Fig. 5) or in permanent human cell lines (26, 32, 44, 46, 48, 52–54, 68).

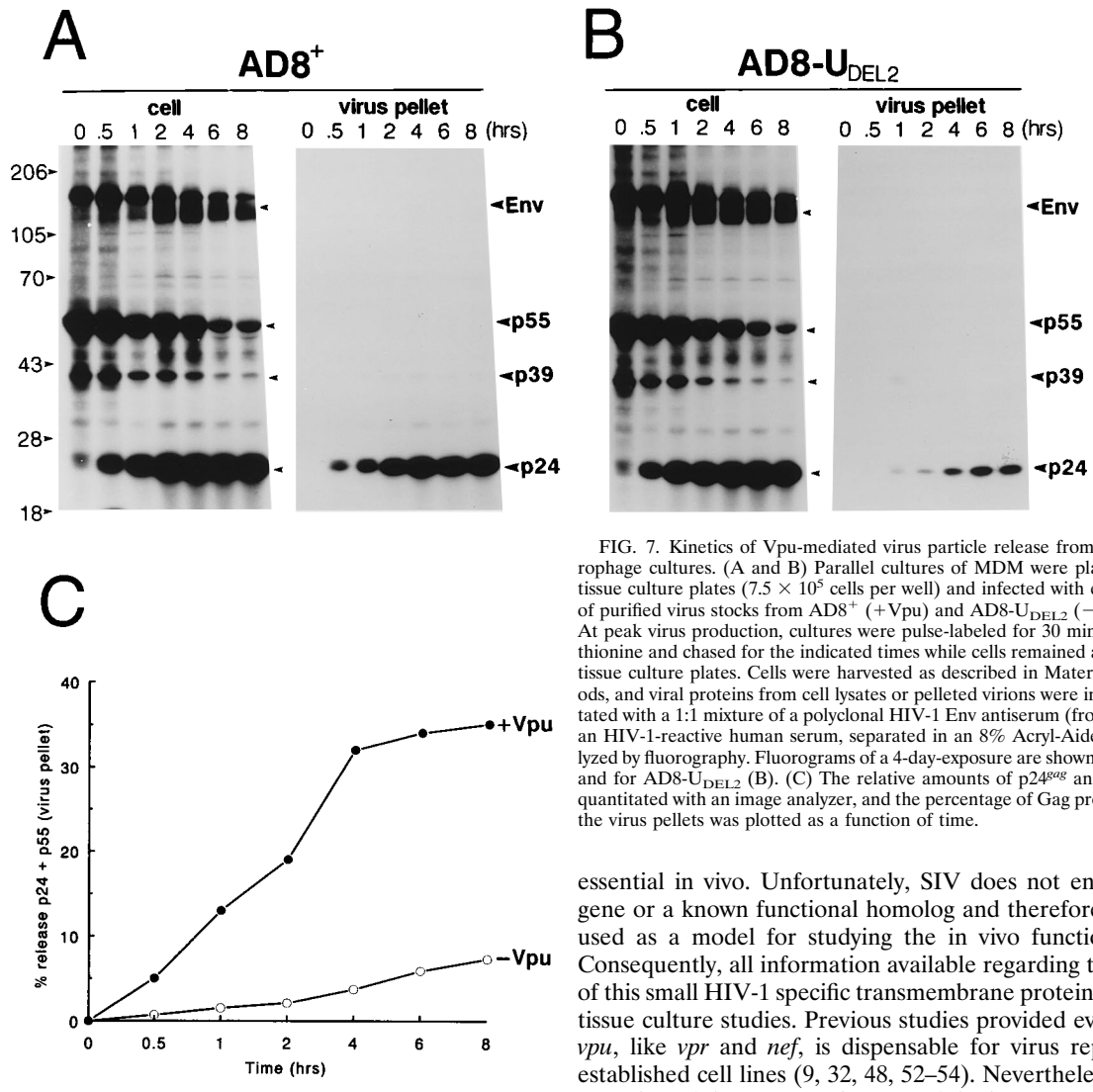

FIG. 7. Kinetics of Vpu-mediated virus particle release from infected macrophage cultures. (A and B) Parallel cultures of MDM were plated in 24-well tissue culture plates (7.5×10^5 cells per well) and infected with equal RT units of purified virus stocks from $AD8^+$ (+Vpu) and AD8-U_{DEL2} (−Vpu) variants. At peak virus production, cultures were pulse-labeled for 30 min with [^{35}S]methionine and chased for the indicated times while cells remained attached to the tissue culture plates. Cells were harvested as described in Materials and Methods, and viral proteins from cell lysates or pelleted virions were immunoprecipitated with a 1:1 mixture of a polyclonal HIV-1 Env antiserum (from rabbits) and an HIV-1-reactive human serum, separated in an 8% Acryl-Aide gel, and analyzed by fluorography. Fluorograms of a 4-day-exposure are shown for $AD8^+$ (A) and for AD8-U_{DEL2} (B). (C) The relative amounts of $p24^{gag}$ and $Pr55^{gag}$ were quantitated with an image analyzer, and the percentage of Gag proteins found in the virus pellets was plotted as a function of time.

DISCUSSION

Over the past years, an immense effort has been put into the investigation of the function of HIV accessory proteins. The biological phenotypes attributed to the activity of some of these factors in vitro are rather subtle, and mutations in several of the accessory genes do not significantly interfere with virus replication in tissue culture systems. This had led to an intense discussion concerning the relevance of these factors for HIV replication in vivo. Insights into the in vivo importance of some of the accessory genes have been gained from the analysis of SIV strains, which are similar to human lentiviruses and encode homologous *vif*, *vpr*, and *nef* genes and for which an animal model is available (for a review, see reference 50). For example, studies with rhesus monkeys infected with SIV_{mac} demonstrated that high virus load and full pathologic potential require a functional *nef* gene, for which a strong selective pressure occurs in vivo (31). Similarly, selective pressure for a functional *vpr* gene was observed in SIV_{mac}-infected rhesus monkeys, suggesting an important role of Vpr in virus persistence and disease progression (35). These results imply that the functions of accessory genes, while dispensable in vitro, are essential in vivo. Unfortunately, SIV does not encode a *vpu* gene or a known functional homolog and therefore cannot be used as a model for studying the in vivo function of Vpu. Consequently, all information available regarding the function of this small HIV-1 specific transmembrane protein is based on tissue culture studies. Previous studies provided evidence that *vpu*, like *vpr* and *nef*, is dispensable for virus replication in established cell lines (9, 32, 48, 52–54). Nevertheless, the conservation of the *vpu* gene in most of the known HIV-1 isolates suggests an important role in the virus life cycle in vivo. Furthermore, it has been generally accepted that Vpu augments virus secretion from a variety of human cells, including the $CD4^+$ T-cell lines A3.01 (14, 32, 44, 46, 48, 52, 53), Jurkat (54), and MT4 (68), as well as non-T-cell lines such as epithelial HeLa cells (23, 26, 44, 46, 48, 52, 53, 68) and SW480 colon carcinoma cells (26). However, Geraghty et al. (23) and Göttlinger et al. (27), who analyzed the function of Vpu in Cos-1, Cos-7, and CV1 cells, were unable to detect Vpu-mediated enhancement of virus secretion in simian cell lines. These findings could suggest a cell-type-specific function of Vpu. However, inasmuch as Cos and CV1 cells are nonhuman cell lines, it is also possible that the absence of detectable Vpu activity in these cells reflects a species specificity of Vpu rather than a specificity for distinct human cell types. Nevertheless, the absence of detectable Vpu activity on virus release in Cos cells suggests the involvement of cellular factors. Indeed, Geraghty et al. have reported on a 60-kDa Vpu-interacting protein, VIP, identified from a human lymphocyte cDNA library by using the yeast two-hybrid system (23). On the basis of our recent finding that Vpu-mediated virus release is correlated with an ion channel activity of Vpu (46), a cell- or species-specific activity of Vpu would imply that its ion channel activity

is regulated by cellular factors or, alternatively, that the Vpu ion channel activity regulates virus release indirectly by influencing the transport of cellular chaperones involved in the assembly and/or secretion of HIV virions.

Because of the lack of an animal model for Vpu, primary human cells, i.e., PBMC or macrophages, are currently the most relevant model system available to study the function of Vpu. Although several groups have already investigated the function of Vpu in human PBMC or macrophage cultures, their results are conflicting. For example, Balliet et al. (4) and Kawamura et al. (30), using monocyte-tropic HIV-1, and Akari et al. (2), using T-cell-tropic HIV-1, were unable to detect a significant increase in virus secretion from PBMC in the presence of Vpu. In contrast, our own work supports the notion that Vpu, in the context of either the T-cell-tropic (44) or monocyte-tropic (Fig. 5) viruses, is fully active on virus release in PBMC. Furthermore, Westervelt et al. (59) and Kawamura et al. (30), using chimeric monocyte-tropic isolates, reported a rather moderate, i.e., two- to threefold, effect of Vpu in monocytes, while Balliet et al. (4), using the primary monocyte-tropic isolate HIV-1/89.6 (10), reported an up to 1,000-fold increase in virus production in macrophages. In contrast, Balliet et al. reported no differences between *vpu*$^+$ and *vpu*-deficient forms of HIV-1/89.6 in terms of virus release from PBMC and the T-cell–B-cell hybrid cell line CEMX174 (4). In addition, Westervelt et al. (59) demonstrated that Vpu and Vpr proteins are capable of functional complementation in monocytes, a result which was not supported by findings of Kawamura et al. (30). In our own studies, Vpu was able to rescue neither a donor-dependent impairment of virus replication in the absence of Vpr nor the negative effect of *vif*-deficient mutants in macrophages (data not shown).

Because of the controversial results concerning the function of Vpu in primary cell systems, the goal of our study was to perform a side-by-side analysis of *vpu*$^+$ and *vpu*-deficient primary and chimeric monocyte-tropic HIV-1 isolates in PBMC and macrophages. In addition to studying virus secretion by RT assay or Western blot, we extended our study to include pulse-chase analyses to evaluate the Vpu-mediated augmentation of virus release kinetics in both PBMC and macrophage cultures.

Using a set of two different chimeric isolates and one primary monocyte-tropic isolate, we tested the function of Vpu in cultures of PBMC and macrophages from multiple donors. In agreement with Vpu effects determined previously in human cell lines (32, 44, 46, 48, 52, 53), we generally observed a significant augmentation of virus release in the presence of wild-type Vpu which was approximately four- to sixfold in macrophages and approximately two- to threefold in PBMC. In addition to wild-type Vpu, we analyzed partially active mutants: Vpu_{35}, encoding the N-terminal membrane anchor, and $Vpu_{2/6}$, encoding nonphosphorylated Vpu. Both mutants had residual activities in primary cells similar to those observed in HeLa cells and CD4$^+$ T cells (44, 48).

One explanation for the difference between our results and those of previous Vpu studies with primary human cells could be the differences in the experimental setups used by individual investigators in terms of macrophage culture, preparation of virus stocks, *vpu* mutants used, and biochemical analysis of virus release. While macrophages were precultured in the presence of granulocyte-macrophage colony-stimulating factors in previous analyses (4, 30, 59), we examined virus replication in macrophages cultured in medium supplemented solely with pooled human serum without exogenous cytokines. Furthermore, other authors investigated Vpu function in macrophages (4, 30, 59) infected with cell culture supernatant, i.e., unpurified virus stocks. The results of our studies indicate that the use of unpurified virus stocks can significantly interfere with the infection of macrophage cultures and produce aberrant results. Although the nature of this inhibitory effect is not understood, it is likely that soluble gp120, which is shed into HeLa supernatants in significant amounts during the production of virus stocks (48, 65), contributes to the observed inhibition of virus replication. It is also conceivable that in the process of virus purification, potential aggregates of virions are dispersed, thus increasing virus infectivity. Finally, all data addressing the function of Vpu in primary cell systems, either macrophages or PBMC, have so far been restricted to the analysis of extracellular viral proteins, examined by means of either RT activity (2, 30, 59) or p24 antigen capture assay (4). These methods do not necessarily distinguish between nonspecific secretion of viral proteins and release of virus particles. Therefore, in addition to RT assay and Western blot analysis of pelleted virions, we assayed Vpu-regulated release kinetics of virions by steady-state and pulse-chase metabolic labeling in PBMC and MDM. Our results from the RT, Western blot, and pulse-chase analyses with purified virus stocks for infection of PBMC and MDM are consistent and support the conclusion that Vpu is active not only in human cell lines but also in primary lymphocytes and macrophages. The Vpu effect observed was independent of the donor used for PBMC and MDM preparation and of the virus isolate used for infection. Furthermore, in all cases, mutations in *vpu* had effects in primary cells which were similar to those observed in HeLa cells or A3.01 cells, for which all of these mutants have been characterized previously (32, 44, 48, 52, 53). Therefore, our data do not support the idea of a cell-type-specific function of Vpu.

In agreement with other reports (4, 20, 30, 59), we were unable to detect a significant contribution of Vpu to the cytopathicity of HIV in infected macrophages. Both *vpu*$^+$ and *vpu*-deficient viruses showed characteristic cytopathic effects such as the formation of multinucleated giant cells, often containing 10 or more nuclei per cell, followed by cell lysis. In contrast to the phenotype of Vpu in macrophages, Vpu clearly affected the onset of cytopathic changes in infected PBMC; Vpu-deficient cultures exhibited higher cytopathic effects in terms of both number and size of syncytia as well as the onset of cytopathic changes, which generally were observed 2 days earlier than in the corresponding *vpu*$^+$ cultures. This phenomenon is consistent with the previous observation that Vpu reduces cytopathic effects in CD4$^+$ T-cell lines and could be explained by the more efficient export of cytotoxic viral factors as a result of the Vpu-mediated augmentation of virus release (32, 48, 52, 53, 67).

ACKNOWLEDGMENTS

We are indebted to Malcolm Martin for his support, and we thank Malcolm Martin, Eric Freed, Stephan Bour, Ronald Willey, and Frank Maldarelli for critical comments and helpful discussions. We thank Eric Freed for plasmids pNL4-3(AD8) and pNL(SF), Ted Theodore for plasmid pAD8$^+$, Tomas Porstmann for Vpu antiserum, and Karis Faust-Weih for PBMC and MDM preparation. We thank Ronald Willey for HIV-1 Env antisera, for technical support with pulse-chase analysis in macrophages, and for sharing unpublished data.

U.S. was the recipient of a fellowship from the AIDS research program of the Deutsches Krebsforschungszentrum Heidelberg, Heidelberg, Germany. Part of this work was supported by grant Po-396/3-1 from the Deutsche Forschungs Gesellschaft, Germany, to U.S. and by a grant from the NIH Intramural AIDS Targeted Antiviral Program to K.S.

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