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The Human Immunodeficiency Virus (HIV) Type 2 Envelope Protein Is a Functional Complement to HIV Type 1 Vpu That Enhances Particle Release of Heterologous Retroviruses

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We have recently shown that the envelope glycoprotein of the ROD10 isolate of human immunodeficiency virus type 2 (HIV-2) has the ability to positively regulate HIV-2 viral particle release. The activity provided by the ROD10 Env was remarkably similar to that of the HIV-1 Vpu protein, thus raising the possibility that the two proteins act in a related fashion. We now show that the ROD10 Env can functionally replace Vpu to enhance the rate of HIV-1 particle release. When provided in *trans*, both Vpu and the ROD10 Env restored wild-type levels of particle release in a Vpu-deficient mutant of the NL4-3 molecular clone with indistinguishable efficiencies. This effect was independent of the presence of the HIV-1 envelope protein. The ROD10 Env also enhanced HIV-1 particle release in the context of HIV-2 chimeric viruses containing the HIV-1 *gag-pol*, indicating a lack of need for additional HIV-1 products in this process. In addition, we show for the first time that HIV-1 Vpu, as well as ROD10 Env, has the ability to enhance simian immunodeficiency virus (SIV) particle release. The effects of Vpu and ROD10 Env on SIV particle release were indistinguishable and were observed in the context of full-length SIVmac239 and simian-human immunodeficiency virus chimeras. These results further demonstrate that ROD10 Env can functionally complement Vpu with respect to virus release. In contrast, we found no evidence of a destabilizing activity of ROD10 Env on the CD4 molecule. HIV-1 and HIV-2 thus appear to have evolved genetically distinct but functionally similar strategies to resolve the common problem of efficient release of progeny virus from infected cells.

Vpu is an accessory protein specifically encoded by human immunodeficiency virus type 1 (HIV-1) (8, 26, 43). Expression of Vpu has been shown to enhance viral particle release from infected human cell lines (42–44), a phenomenon that correlates with reduced cytopathicity compared with *vpu*-deficient isolates (19, 48). The positive effect of Vpu on viral particle release can be observed in established human cell lines, primary human macrophages, and peripheral blood mononuclear cells infected with HIV-1 (34) and is thus cell type independent. Data obtained in transient expression systems uncovered a second biological activity of the Vpu protein that results in the specific intracellular degradation of the CD4 receptor (22, 45–47).

Although the molecular mechanisms underlying the two biological activities of Vpu are not fully understood, extensive biochemical characterization of Vpu has led to a better understanding of the structure-function relationships in that protein. Vpu is an 81-residue homo-oligomeric integral membrane phosphoprotein translated from a bicistronic mRNA that also contains the *env* open reading frame (ORF) (24, 38, 42). Vpu consists of two main structural domains which correlate with biological activities of the protein: the N-terminal hydrophobic domain, which anchors the protein to membranes, and the C-terminal polar cytoplasmic domain (13, 33, 34). The latter domain appears to be directly involved in CD4 degradation (7, 46, 47); it contains two essential phosphorylated serine residues (12, 35, 36) and is likely to mediate the physical interactions between Vpu and the CD4 cytoplasmic tail (6). On the other hand, the transmembrane domain is sufficient to partially support viral particle release although regulatory elements provided by the cytoplasmic domain are essential for the full activity of the protein (33).

In light of the multiple biological activities provided by the Vpu protein during the HIV-1 life cycle, it is intriguing that except for the chimpanzee simian immunodeficiency virus (SIV) isolate SIVcpz (16), no functional equivalent to Vpu is found in related viruses such as HIV-2 or SIV. This fact seemed especially paradoxical since Vpu was shown to augment the release of chimeric viruses bearing the *gag-pol* regions of retroviruses that naturally lack a *vpu* ORF, such as HIV-2, visna virus, and Moloney murine leukemia virus (15). By examining the efficiency of particle release and Vpu responsiveness of the ROD10 full-length molecular clone of HIV-2, we recently showed that mutations that disrupted the pROD10 *env* ORF, but not the *vif*, *vpr*, *vpx*, or *nef* ORF, had a profound negative effect on virus particle release (5). Concomitantly, the pROD10 envelope glycoprotein provided in *trans* could rescue the envelope mutants and restore wild-type levels of particle release (5). The efficiency with which the HIV-2 Env enhanced HIV-2 particle release was very similar to that of Vpu. Both activities could be provided in *trans* and were sensitive to treatment of cells with brefeldin A (BFA), suggesting that they both operate in a post-endoplasmic reticulum (ER) compart-

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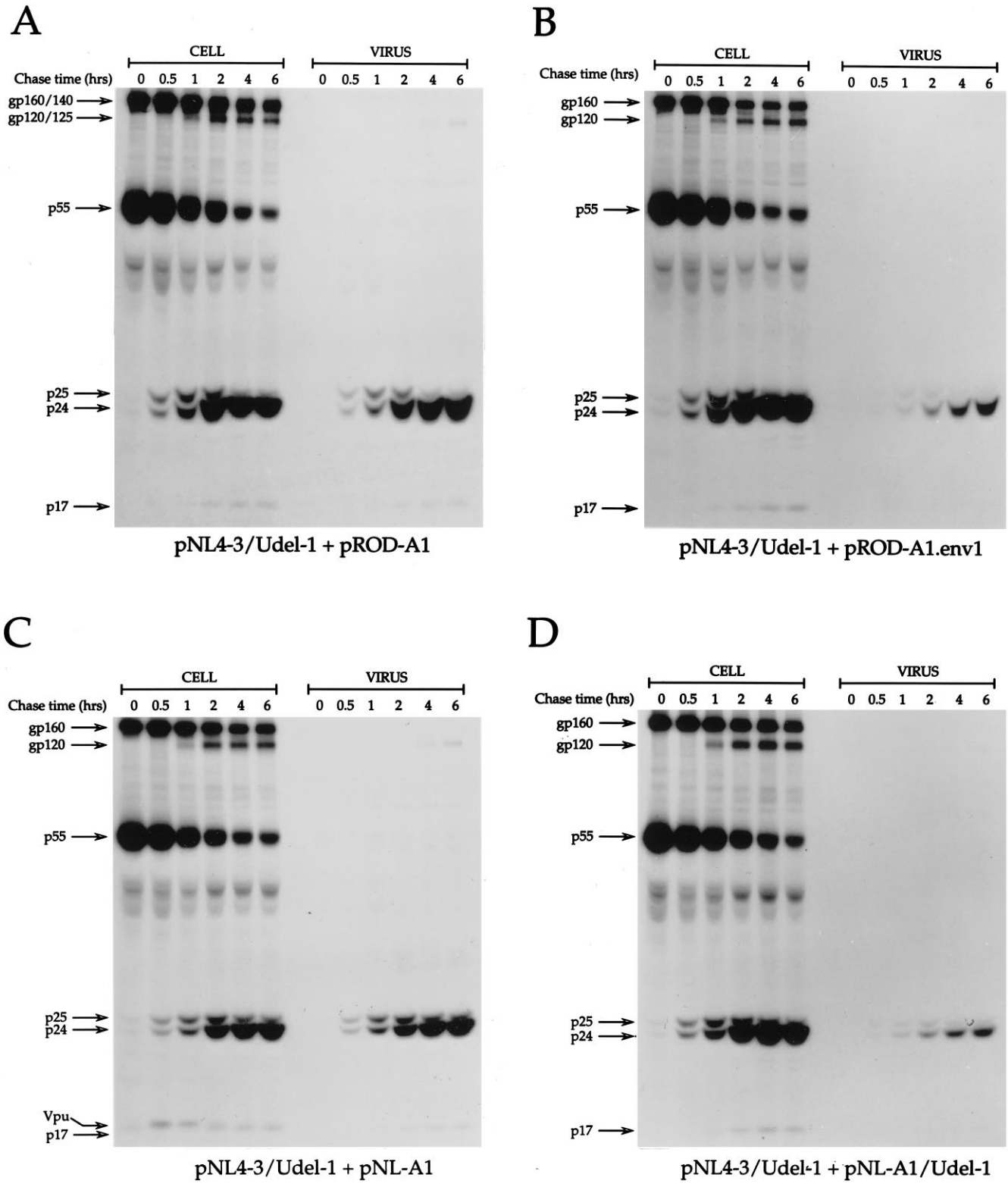


ment (5). Despite these similarities, the question remained as to whether the particle release activity of ROD10 Env was restricted to HIV-2 or whether it could also enhance particle release by other retroviruses, thus representing a functional counterpart to HIV-1 Vpu.

In this report, we examine whether the HIV-2_{ROD10} envelope glycoprotein is a genuine Vpu-like factor through its ability to complement Vpu in enhancing HIV-1 particle release and to act on other retroviral Gag-Pol. We show that identical enhancement of HIV-1 viral particle release is observed when

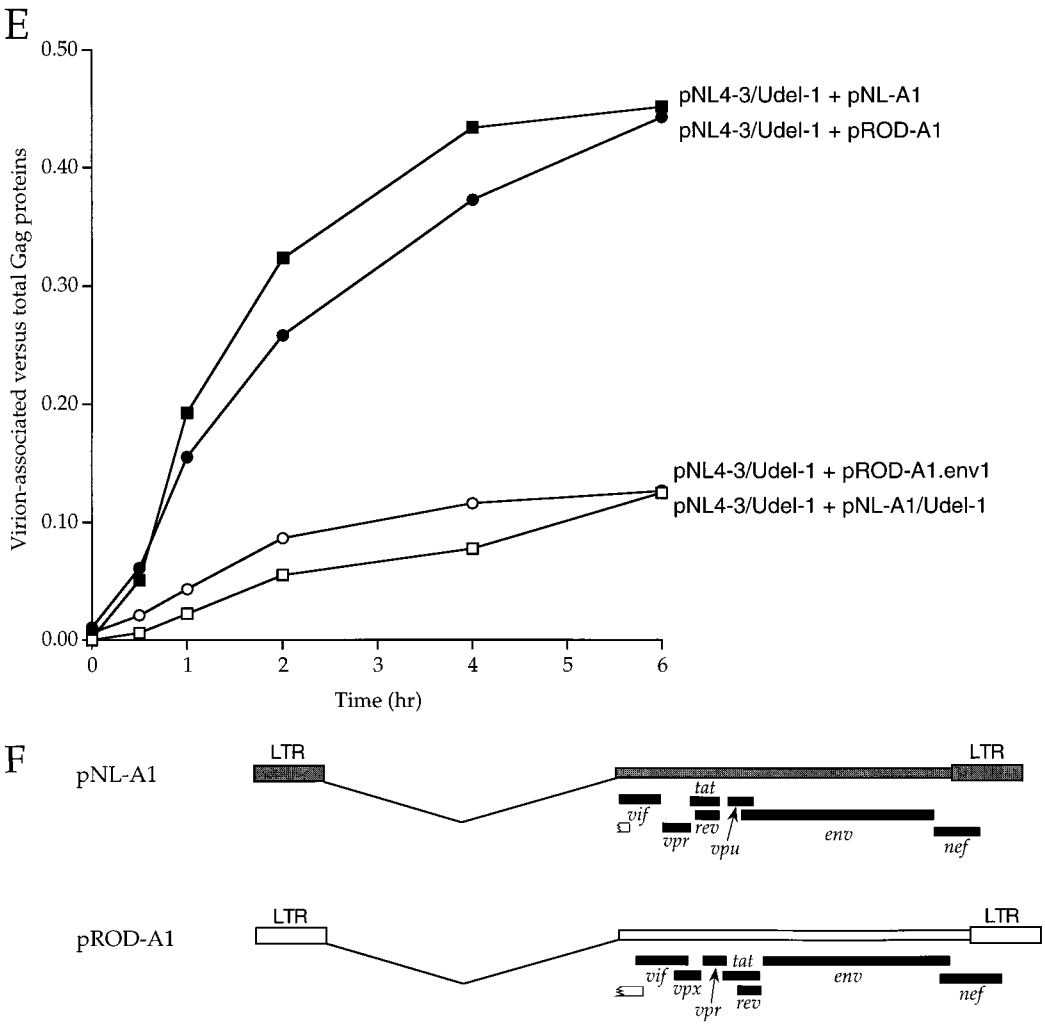


FIG. 1. The HIV-2_{ROD10} envelope glycoprotein enhances HIV-1 particle release. (A to D) Kinetic analysis of viral particles release by the Vpu-deficient pNL4-3/Udel-1 in the presence or absence of Vpu and the ROD10 envelope protein. HeLa cells were transfected with 20 μg of pNL4-3/Udel-1 DNA together with 10 μg of the $gp140^+$ pROD-A1 vector (A), 10 μg of the $gp140^-$ pROD-A1.env1 vector (B), 10 μg of the Vpu^+ pNL-A1 vector (C), and 10 μg of the Vpu^- pNL-A1/Udel-1 vector (D) and subjected to pulse-chase analysis. Cells were pulse-labeled for 30 min with Trans^{35}S-Label (1 mCi/ml) and chased for the indicated times. At each time point, aliquots of the cells and the virus-containing supernatants were harvested, and virus particles present in the culture medium were pelleted. Cells and viral pellets were lysed in NP-40–DOC buffer. Viral proteins were recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions with a mixture of anti-HIV-1 patient serum and anti-HIV-2 Env rabbit serum. Viral proteins were separated by SDS-PAGE (12% gel) and visualized by fluorography. The HIV-1 major Gag proteins $p55^{gag}$, $p24\text{-}25^{CA}$, and $p17^{MA}$ are identified on the left. The HIV-2 envelope glycoprotein precursor gp140 is identified on the left in panels C and D as a band comigrating with the gp160 envelope precursor of HIV-1. (E) Bands corresponding to the HIV-1 major Gag proteins in panels A to D were quantified with an image analyzer. The efficiency of particle release at each time point was calculated by dividing the amount of Gag proteins present in the pelleted virus fraction by the total of cell- and virus-associated Gag proteins. The ratio of virion-associated to total Gag proteins was then plotted as a function of time. (F) Schematic representation of the pNL-A1 and pROD-A1 constructs. Plasmids were derived from the pNL4-3 (HIV-1) and pROD10 (HIV-2) molecular clones as previously described (5, 43).

either Vpu or ROD10 Env is provided in *trans* to a Vpu mutant of the NL4-3 (1) molecular clone of HIV-1. The positive effect of ROD10 Env on HIV-1 particle release was independent of HIV-1 proteins outside the Gag-Pol region, including the HIV-1 Env, further suggesting similarities with the mechanism of action of Vpu (14, 49). In contrast, no evidence of CD4 degradation was observed in the presence of the ROD10 envelope, indicating that this biological activity remains Vpu specific. Another feature of Vpu is its ability to enhance viral particle release from heterologous retroviruses such as HIV-2, murine leukemia virus, and visna virus (5, 15). We now extend these finding by showing that Vpu can also enhance viral particle release from the mac239 isolate of SIV (29). Consistent with its effect on HIV-1 and HIV-2 particle release, HIV-2_{ROD10} envelope induced an enhancement of SIV particle release indistinguishable from that observed with Vpu. Taken together, our data indicate that the HIV-2_{ROD10} envelope glycoprotein is a genuine Vpu-like factor that positively regulates viral particle release from different retroviruses.

MATERIALS AND METHODS

Recombinant plasmid DNA. Construction of the pROD10 molecular clone of HIV-2 has been described elsewhere (5, 32).

The pNL-A1 and pROD-A1 plasmids are derivatives of pNL4-3 (1) and pROD10, respectively, lacking the *gag* and *pol* genes but expressing all other viral genes (5, 43). The pNL-A1/Udel-1 construct is a derivative of pNL-A1 and carries a deletion mutation that inactivates the *vpu* gene (5, 19). The pROD-

A1.env1 plasmid is derived from pROD-A1 and carries a frameshift mutation that allows the expression of only the first 60 N-terminal amino acids of the Env protein followed by 15 C-terminal missense residues (5).

pNL43-K1 and pNL43-K1/Udel-1 are *env*-deficient variants of pNL4-3 and pNL4-3/Udel-1, respectively. The mutants were constructed in pNL-A1 and pNL-A1/Udel-1 by partial digestion with *Kpn*I followed by blunt-end ligation. Clones were selected for the loss of the *Kpn*I site located at the 5′ end of the *env* gene. The envelope mutation was then transferred into pNL4-3 as an *Eco*RI-*Bam*HI fragment from pNL-A1 and pNL-A1/Udel-1.

The pHIV-2gp1 chimeric proviral construct was obtained by replacing the *gag-pol* region of the HIV-2 molecular clone pROD10 with the corresponding pNL4-3 sequences. The construction involved a multistep cloning strategy which ultimately introduced the NL4-3 *Nar*I-*Nde*I fragment (positions 637 in the 5′ leader region and 5122 in *vif*, respectively) into pROD10 between the common *Nar*I site at position 860 and the *Bcl*I site at position 5221 at the end of the *pol* ORF. Base numbering for both pNL4-3 and pROD10 starts at the first nucleotide in the 5′ long terminal repeat (LTR). An *env*-deficient variant, pHIV-2gp1.env1, was constructed by introducing a 2,341-bp *Bsa*A1-*Bam*HI fragment from pROD-A1.env1, containing the envelope frameshift mutation described above, into pHIV-2gp1.

pMA239 is an SIV infectious molecular clone (41) derived from the lambda phage clone of SIVmac239 (29). The *env*-deficient variants SIVmac.env1 and SIVmac.env2 were obtained by introducing frameshift mutations in the pMA239 molecular clone. SIVmac.env1 was obtained by partial digestion with *Spe*I followed by Klenow fill-in of the site at position 6757 in the *env* gene, resulting in the synthesis of an envelope protein truncated after the N-terminal 149 residues. The SIVmac.env2 construct encodes an envelope protein that terminates 34 residues upstream of the SU/TM cleavage site and was obtained by fill-in of a unique *Cla*I site at position 7784. The MD-1 and MD-1ΔU SIV/HIV-1 (SHIV) chimeric viruses are a generous gift of Riri Shibata (39). The constructs consist of the SIVmac239 *gag*, *pol*, *vif*, and *vpx* genes and part of the *vpr* gene in the context of the DH125 molecular clone of HIV-1 (40). MD-1 and MD-1ΔU differ only by the presence or absence of the *vpu* initiation codon.

The pHIV-CD4ΔBam vector expresses the full-length CD4 molecule under the transcriptional control of the HIV-1 LTR and has been previously described (6, 47).

Cells and transfection. HeLa cells (ATCC CCL2) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). For transfection, HeLa cells were grown to near confluence in 25-cm^2 flasks (5×10^6 cells per flask). Two hours prior to transfection, the medium was replaced with 5 ml of fresh DMEM-FBS. Calcium phosphate-precipitated plasmid DNA (25 to 30 μg) was added to the cells. The medium was removed after 4 h, and the cells were subjected to a glycerol shock for 2.5 min. The cultures were then washed once with phosphate-buffered saline (PBS) and maintained in 5 ml of DMEM-FBS.

Antisera and antibodies. Serum from an asymptomatic HIV-1-seropositive patient (TP serum) was used to detect HIV-1-specific proteins, including Vpu, by immunoprecipitation. The TP serum also recognizes the HIV-2_{ROD} and SIV mature core and matrix proteins and to a lesser extent the Gag precursor or the envelope proteins.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. A pool of HIV-2 patient sera (HIV-2 serum reference panel, contributed by Saladin Osmanov) was used for immunoprecipitation of HIV-2_{ROD} and SIV proteins not recognized by the TP serum (2, 25, 27, 28). A rabbit antiserum to HIV-2_{ST} gp120 (contributed by Raymond Sweet) was used in some cases for immunoprecipitation of the HIV-2_{ROD} envelope proteins (17, 27). A CD4 rabbit antiserum (T4-4, contributed by Raymond Sweet) was used for CD4 immunoprecipitation (11, 47).

Pulse-chase and immunoprecipitation. Viral particle release was assessed by pulse-chase analysis and immunoprecipitation of viral proteins secreted in the culture medium in the form of pelletable virions as recently described (5). Briefly, transfected HeLa cells were pulse-labeled with 1 mCi of Trans^{35}S-Label (ICN Biomedical, Inc., Costa Mesa, Calif.) per ml for 30 min. Cells were subjected to a chase at 37°C in 300 μl of prewarmed DMEM-FBS for the indicated chase periods. At each time point, cells were collected and lysed in 400 μl of Nonidet P-40 (NP-40)–deoxycholate (DOC) buffer (20 mM Tris-HCl [pH 8], 120 mM NaCl, 2 mM EDTA, 0.5% DOC, 1% NP-40). The culture supernatants were filtered through 0.45-μm-pore-size cellulose acetate Spin-X centrifuge tube filters (Corning Costar Corporation, Cambridge, Mass.) to remove remaining cells and cell debris. Virus particles were then pelleted from cell-free supernatants in a refrigerated Eppendorf microcentrifuge (4°C, 90 min, 16,000 × *g*). Pelleted virions were lysed in 400 μl of NP-40–DOC buffer. Cell lysates were precleared by incubation at 4°C for 1 h with protein A-Sepharose beads (Sigma Chemical, St. Louis, Mo.) and immunoprecipitated with either the TP serum or a 1:1 mixture of TP and HIV-2 patient serum. Immunoprecipitates were solubilized by boiling in sample buffer containing 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 1% glycerol, and 65 mM Tris-HCl (pH 6.8) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 12% polyacrylamide gels. Gels were fixed, incubated for 20 min in Enlightning (NEN Research Products, Boston, Mass.), and dried. Radioactive bands were visualized by fluorography using Bio-Max MR films (Eastman Kodak, Rochester, N.Y.). Quantitation of the relevant bands was performed with a Fujix BAS 2000 Bio-Image Analyzer.

CD4 degradation assay. HeLa cells were scraped off the culture flasks 16 h posttransfection, rinsed once in PBS (10 mM phosphate buffer [pH 7.4], 100 mM NaCl), and starved for 30 min in methionine- and cysteine-free RPMI 1640 medium (Specialty Media, Inc., Lavallette, N.J.). Cells were pulse-labeled for 6 min with 2 mCi of Trans^{35}S-Label (ICN Biomedical) per ml and subjected to a chase for 1 h at 37°C in 400 μl of prewarmed DMEM-FBS for the indicated chase periods. Cells were collected and lysed in 400 μl of NP-40–DOC buffer. For cells treated with BFA, the starving, pulse, and chase steps were performed in the presence of 2.5 μg of BFA per ml. Immunoprecipitation was performed as described above, using the anti-CD4 polyclonal serum T4-4.

RESULTS

The HIV-2_{ROD10} envelope glycoprotein enhances HIV-1 particle release. We have recently reported that the HIV-2_{ROD10} envelope glycoprotein has the ability to enhance HIV-2 particle release. Such a putative Vpu-like activity of the HIV-2 Env was observed either in the context of the ROD10 molecular clone or when provided in *trans* to HIV-1/HIV-2 chimeric viruses bearing the ROD10 *gag-pol* region. To show that this activity is indeed functionally equivalent to the activity of Vpu, we examined whether the ROD10 Env could substitute for Vpu in the context of HIV-1. HeLa cells were transfected with the Vpu$^-$ pNL4-3/Udel-1 molecular clone and the gp140 ROD10 Env, or Vpu were provided in *trans* by cotransfection of pROD-A1 and pNL-A1, respectively. We have previously shown that both Vpu and gp140 were able to enhance HIV-2 particle release under similar experimental conditions (5). In control experiments, pNL43/Udel-1 was cotransfected with either the HIV-2 Env mutant pROD-A1.env1 or the Vpu mutant pNL-A1/Udel-1. Cells were pulse-labeled and subjected to a chase for up to 6 h as described in Materials and Methods. At each time point, equal aliquots of cells were harvested and virions released into the supernatant were collected by centrifugation. Each fraction was lysed in lysis buffer, and viral proteins were subjected to immunoprecipitation with an HIV-1 patient serum, containing antibodies against all major HIV-1 proteins, supplemented with a polyclonal serum directed against the HIV-2 envelope glycoprotein. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography (Fig. 1A to D). Results of the pulse-chase experiments show that providing either ROD10 Env or Vpu in *trans* resulted in an increase of viral proteins secreted into the culture supernatant in the form of pelletable virions (compare Fig. 1A with Fig. 1B or Fig. 1C with Fig. 1D). The presence of HIV-2 Env or Vpu did not alter the processing kinetics of the HIV-1 Gag or Env proteins over time. Rather, the presence of the HIV-2 Env generated a redistribution of Gag proteins from the cellular compartment to the virus fraction, as previously reported for Vpu (19) (Fig. 1A and B). To estimate the efficiency of particle release, radioactive bands corresponding to the $p55^{gag}$, $p25/24^{CA}$, and $p17^{MA}$ proteins were quantified, and the ratio of viral Gag proteins present in the culture supernatant to the total pool (cell and supernatant) was calculated and plotted as a function of time (Fig. 1E). The effect of ROD10 Env on pNL4-3/Udel-1 was manifested by a four-fold increase in the efficiency of particle release. This effect was strikingly similar to that observed when Vpu was provided in *trans* (Fig. 1E). Results from these experiments indicate that the HIV-2_{ROD10} Env is a Vpu-like factor that can functionally replace the Vpu protein to enhance HIV-1 particle release.

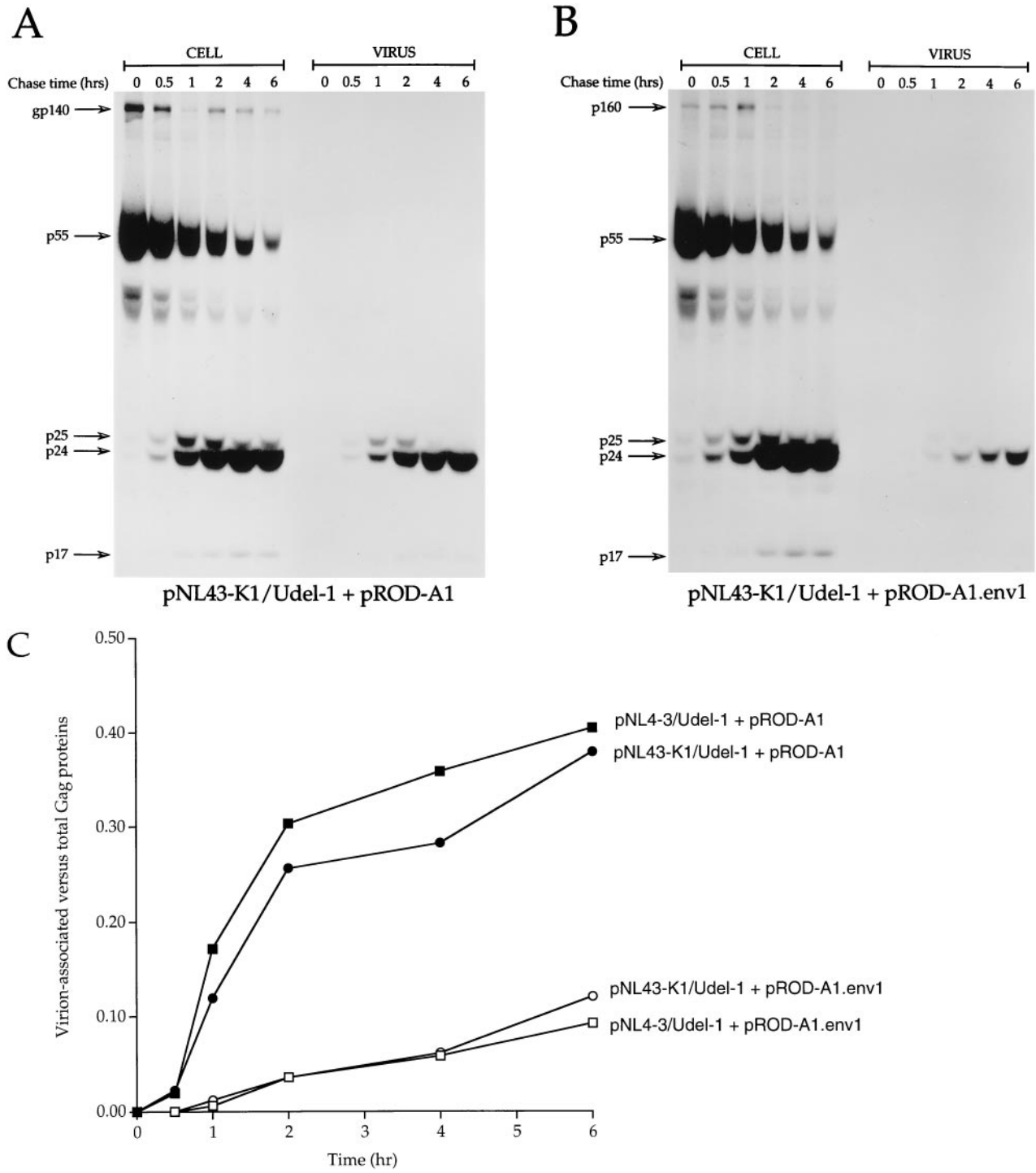
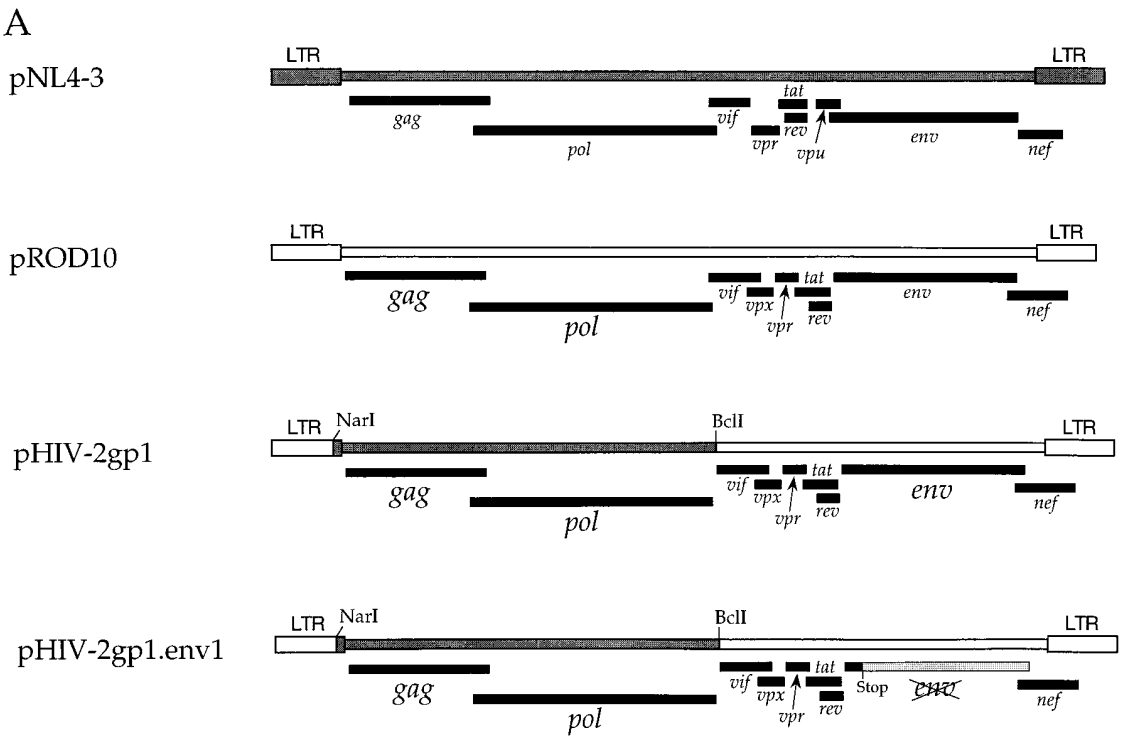


FIG. 2. The Vpu-like effect of ROD10 Env on HIV-1 particle release is independent of HIV-1 envelope glycoprotein. (A and B) HeLa cells were transfected with 20 μg of pNL4-3/Udel-1 (not shown) or the HIV-1 envelope-deficient mutant pNL43-K1/Udel-1 together with either 10 μg of pROD-A1 or 10 μg of pROD-A1.env1. Viral particle release was assessed by pulse-chase analysis as described for Fig. 1. The HIV-1 major Gag proteins $p55^{gag}$, $p24\text{-}25^{CA}$, and $p17^{MA}$ are identified on the left. The p160 Gag-Pol precursor protein visible in the absence of envelope proteins is identified in panel B. The HIV-2 envelope glycoprotein precursor gp140 is identified on the left in panel A. (C) Gag proteins recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions in panels A and B were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time.



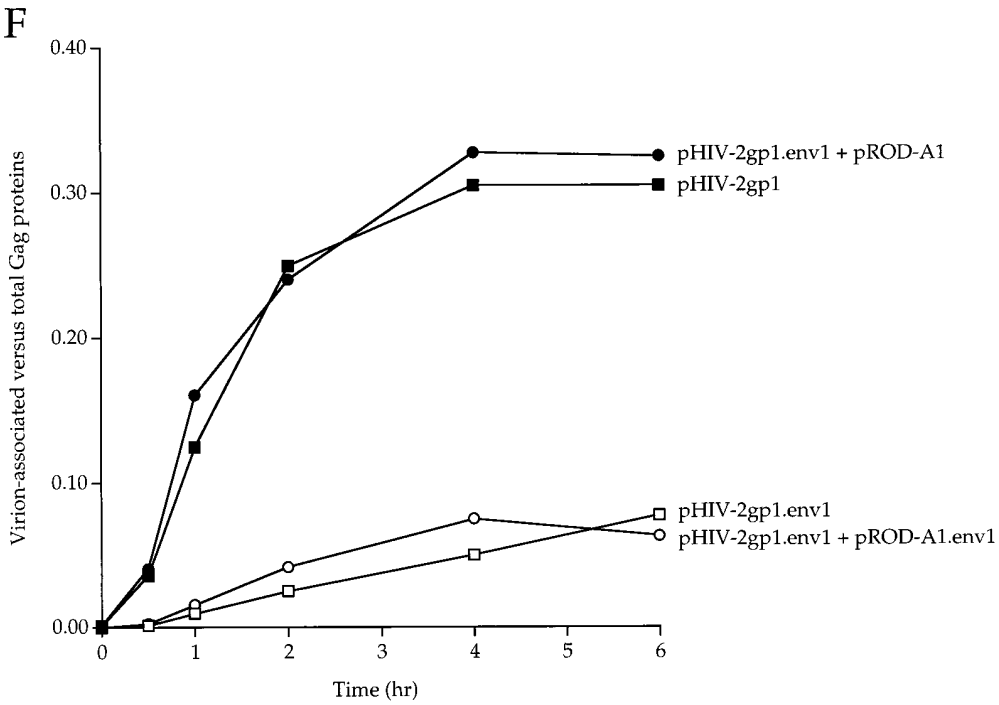


FIG. 3. The ROD10 envelope protein enhances HIV-1 particle release in the context of an HIV-2/HIV-1 chimeric virus. (A) Schematic representation of the pHIV-2gp1 and pHIV-2gp1.env1 HIV-2/HIV-1 chimeric constructs. The two constructs are isogenic except for the presence of a mutation that inactivates the HIV-2_{ROD10} *env* gene in pHIV-2gp1.env1. (B to E) HeLa cells were transfected with 20 μg of either pHIV-2gp1 (B) or pHIV-2gp1.env1 alone (C) or in the presence 10 μg of either pROD-A1 (D) or pROD-A1.env1 (E). Viral particle release was assessed by pulse-chase analysis as described for Fig. 1. (F) Gag proteins recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions in panels B to E were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time.

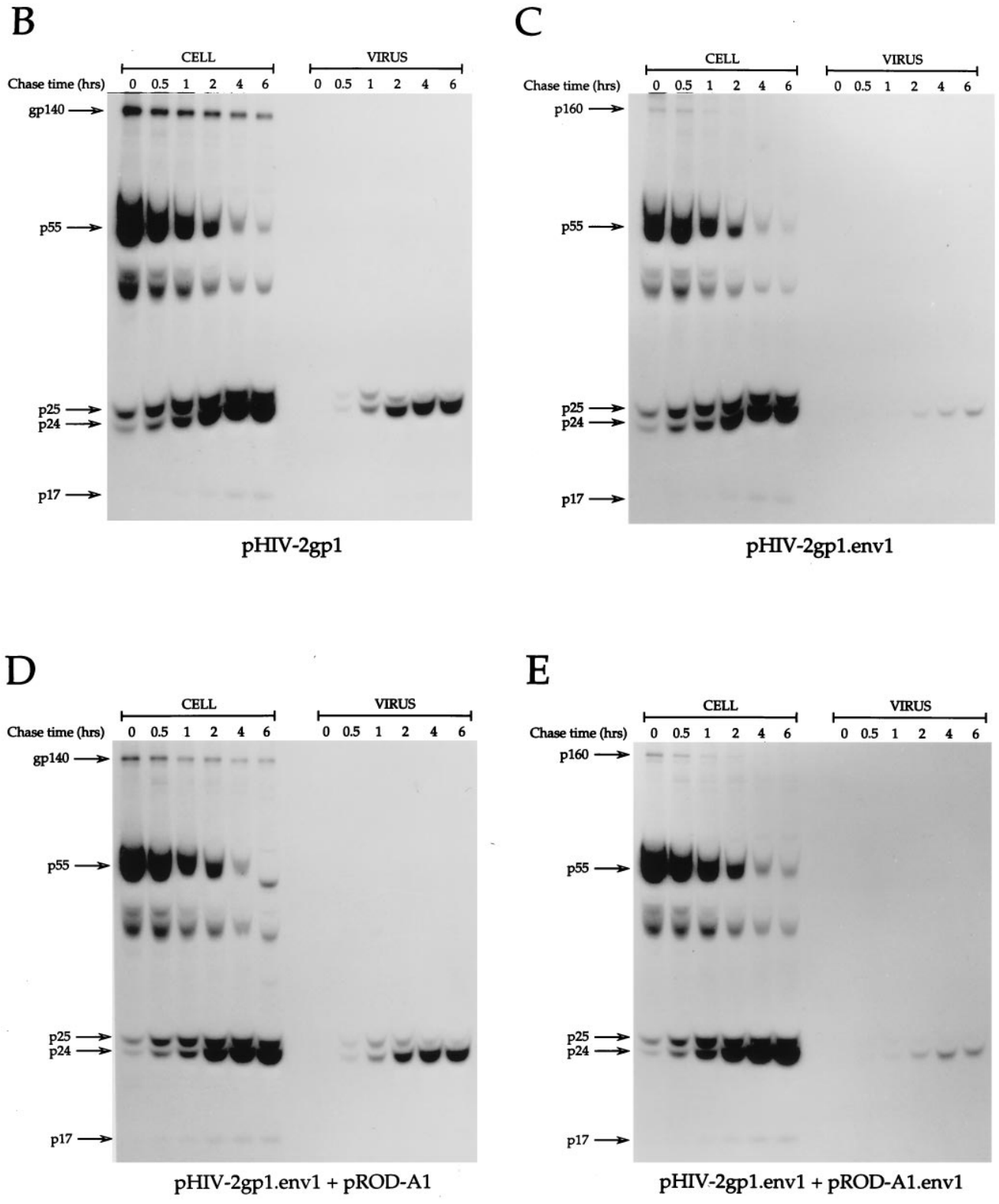


FIG. 3—*Continued.*

The Vpu-like effect of ROD10 Env on HIV-1 particle release is independent of HIV-1 envelope and nonstructural genes. Even though Vpu and the HIV-1 Env are expressed from a common bicistronic mRNA (38), the envelope glycoprotein of HIV-1 is not directly involved in either of the biological activities of Vpu, i.e., CD4 degradation and viral particle release (7, 14, 46, 49). To further examine similarities between the Vpu and ROD10 Env activities, we addressed whether the HIV-1 envelope played a role in the ROD10 Env-mediated enhancement of HIV-1 particle release observed in Fig. 1. HeLa cells were cotransfected with either pNL4-3/Udel-1 (not shown) or the pNL43-K1/Udel-1 envelope mutant in the presence of the ROD10 Env-expressing plasmid pROD-A1. As a control, the effect of the Env-deficient pROD-A1.env1 mutant was analyzed in parallel. The efficiency of HIV-1 particle release under these experimental conditions was assessed by pulse-chase analysis followed by immunoprecipitation of cell and virus fractions, SDS-PAGE, and fluorography (Fig. 2A and B). Radioactive bands corresponding to the $p55^{gag}$, $p25/24^{CA}$, and $p17^{MA}$ proteins were quantified, and the efficiency of particle release was calculated as described for Fig. 1 (Fig. 2C). As shown before (Fig. 1), cotransfection of pNL4-3/Udel-1 with the ROD10 Env-expressing plasmid pROD-A1 led to a fourfold enhancement of HIV-1 particle secretion compared with the Env-deficient mutant pROD-A1.env1. Cotransfection of the Env- and Vpu-deficient pNL43-K1/Udel-1 plasmid with pROD-A1 or pROD-A1.env1 revealed that the ROD10 Env has the ability to enhance HIV-1 particle release independent of the presence of the HIV-1 envelope protein (Fig. 2A and B). Very similar fourfold-enhancing effects of the ROD10 Env on either pNL4-3/Udel-1 or pNL43-K1/Udel-1 particle release were observed, indicating that the HIV-1 envelope is not involved in the mechanism by which the ROD10 Env promotes HIV-1 particle release (Fig. 2C). Taken together, results of Fig. 1 and 2 indicate that Vpu and the HIV-2_{ROD10} envelope glycoprotein are functionally interchangeable; the two activities enhance HIV-1 particle release with identical efficiencies, they are both independent of the HIV-1 envelope, and they are similarly sensitive to BFA treatment (5).

We next wished to address whether the positive effect of the ROD10 Env on HIV-1 particle release observed with full-length NL4-3 was context dependent. For that purpose, HIV-2/HIV-1 chimeras containing the *gag* and *pol* regions of NL4-3 in the context of the HIV-2_{ROD10} molecular clone were constructed (Fig. 3A). Virus release by the pHIV-2gp1 chimeric construct was compared with that by an isogenic variant, pHIV-2gp1.env1, lacking a functional *env* gene (Fig. 3B and C). Both the pHIV-2gp1 and pHIV-2gp1.env1 chimeras expressed the major HIV-1 Gag precursor p55, which was processed with similar kinetics as in the case of wild-type NL4-3 (compare Fig. 3B and C with Fig. 1). The ROD10 envelope precursor, detected in pHIV-2gp1 as a 140-kDa band, was absent in pHIV-2gp1.env1 (Fig. 3B and C). Inactivation of the ROD10 envelope gene in pHIV-2gp1 resulted in significantly reduced virus secretion. However, supplementing Env in *trans* to pHIV-2gp1.env1 by cotransfection of plasmid pROD-A1 restored normal levels of virus production (Fig. 3D). No such effect was observed when the envelope-deficient pROD-A1.env1 plasmid was cotransfected with pHIV-2gp1.env1 (Fig. 3E). Thus, the decrease of particle release observed in pHIV-2gp1.env1 compared with pHIV-2gp1 was entirely attributable to the inactivation of the ROD10 envelope in pHIV-2gp1.env1. Quantitation of the Gag proteins present in Fig. 3A to D showed that the ROD10 Env, whether encoded by pHIV-2gp1 or provided in *trans* to pHIV-2gp1.env1, resulted in a fourfold increase in HIV-1 particle release relative to the Env-deficient chimera (Fig. 3F). This effect was identical to that observed with pNL4-3/Udel-1 and pNL43-K1/Udel-1 (Fig. 1E and 2C, respectively). The Vpu-like activity provided by the HIV-2_{ROD10} envelope glycoprotein is thus able to increase HIV-1 viral particle release in a manner that is indistinguishable from that of Vpu and is independent of the presence of the HIV-1 Env protein or HIV-1 sequences other than the *gag* and *pol* genes.

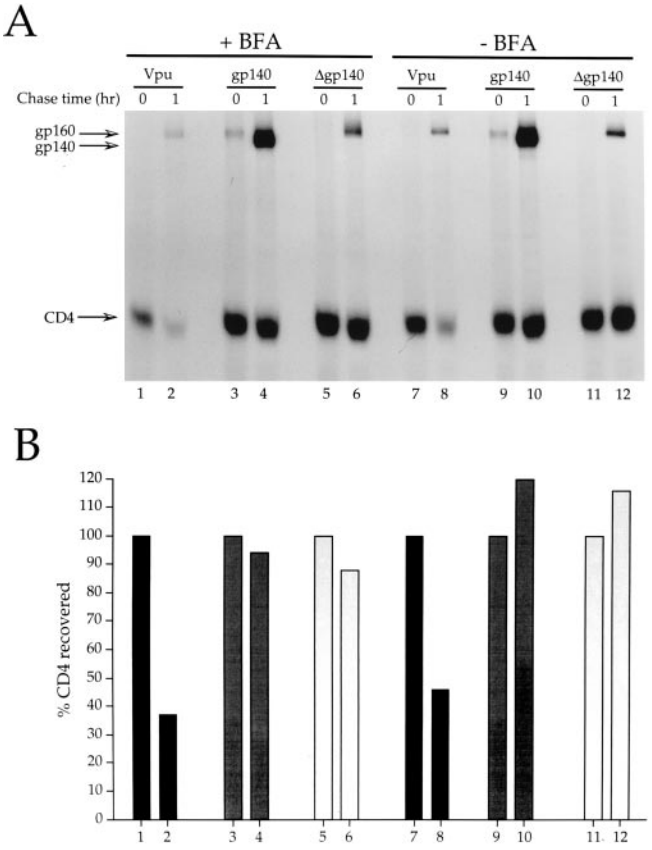


FIG. 4. The ROD10 Env does not induce intracellular degradation of CD4. (A) HeLa cells were transfected with 4 μg of the CD4 expression vector pHIV-CD4ΔBam in the presence of 20 μg of either pNL-A1 (Vpu), pROD-A1 (gp140), or pROD-A1.env1 (Δgp140). A small quantity (4 μg) of the Vpu^-, Tat-expressing pNL-A1/Udel-1 plasmid was added to all samples to ensure consistent expression of CD4 under the control of the HIV-1 LTR. Cells were labeled for 6 min in the presence of Trans^{35}S-Label and subjected to a 1-h chase. Samples recovered by immunoprecipitation with anti-CD4 polyclonal antibodies were separated on SDS 10%–polyacrylamide gels and visualized by fluorography. Bands corresponding to CD4, the gp160 envelope precursor of HIV-1, and the gp140 envelope precursor of HIV-2_{ROD10} are identified on the left. (B) The amount of CD4 proteins recovered at each time point was quantified in an image analyzer, and the rate of CD4 degradation was expressed in each case as the ratio of CD4 recovered after 1 h versus the amount present at time = 0.

The HIV-2_{ROD10} envelope glycoprotein does not induce CD4 degradation. A recent report suggests that despite the absence of a *vpu* gene, cells infected by the HIV-2_{ROD10} isolate may be subjected to intracellular degradation of the CD4 receptor in a manner similar to that observed in cells infected with Vpu-positive HIV-1 (10). Since the ROD10 Env can functionally replace Vpu in its action on HIV-1 particle release, we addressed whether this phenomenon could be explained by a destabilizing activity of the envelope on CD4. HeLa cells were transfected with the pHIV-CD4ΔBam plas-

mid, expressing CD4 under the control of the HIV-1 promoter (6, 47), in the presence of either pNL-A1, pROD-A1, or pROD-A1.env1. Although pROD-A1 and pROD-A1.env1 both express the HIV-2 Tat protein, low levels of CD4 expression were obtained as a result of poor transactivation of the HIV-1 LTR (data not shown). To ensure consistent levels of CD4 expression, the HIV-1 Tat protein was provided by including plasmid pNL-A1/Udel-1. All samples thus contained the HIV-1 envelope glycoprotein, which promotes Vpu-mediated degradation by blocking CD4 in the ER through formation of intracellular complexes (4, 9, 18, 46, 47). Experiments were also performed in the presence or absence of BFA, which was previously reported to enhance Vpu-mediated degradation of CD4 (46). Cells were pulse-labeled for 6 min in methionine- and cysteine-free RPMI containing 2 mCi of Trans^{35}S-Label per ml, the labeled medium was then removed, half of the cells were immediately harvested (time = 0), and the remaining cells were transferred to normal DMEM and chased at 37°C for 1 h (time = 1). Cells were lysed, and CD4 recovered by immunoprecipitation was separated by SDS-PAGE and visualized by fluorography as described in Materials and Methods (Fig. 4A). In the presence of Vpu, the amount of CD4 recovered after 1 h was significantly reduced relative to the time = 0 control, indicating degradation of CD4 (Fig. 4A, lanes 1 and 2). We observed similar degradation of CD4 in the absence of BFA when CD4 was trapped in the ER through complexes with the HIV-1 envelope glycoprotein gp160, as evidenced by the coimmunoprecipitation of gp160 (Fig. 4A, lanes 7 and 8). In contrast, CD4 remained stable over the 1-h chase period in the presence of ROD10 Env, independent of the presence or absence of BFA (Fig. 4A, lanes 3, 4, 9, and 10). The presence of large amounts of gp140 envelope precursor coimmunoprecipitated with CD4 indicates that both proteins were coexpressed in cells and that the presence of small quantities of HIV-1 envelope in this assay did not lead to competition with the ROD10 Env for binding to CD4. The lack of CD4 degradation observed in the presence of the ROD10 Env is therefore not due to an inability of the two proteins to associate intracellularly (Fig. 4A, lanes 3, 4, 9, and 10). CD4 was also stable when coexpressed with the ROD10 Env-deficient plasmid pROD-A1.env1 (Fig. 4A, lanes 5, 6, 11, and 12). In the absence of ROD10 Env, the HIV-1 envelope precursor gp160 encoded by pNL-A1/Udel-1 was detected as a 160-kDa band that coimmunoprecipitated with CD4 (Fig. 4A, lanes 6 and 12). Such coimmunoprecipitation was observed only after 1 h of chase, as previously reported (46), while association between CD4 and the ROD10 Env occurred faster and was detected as early as 6 min after synthesis (Fig. 4A, lanes 3 and 9). For a quantitative assessment of the results shown in Fig. 4A, bands corresponding to CD4 were quantified and the stability of CD4 was expressed as the percentage of CD4 recovered after 1 h of chase relative to the amount present at the end of the pulse (time = 0) (Fig. 4B). Under the experimental conditions used here, the presence of Vpu led to a loss of over 50% of CD4 within 1 h (Fig. 4B, lane 8). In the presence of BFA, approximately 60% of the initial CD4 was lost (Fig. 4B, lane 2). Regardless of the presence or absence of BFA, neither pROD-A1 nor pROD-A1.env1 induced destabilization of CD4 (Fig. 4B, lanes 3 to 6 and 9 to 12). These results thus show that although ROD10 Env can functionally complement Vpu with respect to HIV-1 particle release, the ability to induce intracellular degradation of CD4 is restricted to Vpu.

HIV-1 Vpu and HIV-2$_{ROD10}$ Env enhance SIVmac239 particle release. A characteristic of the HIV-1 Vpu protein is its ability to augment the release of chimeric viruses bearing the *gag-pol* regions of retroviruses that naturally lack a *vpu* ORF, such as HIV-2, visna virus, and Moloney murine leukemia virus (5, 15). Although a recent report suggests that Vpu may contribute to higher viral loads in macaques infected with SHIV (23), no information pertaining to the effect of Vpu on SIV particle release has been reported. We assessed the potential effects of Vpu on SIV particle release by using Vpu$^+$ and Vpu$^-$ SHIV constructs, designated MD-1 and MD-1ΔU, respectively (39). These recombinant viruses contained the *gag*, *pol*, *vif*, and *vpx* genes and part of the *vpr* gene from SIVmac239 in the context of the HIV-1 DH125 isolate (40) (Fig. 5A). The ability of Vpu to enhance SIV particle release when expressed in the context of SHIV was assessed by pulse-chase experiments performed in transfected HeLa cells as described in Materials and Methods. Viral proteins immunoprecipitated from cell and viral pellet extracts were separated by SDS-PAGE and visualized by fluorography (Fig. 5B and C). The presence of Vpu in MD-1 resulted in greater secretion of viral particles in the culture supernatant than in the Vpu-deficient MD-1ΔU SHIV (Fig. 5B and C). The presence of Vpu had no effect on synthesis and maturation kinetics of SIV Gag proteins. A similar enhancement of viral particle release was observed when Vpu was provided in *trans* by cotransfection of HeLa cells with MD-1ΔU and the Vpu$^+$ plasmid pNL-A1 (Fig. 5D and E). Taken together, these results indicate that Vpu has the ability to increase the release of chimeric viruses expressing the SIVmac239 Gag-Pol proteins. Similarly, the effects of HIV-2$_{ROD10}$ SHIV release were investigated by pulse-chase analysis following cotransfection of the Vpu$^-$ SHIV chimera, MD-1ΔU, with the Env producer pROD-A1 or its Env-deficient isogenic variant, pROD-A1.env1. As shown in Fig. 5F and G, increased amounts of viral particles were secreted into the medium in the presence of pROD-A1 compared with pROD-A1.env1. As observed earlier with HIV-1, the presence of ROD10 Env led to an increased secretion of virus particles without affecting the synthesis or maturation of the SIV Gag proteins (Fig. 5F and G). The effects of Vpu or ROD10 Env on SHIV particle release were quantified as described for Fig. 1 and plotted as a function of time (Fig. 5H). The effect of Vpu, whether provided in *cis* or in *trans*, were indistinguishable from those observed for ROD10 Env and led to an approximately twofold increase of SHIV particles (Fig. 5H). Together with previous findings (5, 15), these data indicate that Vpu can enhance the rate of particle release from the three closely related viruses HIV-1, HIV-2, and SIV. In all cases, we could demonstrate a similar activity of the HIV-2 Env.

We have previously reported that in the case of HIV-2, Vpu could enhance viral particle release in the context of HIV-1/HIV-2 chimeric constructs but not in the context of the HIV-2$_{ROD10}$ length molecular clone (5). This context-dependent response of the HIV-2 *gag-pol* to the enhancing effect of Vpu was attributed to the presence of a Vpu-like activity of the ROD10 Env protein (5). To assess a potential Vpu-like activity of the SIVmac239 Env, we examined the abilities of Vpu and the ROD10 Env to also enhance SIV particle release in the context of the full-length SIVmac239 proviral clone. HeLa cells were transfected with plasmid pMA239, expressing full-length SIVmac239, in the presence of either pNL-A1 or the Vpu-deficient pNL-A1/Udel-1 vector. Pulse-chase experiments were performed as described above, and viral proteins in the cell and viral pellet extracts recovered by immunoprecipitation were separated by SDS-PAGE (Fig. 6A and B). In addition to the major SIV Gag proteins, both the HIV-1 gp160 envelope precursor and gp120 mature SU, as well as the SIV gp140

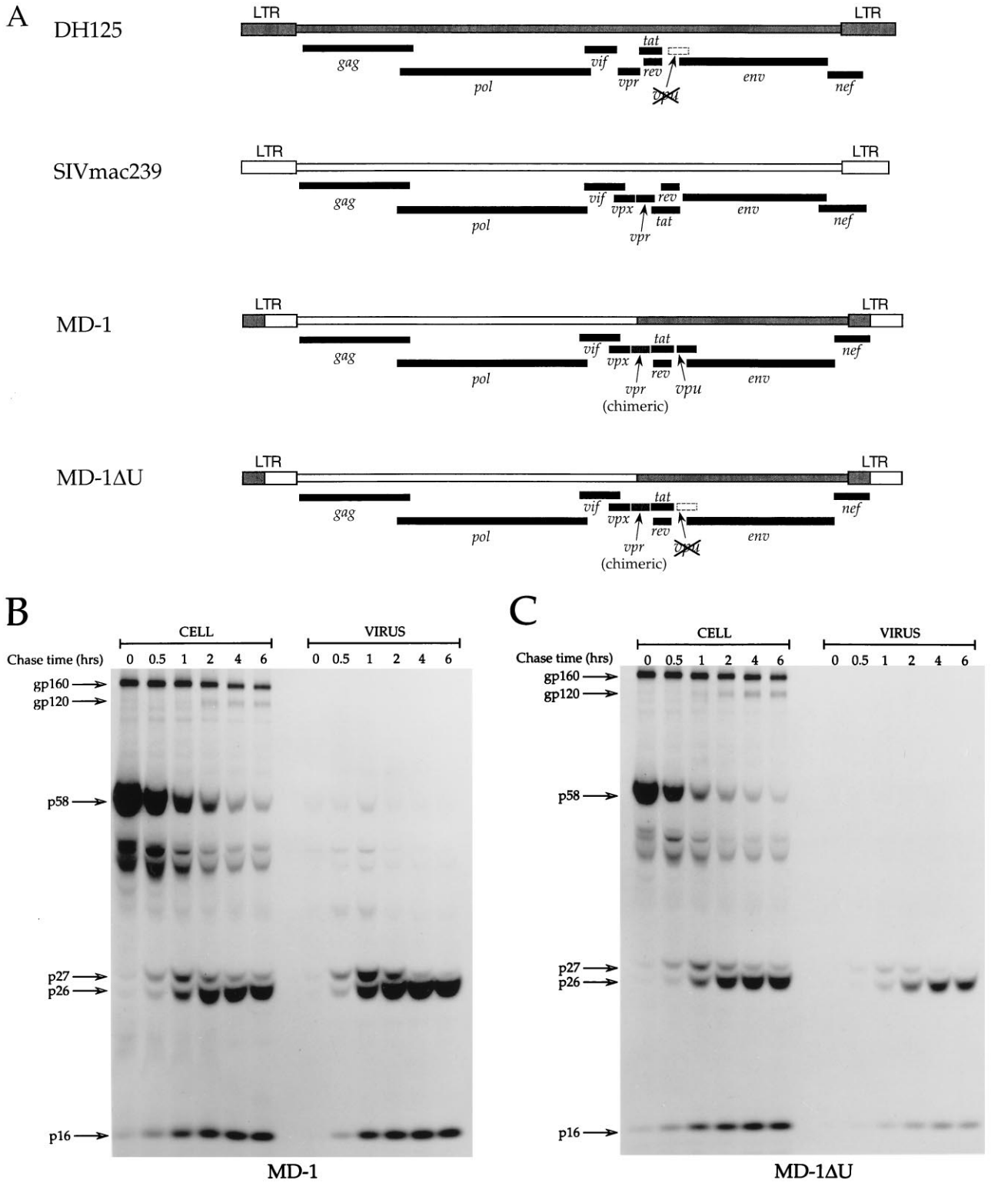


FIG. 5. The ROD10 envelope protein enhances SIV particle release in the context of an SHIV chimera. (A) Schematic representation of the MD-1 and MD-1ΔU SHIV chimeric constructs. The two constructs are isogenic except for the presence of a mutation that inactivates the *vpu* gene in MD-1ΔU. (B to G) HeLa cells were transfected with 20 μg of either MD-1 (B) or MD-1ΔU (C to G) vector DNA. In D to G, MD-1ΔU was cotransfected with 10 μg of either the Vpu^+ pNL-A1 plasmid (D), the isogenic Vpu^- pNL-A1/Udel-1 plasmid (E), the pROD-A1 plasmid (F), or the Env-deficient pROD-A1.env1 plasmid (G). Viral particle release was assessed by pulse-chase analysis as described for Fig. 1. (H) Gag proteins recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions in panels B to G were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time.

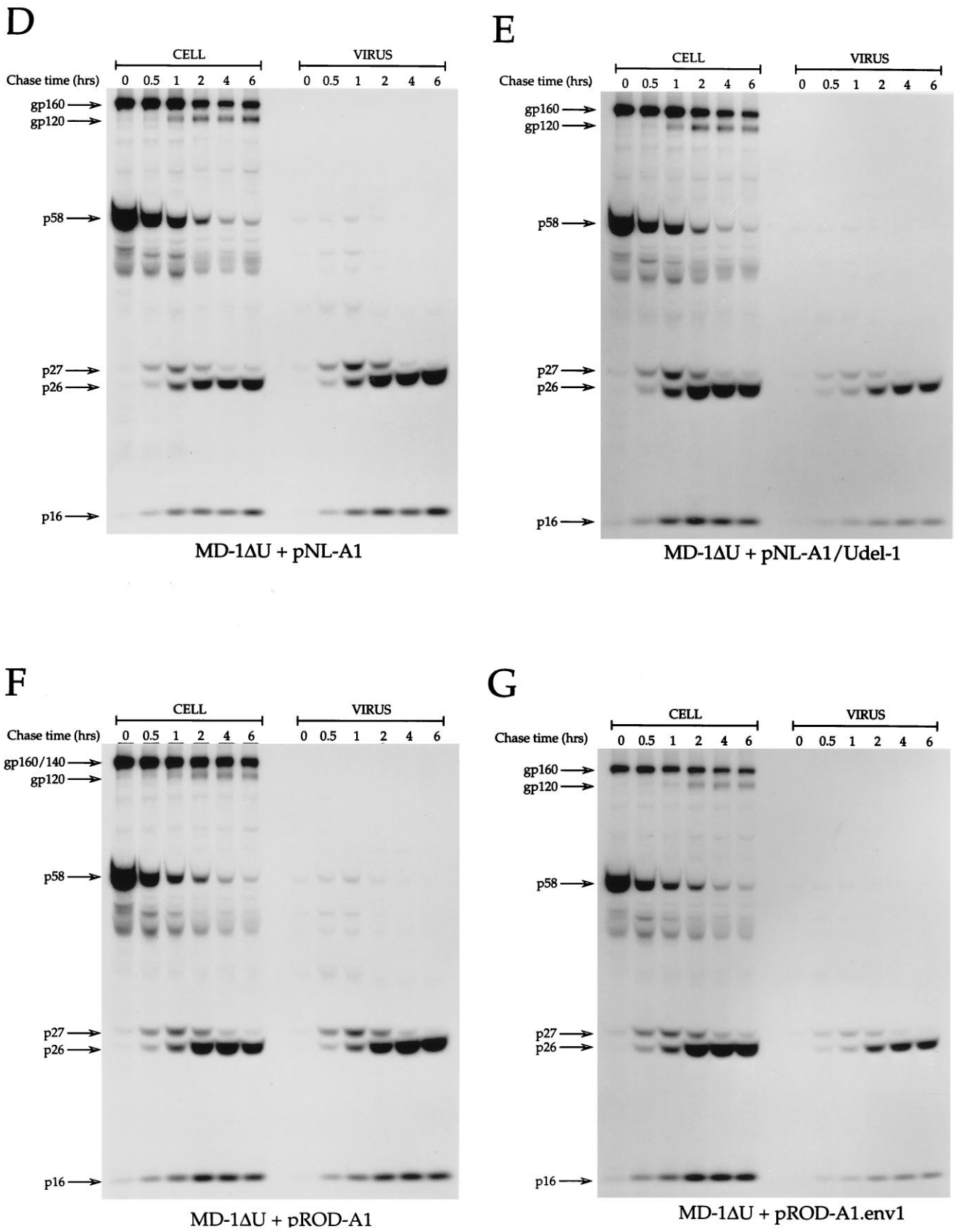


FIG. 5—*Continued.*

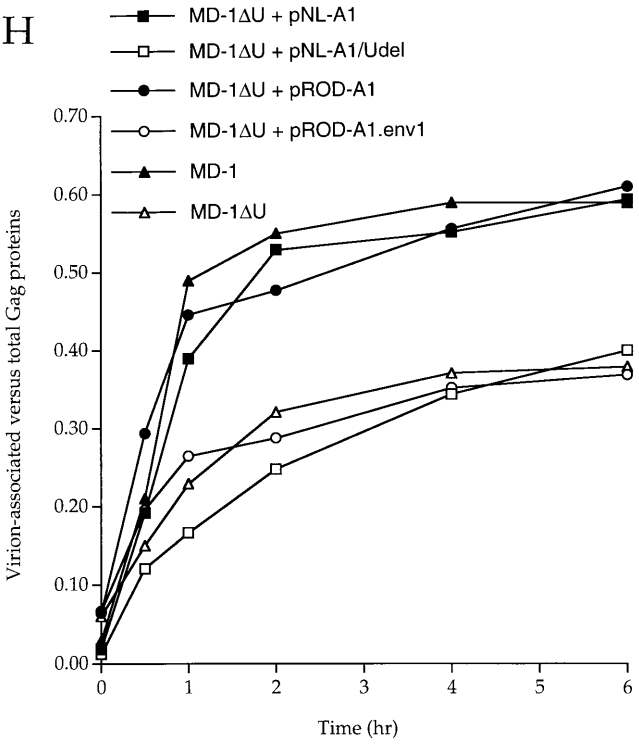


FIG. 5—*Continued.*

envelope precursor and gp125 mature SU, were detected on the gels (Fig. 6A and B). Consistent with the data shown in Fig. 5, the presence of Vpu increased the efficiency of SIVmac239 virus release. Similar results were obtained following cotransfection of SIVmac239 with the ROD10 Env expression plasmid pROD-A1 but not with plasmid pROD-A1.env1 (Fig. 6C and D). Quantitation of the results shown in Fig. 6A to D (Fig. 6E) reveals an increase of virus release in response to Vpu or ROD10 Env that is somewhat less than that observed with the SHIV chimera (Fig. 5H). However, the maximal level of particle release reached by SIV in the presence of pROD-A1 was very similar to that previously observed in the case of the HIV-2_{ROD10} isolate (5). This finding suggests that the reduced enhancing effect of pROD-A1 is not due to a lack of effect of the ROD10 Env on SIVmac239 particle release but rather attributable to the high release activity of SIVmac239 (Fig. 6E). A similarly efficient release of viral particles was observed in the case of the MD-1ΔU chimera (Fig. 5H), suggesting that it is due to an intrinsic ability of the SIVmac239 Gag-Pol proteins to form and release viral particles. However, given the similarities between SIV and HIV-2 envelope proteins, we wished to address whether this high basal rate of particle release could also reflect the presence of a Vpu-like activity in SIVmac239. For this purpose, the *env* gene of SIVmac239 was inactivated and its effect on particle release was assessed. HeLa cells were transfected with either plasmid SIVmac.env1, encoding an envelope protein truncated after the first 149 N-terminal residues, or the SIVmac.env2 construct, which expresses the SU subunit but lacks the TM subunit of Env. Pulse-chase experiments were performed as described above except that the cells were subjected to an 8-h chase. Cell- and virus-associated viral proteins collected at each time point were immunoprecipitated with a 1:1 mixture of HIV-1 and HIV-2 sera, separated by SDS-PAGE, and visualized by fluorography (data not shown). Radioactive bands corresponding to SIV Gag proteins were quantified, and the effects of the envelope mutations on SIVmac239 particle release was calculated as described above. As shown in Fig. 7, a modest but detectable reduction of particle release resulted from inactivation of the SIVmac239 *env* gene. This reduction was identical for both envelope mutants tested, indicating that it was directly attributable to the inactivation of a residual activity of the SIVmac239 envelope protein. Together with results in Fig. 6, these data show that the high level of particle release efficiency of SIVmac239 is attributable to both the Gag-Pol and Env proteins. We next addressed whether the relatively weak enhancing effect of ROD10 Env on SIV particle release observed in Fig. 6 could also be due to the presence of the SIVmac239 envelope in these experiments. Pulse-chase experiments were performed on HeLa cells transfected with the SIVmac.env1 variant in the presence of pROD-A1. As shown in Fig. 7, the absence of the SIVmac239 envelope led to improved enhancement of particle release by the ROD10 Env, which was almost doubled. Nevertheless, the maximal level of particle release attained was remarkably similar to that observed in the presence of SIV envelope (Fig. 6E). This result further demonstrates that the more modest Vpu-like activity of the ROD10 Env observed in Fig. 6 is due not to a lesser activity of that protein on SIV but to an apparent high level of particle release by this virus, even in the absence of enhancing factors.

DISCUSSION

We have recently demonstrated that the activity attributed to HIV-1 Vpu resulting in the enhancement of viral particle release could also be found in the envelope glycoprotein of the HIV2_{ROD10} isolate (5). This result indicated that even though Vpu is unique to HIV-1, at least the closely related HIV-2, but potentially other viral systems as well, encodes a functional homolog that ensures efficient virus production. We had further demonstrated that the efficient activation of HIV-2 particle release by Vpu, which was previously observed in the context of HIV-1/HIV-2 chimera lacking HIV-2 *env* (5, 15), was masked in the presence of ROD10 Env. On the basis of these data, we had speculated that the ROD10 Env product may represent a Vpu-like factor in HIV-2 (5). These findings were recently confirmed in an independent study showing that the envelope glycoprotein of the HIV-2/ST isolate (20, 21) had an enhancing effect on viral particle budding in a vaccinia virus expression system (31). The focus of the current study was to investigate whether the activity of ROD10 Env was restricted to HIV-2 or, similarly to Vpu, could, in a more general fashion, activate the release of heterologous viruses as well. As model systems we chose HIV-1 NL4-3, because of its well-characterized response to Vpu, as well as SIVmac239, for which neither a Vpu response nor a ROD10 Env response had been previously reported. ROD10 Env- or Vpu-mediated virus release was independently assessed for each viral system, using both full-length molecular clones as well as chimeric constructs expressing the HIV-1 *gag-pol* genes in the context of an HIV-2 backbone or the SIV *gag-pol* genes in the context of an HIV-1 backbone.

The results of our studies clearly showed that ROD10 Env was capable of enhancing HIV-1 particle release in a manner indistinguishable from that of Vpu (Fig. 1). This was true not only for full-length, Vpu-deficient HIV-1 but also for the HIV-2/HIV-1 chimera, pHIV-2gp1 (Fig. 3). Furthermore, the effect of ROD10 Env on HIV-1 particle release was inde-

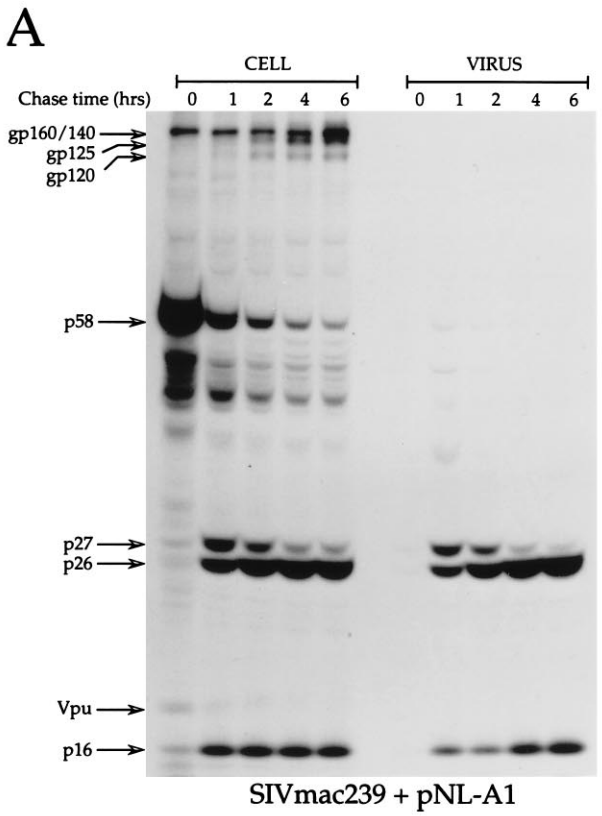
pendent of the presence or absence of the HIV-1 Env protein (Fig. 2), a characteristic previously reported for Vpu (14, 49). Thus, with respect to the regulation of HIV-1 release, Vpu and ROD10 Env are functionally equivalent. Similar results were obtained when the effects of ROD10 Env and Vpu on the release of SIV particles were analyzed (Fig. 5 and 6). The full-length SIVmac239 molecular clone and the SHIV chimera were equally responsive to ROD10 Env and Vpu and exhibited an up to twofold increase in virus secretion. We therefore conclude that the activity of ROD10 Env on virus release is a general characteristic of this protein and that ROD10 Env and Vpu are functionally equivalent. In a recent report, expression of Vpu in SHIV was correlated with higher virus load in infected macaques that may potentially contribute to accelerated disease progression in the affected animals (23). Our finding that Vpu has the ability to enhance SIV particle release in vitro may suggest that the activity of Vpu on particle release observed in cell culture has in vivo relevance and could lead to increased virus loads in infected animals.

The results in Fig. 5 and 6 suggest that ROD10 Env and Vpu each affect SIV virus release to a lesser degree (up to twofold) than HIV-1 or HIV-2 particle secretion, in which case typically a fourfold effect was observed in similar pulse-chase analyses (Fig. 1E, 2E, and 5). However, the maximal rates of particle release for all three viruses reached plateaus at similar levels during the observation period. At the same time, the kinetics of virus secretion in the absence of Vpu or ROD10 Env were significantly higher for SIV than for HIV-1 or HIV-2. Thus, the comparatively small effect of ROD10 Env or Vpu on SIV particle release is not due to a lack of function of these factors but can be explained by the higher basal release kinetics of SIV relative to HIV-1 or HIV-2. In addition, release of SIV particles derived from the full-length SIVmac239 molecular clone and the Vpu-deficient SHIV chimera occurred with equal efficiencies. This result indicates that unlike the case for HIV-2, the efficiency of SIV particle release was context insensitive and is thus likely an inherent feature of the SIVmac239 *gag-pol* products. We nevertheless addressed the question of whether SIV, like HIV-2, encodes a Vpu-like function in its Env product. Examination of two different envelope-deficient variants of SIVmac239 revealed only a modest negative effect on viral particle release resulting from the inactivation of the SIVmac239 envelope gene. This finding suggests that the SIVmac239 envelope protein may have a residual Vpu-like activity that contributes, together with the Gag-Pol proteins, to the relatively high efficiency of viral particle release by this virus. The fact that the envelope protein of the SIVmac239 isolate did not exhibit a strong effect on viral particle release does not exclude the possibility that a Vpu-like activity similar to that of HIV-2_{ROD10} exists in other isolates of SIV.

As with Vpu, the precise subcellular location from which ROD10 Env regulates virus release has yet to be determined. Nevertheless, retention of Vpu or ROD10 Env in the ER following treatment of cells with BFA obliterated the effects of both proteins on virus secretion (5, 37), suggesting that Vpu and ROD10 Env each regulate virus release from a post-ER compartment. Unlike Vpu, ROD10 Env is subject to extensive posttranslational modification by N-linked glycosylation (30). The specific modification of N-linked glycans in defined cellular compartments could provide a useful model with which to define the subcellular compartment from which this protein, and possibly Vpu, mediates viral particle release.

A recent report indicates that the ROD10 isolate of HIV-2 has the ability to induce intracellular degradation of CD4 in an envelope-binding-dependent manner (10). Although we show that ROD10 Env and Vpu have identical effects on viral particle release, we found no evidence to support the notion that ROD10 Env could promote CD4 degradation (Fig. 4). In addition, since our experiments involved pROD-A1, which expresses Vif, Vpr, Vpx, Nef, Rev, and Tat, in addition to Env, the lack of CD4 degradation observed in our studies also rules out the possible involvement of any of these factors in the proposed HIV-2-induced degradation of CD4. The inability of ROD10 Env to induce degradation of CD4 does not imply that ROD10 Env and Vpu regulate particle release through different mechanisms. To the contrary, our previous data show that the two biological activities of Vpu are structurally and mechanistically separable (33, 37). It is thus conceivable that Vpu and ROD10 Env can act on particle release through similar mechanisms without sharing the ability to induce CD4 degradation.

We do not know at present which domain of the ROD10 Env is responsible for the Vpu-like activity. We are in the process of constructing chimeric *env* genes that are designed to address this question. Although it remains to be shown whether Vpu and ROD10 Env regulate particle release through similar molecular mechanisms, the close functional similarities between the two proteins point to the possible involvement of the gp41 transmembrane (TM) subunit of ROD10 Env (5). Indeed, gp41 contains the membrane-spanning domain of Env which could have a role similar to that of the TM domain of Vpu in regulating of virus release (33). In addition, a recent report points to the involvement of the HIV-2 Env cytoplasmic tail in the regulation of its Vpu-like activity (31). The authors showed that the envelope protein of the HIV-2/ST isolate bearing a full-length, 164-amino-acid-long, gp41 cytoplasmic tail was able to augment the release in Gag pseudoparticles from vaccinia virus-infected SupT1 cells (31). No such activity was detected when the envelope protein of the HIV-2/ST#2 variant, bearing a truncation to 17 amino acids in the gp41 cytoplasmic tail, was used (31). On one hand, these results could suggest further similarities between the mechanisms of action of HIV-2 Env and Vpu on particle release. Indeed, although we have shown that the Vpu TM domain by itself has residual activity for the enhancement of particle release, we have also demonstrated that the presence of the cytoplasmic tail of Vpu significantly increases this activity (33). On the other hand, the fact that the truncated HIV-2/ST#2 envelope does not enhance viral particle release is difficult to reconcile with our own data since the envelope glycoprotein of ROD10, used throughout the present study and in our earlier report (5), bears a stop codon that truncates the gp41 cytoplasmic tail to 18 amino acids (3). Since the inactive HIV-2/ST#2 envelope has only 17 cytoplasmic amino acids, these results could suggest that the ROD10 cytoplasmic tail represents the minimal element required for the HIV-2 Env to positively regulate viral particle release. However, there are other differences at the amino acid level between the ROD10 and ST#2 envelopes that may explain the difference in activity and may suggest the involvement of other domains of the envelope protein in regulating particle release. Extensive mutagenesis is under way to resolve these questions and to explain the paradoxical finding that both a full-length (HIV-2/ST) and truncated (ROD10) envelope can regulate release of progeny virus.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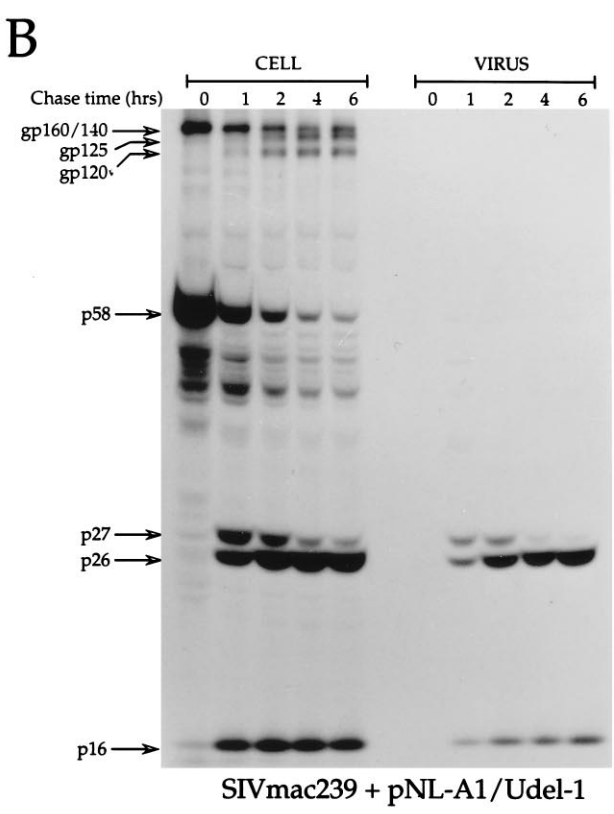
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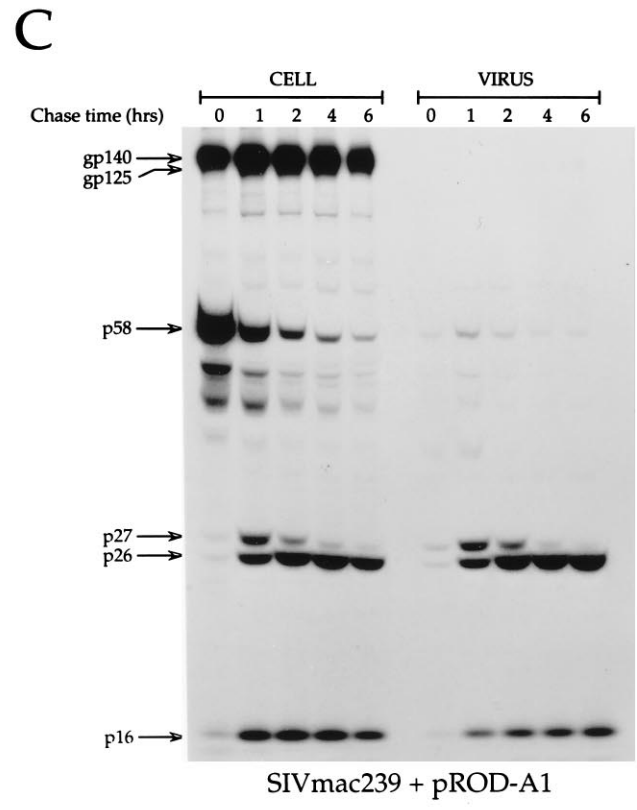
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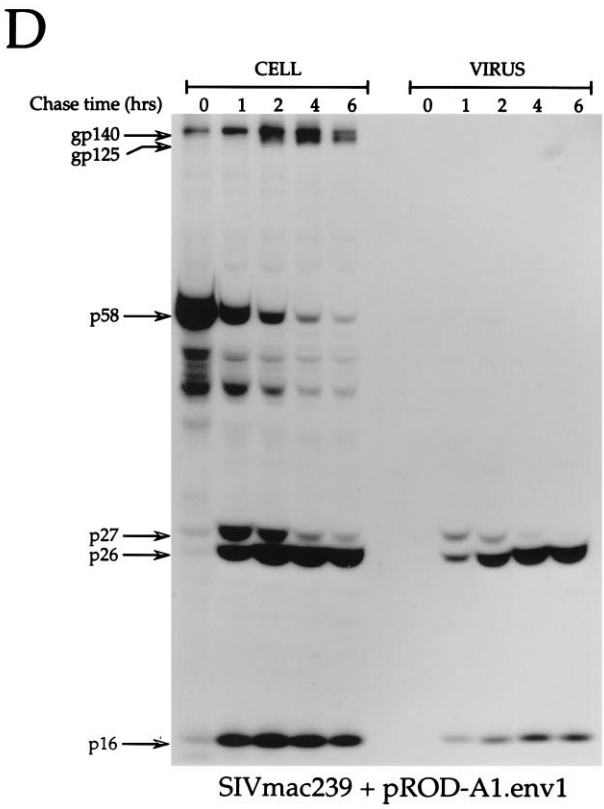
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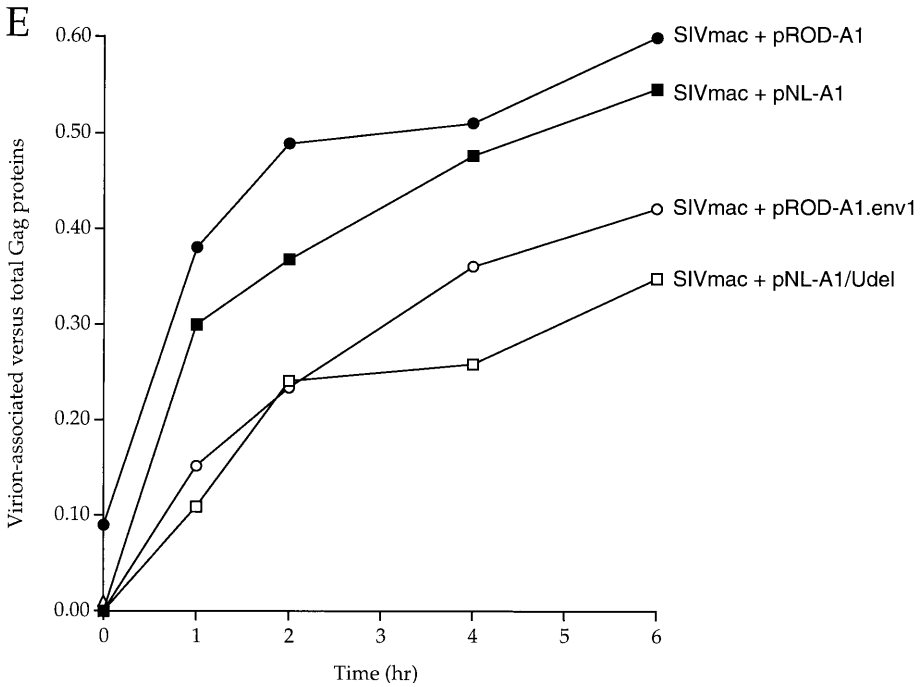

FIG. 6. The ROD10 envelope protein enhances particle release by full-length SIVmac239. (A to D) HeLa cells were transfected with 20 μg of plasmid pMA239, expressing full-length SIVmac239, in the presence of 10 μg of either pNL-A1 (A), pNL-A1/Udel-1 (B), pROD-A1 (C), or pROD-A1.env1 (D). Viral particle release was assessed by pulse-chase analysis as described for Fig. 1. The SIV envelope glycoprotein precursor gp140 as well as the mature 125-kDa SU proteins are identified on the left. The HIV-1 gp160/gp120 envelope proteins are also detected in panels A and B in the presence of plasmid pNL-A1. (E) Gag proteins recovered by immunoprecipitation of both the cell lysate and the pelleted virus fractions in panels A to D were quantified with an image analyzer. The efficiency of particle release at each time point was calculated as described in the legend to Fig. 1, and the ratio of virion-associated to total Gag proteins was plotted as a function of time.

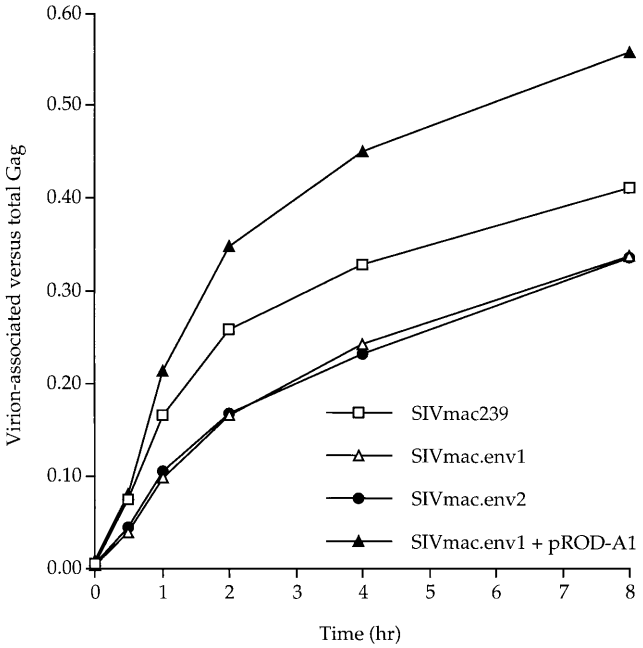

FIG. 7. The SIVmac239 envelope protein has a residual enhancing activity on viral particle release. HeLa cells were transfected with 20 μg of either plasmid pMA239, expressing wild-type SIVmac239, or the SIVmac.env1 or SIVmac.env2 envelope mutant plasmid. The ability of ROD10 Env to enhance SIV particle release was assessed in the absence of SIV envelope by cotransfection of 20 μg of SIVmac.env1 and 10 μg of pROD-A1. Viral particle release was assessed by pulse-chase analysis as described for Fig. 1 (not shown). The efficiency of particle release at each time point was calculated and plotted as described in the legend to Fig. 1.

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