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SHORT COMMUNICATION

HIV-1 Particle Release Mediated by Vpu Is Distinct from That Mediated by p6

MICHAEL D. SCHWARTZ, ROBERT J. GERAGHTY,[1] and ANTONITO T. PANGANIBAN[2]

McArdle Laboratory for Cancer Research, University of Wisconsin, Medical School, 1400 University Avenue, Madison, Wisconsin 53706

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Vpu and the C-terminal peptide of Gag (p6) are both HIV-1-encoded proteins that augment the release of virus particles from cells. We examined the functional relationship between these proteins and their activities during particle release. Our results indicate that efficient HIV-1 particle release from HeLa and Jurkat cells depends on the presence of Vpu. However, Vpu is dispensable for efficient release from Cos cells. In contrast, p6 is required for efficient release from Cos cells but not from Jurkat or HeLa cells. These data suggest that Vpu and p6 have distinct activities in virus exit from different cell lines. Intracellular proteolytic processing of Gag precursor protein is more complete in Cos cells than in HeLa cells. However, this processing has little or no effect on Vpu- or p6-mediated particle release. p6 is required for incorporation of yet another virus protein (Vpr) into cells but our data suggest that Vpr plays no role in p6-dependent particle release. Vpu also facilitates the degradation of CD4 in virus producing cells but, in contrast to particle release, the ability of Vpu to facilitate the degradation of CD4 is not cell line-dependent. © 1996 Academic Press, Inc.

All replication competent retroviruses contain *gag, pol,* and *env* genes. In the case of the oncovirus subfamily, these three genes are sufficient *in trans* for replication. However, lentiviruses, spumaviruses, and the human T-cell leukemia virus (HTLV) and its relatives contain auxiliary genes along with the signature *gag, pol,* and *env* genes. Human immunodeficiency virus Type 1 (HIV-1) is a member of the lentivirus subfamily and contains at least six accessory genes.

One of these accessory genes, viral protein U (*vpu*), is unique to HIV-1 and contains no homolog even in related lentivirus species such as human immunodeficiency virus Type-2 (HIV-2) or simian immunodeficiency virus (SIV). Vpu, an 81-amino-acid, phosphorylated (*1, 2*), type I integral membrane protein (*3*), is not absolutely required for HIV-1 replication in cell culture (*2, 4, 5*). Vpu has been demonstrated to augment the rate of release of viral particles from cells (*4–7*) and acts to degrade CD4 (*8–12*), the cellular receptor for HIV-1.

Although individual Vpu molecules appear to interact with each other (*3*), there is no evidence for direct interaction between Vpu and any other viral proteins. For example, attempts to detect a direct interaction between Vpu and gp160 have not been successful (*10, 11*). Gottlinger *et al.* (*13*) demonstrated that Vpu expression enhances the release of retroviruses both closely (HIV-2 and visna virus) and distantly related (murine leukemia virus or MLV) to HIV-1. Vpu has been shown to interact with and specifically induce the degradation of CD4 (*11, 12, 14*). Taken together, these data do not unequivocally demonstrate that Vpu action is mediated via cellular factors, but the data are consistent with this possibility.

The retroviral core is composed principally of Gag and Gag–Pol proteins. However, in HIV-1 substantial amounts of a protein encoded by a second auxillary gene (*vpr*) are also found in particles (*15*). Many studies have demonstrated that expression of Gag alone is sufficient for the formation and release of immature, noninfectious virus particles (*16*). Mature Gag protein is derived from both the Gag and Gag–Pol polyprotein precursors via proteolytic cleavage catalyzed by a virally encoded protease (PR) (*17*). In both the type-C and lentiviruses, the paradigm of particle assembly is that the Gag and Gag–Pol precursors accumulate at the cytoplasmic side of the plasma membrane (*18*) by way of the hydrophobic, myristolated N-terminus (*19–21*). This aggregation is believed to increase the local concentration of Gag and Gag–Pol proteins leading to intermolecular association between multiple precursors, the concerted formation of viral cores, and the opportunity for proteolytic processing (*18*). Processing can occur both prior to particle release and following liberation of particles from the cell and is not required for efficient virus particle release (*17*). In the primate lentiviruses, the C-terminal product of the Gag precursor is a 52-amino-acid, proline-rich polypeptide, called p6 (*22, 23*). Mutations in p6 can result in abroga-

[1] Present address: Department of Microbiology-Immunology, Northwestern University, 303 E. Chicago Avenue, Ward Memorial Bldg., Chicago, IL 60611.

[2] To whom correspondence and reprint requests should be addressed. Fax: (608) 262-2824. E-mail: Panganiban@oncology.wisc.edu.

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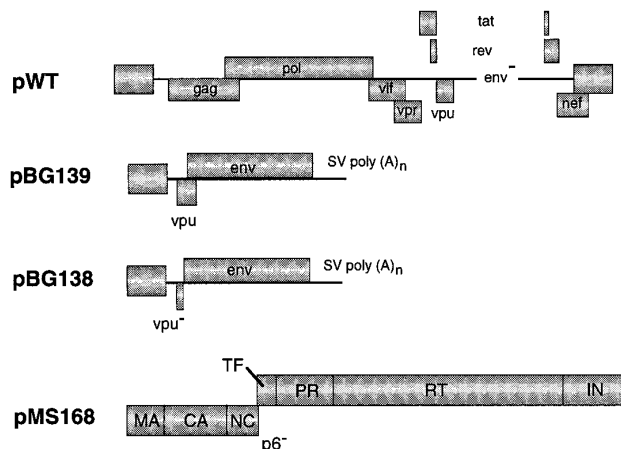
tion of efficient virus particle release from cells (*24–26*). In contrast to results suggesting a role for p6 in virus particle release, several studies have indicated that p6 is not required for efficient particle release in a number of different systems (*27–34*). In addition to its role in particle release p6 is essential, and perhaps sufficient, for association of Vpr with virus particles (*27, 35, 36*). It has also been reported that HIV-1 p6 can functionally substitute in a late budding function for the Rous sarcoma virus (RSV) assembly domain 2 region (*25*).

The fact that the phenotypes of Vpu and p6 mutations have similar features raises the possibility that both proteins promote particle exit through a common mechanism. However, there is no evidence to support such a link. The fact that p6 is required for uptake of Vpr into particles (*27, 35, 36*) also raises the possibility that Vpr is necessary for p6-facilitated particle release.

In this study we attempted to determine whether there is an overt mechanistic connection between the way in which Vpu and p6 promote the exit of HIV-1 particles. Moreover, we wanted to see whether ancillary variables such as cell line, the proteolytic processing of Gag, or incorporation of Vpr into virus particles might also affect Vpu- and p6-mediated particle release. The results of these experiments suggest that there is a fundamental distinction in the way in which these two proteins promote virus release.

HeLa cells have proven to be expedient for the examination of both Vpu-mediated particle release (*4*) and Vpu-mediated CD4 degradation (*14*). However, since the *vpu* gene is present in HIV-1 but absent in SIV, it is reasonable to postulate that Vpu may not be necessary to efficiently produce infectious virus from simian cells. We initially used a *trans*-complementation strategy (*6*) to measure replication, over a single round of replication, in the presence and absence of Vpu for virus produced from Cos cells. Briefly, HIV-1 DNA expression constructs pWT ($env^{-}vpu^{+}$) or pBG135 ($env^{-}vpu^{-}$) (Fig. 1) were cotransfected into Cos cells along with envelope expression constructs that express or lack vpu (pBG138 or pBG139, respectively) (Fig. 1). The virus produced by *trans*-complementation was then used to infect $CD4^{+}$-LTR/β-gal indicator cells (*37*). Virus produced from the initial transfection were capable of only one round of replication since the packaged RNA lacked a functional *env* gene. Successful infection events were quantified by scoring β-gal expression following *de novo* expression of Tat from WT or BG135 proviruses. In contrast to previous results in which virus was produced from HeLa cells (*6*), when virus was derived from Cos cells the titer was equivalent in the presence and absence of Vpu expression (data not shown).

Cos cells contain a replication-defective simian virus 40 (SV40) mutant that expresses T-antigen. It seemed unlikely that T-antigen would obviate the need for Vpu, but we wanted to test this possibility directly. Therefore,



Genotypes of HIV-1 expression constructs

expression construct	genotype
pWT	vpu^{+} $p6^{+}$ vpr^{+} pr^{+}
pBG135	vpu^{-} $p6^{+}$ vpr^{+} pr^{+}
pMS168	vpu^{+} $p6^{-}$ vpr^{+} pr^{+}
pMS161	vpu^{-} $p6^{-}$ vpr^{+} pr^{+}
pMS172	vpu^{+} $p6^{+}$ vpr^{+} pr^{-}
pMS173	vpu^{-} $p6^{+}$ vpr^{+} pr^{-}
pMS176	vpu^{+} $p6^{-}$ vpr^{+} pr^{-}
pMS177	vpu^{+} $p6^{-}$ vpr^{-} pr^{+}
pMS178	vpu^{+} $p6^{+}$ vpr^{-} pr^{+}
pMS179	vpu^{+} $p6^{-}$ vpr^{-} pr^{-}
pMS180	vpu^{+} $p6^{-}$ vpr^{-} pr^{-}

FIG. 1. Diagram and summary of expression constructs used in this study. pWT is a derivative of pNL4-3 that has a deletion in the env gene, rendering virus produced from this expression construct noninfectious (*7*). Vpu^{-} expression constructs contain an 8-bp insertion to create a frameshift mutation in the *vpu* gene. $p6^{-}$ expression constructs contain a base pair substitution which creates a premature stop codon at amino acid 2 of the p6-coding region of the *gag* gene. PR^{-} expression constructs contain a substitution mutation that changes the aspartic acid at amino acid 25 (active site) of HIV-1 protease to an arginine. Vpr^{-} expression constructs contain a substitution mutation that introduces a premature stop codon at amino acid 11 of the Vpr gene. pBG139 expresses the HIV-1 Env-gp and Vpu from the HIV-1 LTR. pBG138 is identical to pBG139 except it contains except that it is Vpu^{-}.

we examined particle release from CV-1 cells, the progenitor cell line to Cos. Although the absolute titer from CV-1 cells was reduced approximately fourfold relative to that from Cos cells, infectious virus production from CV-1 cells was again shown to be Vpu-independent (data not shown).

The titer for virus produced from Cos and CV-1 cells was similar when Vpu was present or absent. One interpretation of this observation is that infectious titer was a direct reflection of the efficiency of particle release. However, it was also formally possible that particle release was still inefficient in the absence of Vpu, but that those particles that were produced could infect and es-

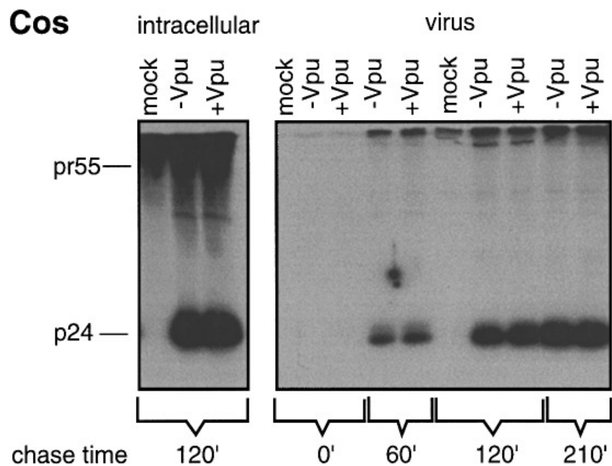


FIG. 2. HIV-1 p24 release from cells transfected with a *vpu*⁻ mutant. Cos cells were mock transfected or transfected with either pBG135 (Vpu⁻) or pWT (Vpu⁺). Forty-eight hours later the cells were pulse labeled with L-[^{35}S]methionine for 60 min, followed by a chase with fresh cell culture media for varying times. After 0, 60, 120, and 210 min, medium was removed and immunoprecipitated using AIDS patient sera and analyzed on a 15% polyacrylamide gel. In addition, the cells from the 120-min time point were lysed and immunoprecipitated to detect intracellular levels of p24.

tablish proviruses more efficiently than particles generated in the presence of Vpu. To test this possibility, and to examine virus release in a more straightforward way, we performed pulse/chase and immunoprecipitation analysis, as previously described (*6*), on viral protein produced from Cos cells transfected with either pBG135 (*vpu*⁻) or pWT (*vpu*⁺). Virus-producing cells were pulsed with L-[^{35}S]methionine for 1 hr and chased with complete media, and the released viral proteins were collected at various times after the chase. Viral proteins were detected by immunoprecipitation with serum from an AIDS patient, gel electrophoresis, and autoradiography (Fig. 2). The results of this experiment demonstrated that the rate of capsid release was similar whether or not Vpu was expressed. Therefore, the efficiency of particle release from this simian cell line is actually Vpu-independent, and infectious titer was indicative of particle production. Furthermore, the data are consistent with the possibility that particle release, mediated by Vpu, is cell line-dependent.

If particle exit from some cell lines does not require Vpu, then what is the role of Vpu in particle egress from human T-cells? To answer this question we also compared particle release from Jurkat cells in the presence and the absence of Vpu with that from HeLa and Cos cells. These three cell lines were transfected with either pWT (*vpu*⁺) or pBG135 (*vpu*⁻), and 48 hr posttransfection the cells were metabolically labeled for 1 hr with L-[^{35}S]-methionine, chased for 3 hr, and intracellular and virion-associated p24 were immunoprecipitated. Following electrophoresis, the amount of intracellular and viral p24 was quantified using phosphorimage analysis. In contrast to the results obtained using Cos cells, and in concordance with the results we obtained using HeLa cells, the release of p24 from transfected Jurkat cells is enhanced approximately 6.5-fold in the presence of Vpu expression (data not shown).

Unlike Vpu, the C-terminal product of the *gag* gene (p6) is found in both HIV-1 and SIV. Since the phenotype of some p6⁻ mutants is very similar to that of Vpu mutations, and since Vpu appears to be dispensable for efficient particle release from Cos cells, we wanted to determine whether p6-mediated particle release is also cell line-dependent. We constructed pMS168, a p6⁻ derivative of an Env⁻ molecular clone pWT (Fig. 1), which has a premature stop codon at the second amino acid position of p6. Thus, expression of pMS168 would be expected to result in the synthesis of a truncated Gag precursor protein with a mass of approximately 49 kDa.

We directly examined the efficiency of particle release from both Cos and HeLa cells in the presence and the absence of p6. Cells were transfected in parallel with pWT (p6⁺) and pMS168 (p6⁻) and cellular and virion-associated Gag protein was detected and quantified by immunoblotting using an anti-Gag serum, a radiolabeled secondary antibody, and subsequent phosphorimage analysis. Expression of *gag* from pWT resulted in the intracellular accumulation of the full-length Gag precursor (pr55^{gag}) as well as fully processed capsid protein (p24^{gag}) in both Cos and HeLa cells (Figs. 3A and 3B). Moreover, expression of pMS168 (p6⁻) resulted in the generation of a truncated Gag precursor with an approximate mass of 49 kDa in both cell lines (Figs. 3A and 3B). Although fully processed p24 was observed in pMS168-transfected cells, proteolytic processing of the truncated Gag derivative was reduced as evidenced by the appearance of two intermediate processing products (p41, composed of matrix (p17^{gag}) and capsid (p24^{gag}) and p25, a penultimate intermediate in the formation of p24) and the reduction of the amount of p24. In concordance with published work (*24*) the absence of p6 profoundly impeded particle release from Cos cells. However, the lack of p6 had little or no effect on particle release from HeLa cells (Fig. 3B), as evidenced by measurement of the total Gag-derived products in viruses resulting from transfection of HeLa cells with pWT or pMS168. This apparent cell line-dependence of p6-enhanced particle release was the opposite of that observed for Vpu-facilitated release. A summary of the data from several transfections of pWT and pMS168 into Cos and HeLa cells is provided in Fig. 3C. These data are reported as the relative efficiency of virus particle release and are derived by calculating the ratio of total virion-associated Gag protein and the total cytoplasmic levels of Gag protein. The relative efficiency of virus particle release from pWT is normalized to 1.0. We also constructed a double mutant containing lesions in both p6 and Vpu (pMS161). The pheno-

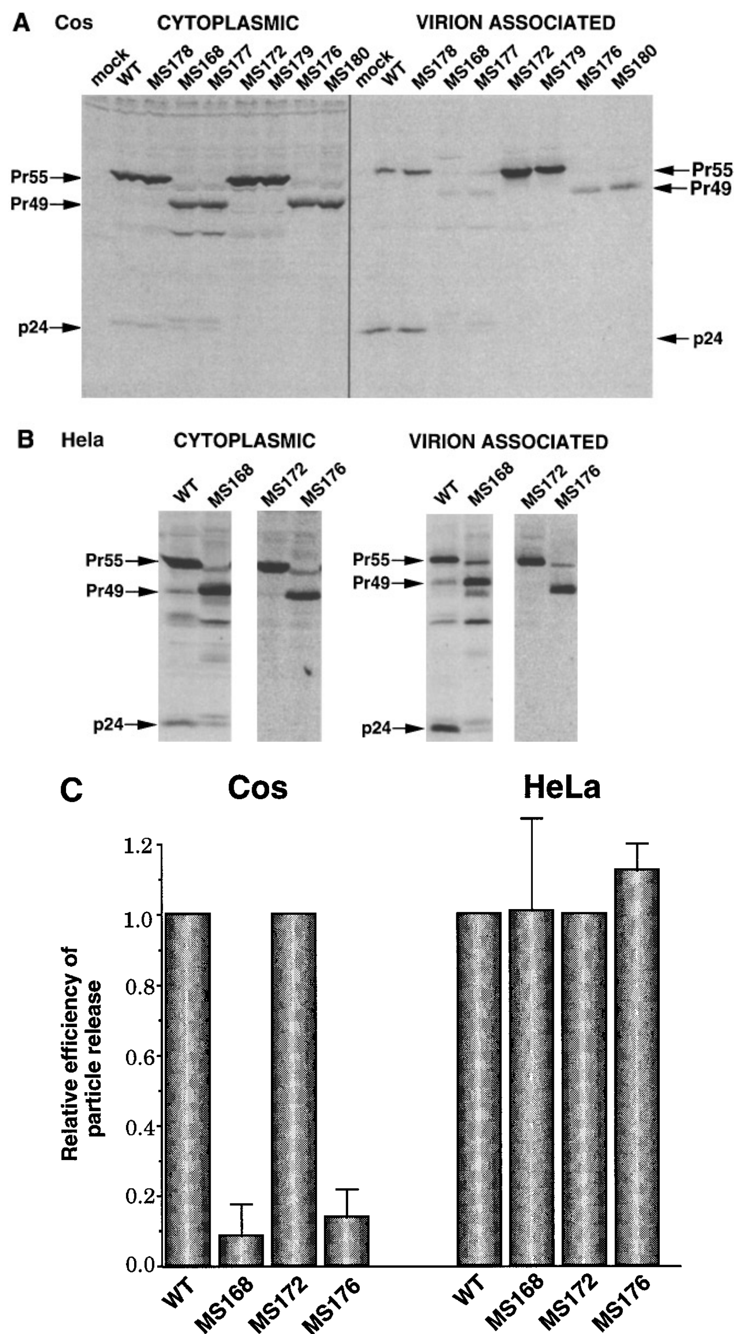


FIG. 3. Immunoblot analysis of cytoplasmic and virion-associated proteins from p6 and Vpr mutant DNAs. Cos (A) or HeLa (B) cells were transfected with various expression plasmids. Forty-eight hours posttransfection, cytoplasmic proteins were isolated. Medium from cells was subjected to high-speed centrifugation and viral particles were pelleted through a 20% sucrose cushion. Both cytoplasmic and virion-associated proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was probed with a rabbit anti-Gag serum and bound antibodies were visualized by incubation with an anti-rabbit ^{35}S-conjugated antibody. Gag protein was visualized and quantified by phosphorimage analysis. The relative ratio of virion-associated to intracellular gag protein for relevant p6^{+} and p6^{-} virus pairs is presented in C. In each case, the ratio for the p6^{+} virus has been normalized to 1.0.

type of this mutant, in HeLa cells, was similar to that of a Vpu mutant (data not shown).

Vpu was necessary for efficient particle release from Jurkat cells. Given our results with Cos cells, we wanted to see whether p6 was also necessary for exit of particles from this T-cell line or whether virus production occurred efficiently even in the absence of p6. Jurkat cells were transfected in parallel with pWT (p6^{+}) or pMS168 (p6^{-}) and particle production was assessed in a manner similar to that for particle production from Cos and HeLa cells. The results of this experiment indicated that, as with HeLa cells, virus particle release in the absence of

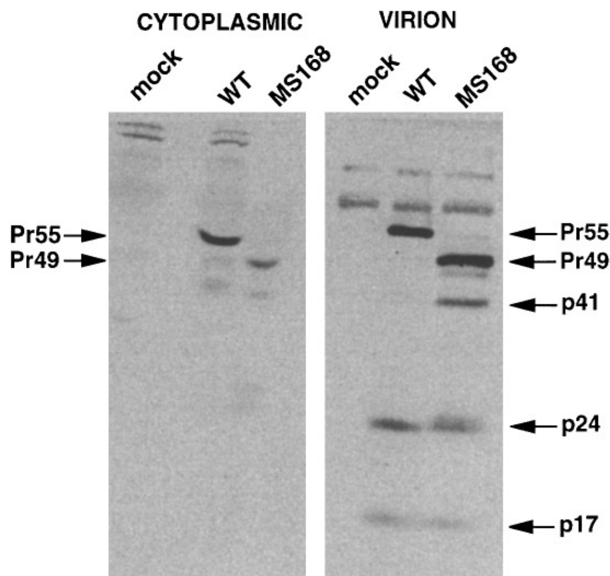


FIG. 4. Immunoblot analysis of cytoplasmic and virion-associated proteins from Jurkat cells. Jurkat cells were transfected with the indicated expression constructs. Forty-eight hours posttransfection, cytoplasmic proteins were isolated. Medium from cells was subjected to high-speed centrifugation and viral particles were pelleted through a 20% sucrose cushion. Both cytoplasmic and virion-associated proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was probed with a rabbit anti-Gag serum and bound antibodies were visualized by incubation with an anti-rabbit ^{35}S-conjugated antibody. Virus-specific protein was visualized and quantified by phosphorimage analysis.

p6 (pMS168) was at least as efficient as release in the presence of p6 (pWT) (Fig. 4). Thus, Jurkat (or HeLa) and Cos cells display inverse dependence on Vpu and p6 for efficient particle release.

The extent of intracellular processing is different in HeLa and Cos cells. Intracellular processing of Gag precursor protein is extensive in Cos cells; in pulse–chase analysis, by 120 min after initiation of the chase, abundant p24 is observed in cells (Fig. 2). In contrast, analysis of intracellular Gag processing at 120 min using an identical protocol indicates relatively less proteolytic processing in HeLa cells (*6*). Since proteolytic processing is an event that temporally overlaps with particle release, we wanted to determine whether the difference in the extent of intracellular proteolytic processing was responsible for the cell line-dependence of Vpu and p6. In particular, we wanted to test the possibility that extensive intracellular processing obviates the need for Vpu. Therefore, viral protein was produced in Cos cells, in the presence and the absence of Vpu, from a construct containing an amino acid substitution in the active site of the viral protease. These constructs, pMS172 (vpu^+pr^-) and pMS173 (vpu^-pr^-) (Fig. 1) contain or lack an intact *vpu* gene, respectively, and would be expected to produce Gag and Gag/Pol polyprotein precursors that are not proteolytically processed.

We transfected Cos cells with either pMS172 (pr^-vpu^+) or pMS173 (pr^-vpu^-) and 48 hr after transfection the cells were metabolically labeled for 1 hr with L-[^{35}S]-methionine. Intracellular and extracellular Gag were then immunoprecipitated after a 3-hr chase period. The results of this experiment demonstrate that, as expected, the released viral cores were composed of unprocessed Gag precursor protein. Further, the proficiency of particle release from Cos cells was similar in the presence or the absence of protease activity (data not shown). Thus, the Vpu-independent release of viral protein occurs even in the absence of processing and indicates that the variance in intracellular proteolytic processing of Gag is unlikely to account for the differential dependence of particle release on Vpu in Cos and HeLa cells.

We also determined whether lack of Gag processing affects the phenotype of a p6$^-$ virus. We transfected pMS172 ($p6^+pr^-$) and pMS176 ($p6^-pr^-$) (Fig. 1) in parallel into Cos and HeLa cells and virus particle release was monitored as described above. The results of that analysis indicated that the absence of processing also had no effect on p6-enhanced particle release from either cell-type (Figs. 3A and 3B). A summary of the data from several transfections of pMS172 and pMS176 into Cos and HeLa cells is provided in Fig. 3C. These data are reported as the relative efficiency of virus particle release and are derived by calculating the ratio of total virion-associated Gag protein and the total cytoplasmic levels of Gag protein. The relative efficiency of virus particle release from pMS172 is normalized to 1.0.

Transport and exit of type-D retroviruses, such as Mason–Pfizer monkey virus (MPMV), is fundamentally different from that of type-C and lentiviruses. MPMV can replicate in Cos cells and virus budding is achieved by formation of intracellular capsids, the entry of those capsids into internal vesicles, and release of virus by exocytosis (*38*). This mode of release can also be accompanied by relatively extensive proteolytic processing of Gag. Thus, we wanted to see whether the primary route of HIV-1 out of Cos cells is via a pathway similar to that used by type-D retroviruses and whether this pathway bypasses the need for Vpu.

To determine whether intracellular core formation occurs more efficiently in Cos cells than in HeLa cells we employed an intracellular capsid formation assay adapted from Rhee *et al.* (*39*). Briefly, Cos or HeLa cells that had been transfected with the pWT were metabolically labeled with L-[^{35}S]methionine and lysed at various time points with a Triton X-100 lysis buffer. This lysate was then subjected to centrifugation. The supernatant and pellet were then independently immunoprecipitated with a rabbit anti-Gag serum and the viral proteins were separated by 10% SDS–PAGE and analyzed by phosphorimage analysis. This analysis indicated that in both

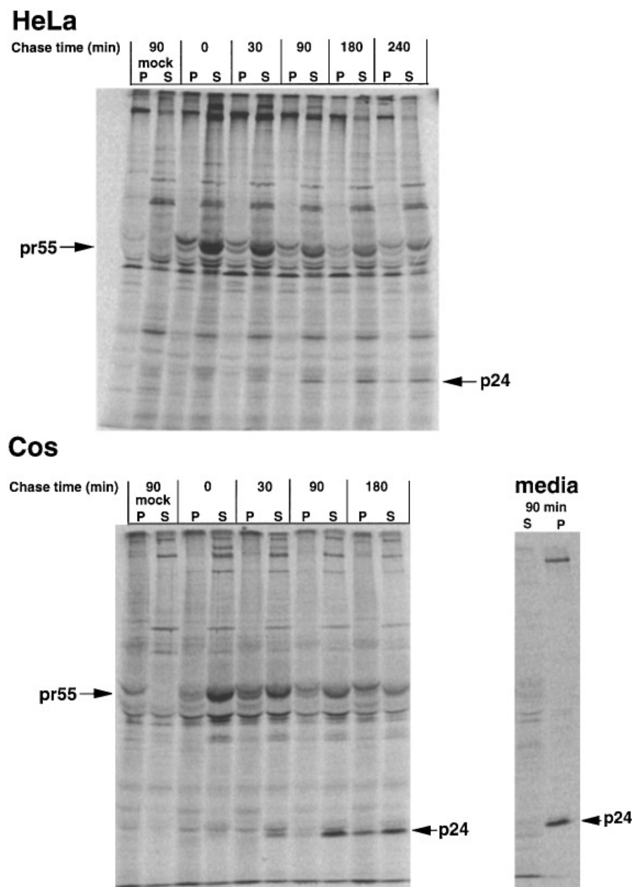


FIG. 5. Intracytoplasmic capsid formation assay. Cos or HeLa cells were transfected with pWT and 36 hr posttransfection cells were pulsed labeled with L-[^{35}S]methionine for 60 min and chased for various times. Following the chase periods cells were lysed with Triton X-100 lysis buffer and subjected to centrifugation through a 30% sucrose cushion. The pellet and supernatant from the spins were then independently immunoprecipitated with a rabbit anti-Gag serum, subjected to 10% SDS–PAGE, and visualized by phosphorimage analysis. Media from cells of the 90-min chase were also subjected to the same virus isolation and immunoprecipitation protocol to demonstrate that this procedure is appropriate for the isolation of HIV-1 particles (media).

Cos and HeLa cells the majority of the viral protein is found in the soluble fraction, suggesting that intracellular particles do not form efficiently (Fig. 5). These results are consistent with coupled particle assembly and release at the cell surface, a mode typical for type-C and lentiviruses rather than type-D retroviruses. When medium from transfected cells was subjected to the same assay as the cell lysates, we found that fully processed p24 can be pelleted, indicating that the conditions used for fractionation of soluble and insoluble viral protein from within cells are appropriate for the isolation of HIV-1 particles (Fig. 5, media).

In addition to facilitating particle release from HeLa cells, Vpu promotes the degradation of CD4 in HeLa cells (*11, 12*). Evidence to date indicates that these two activities are separate. However, since Vpu was not required for efficient release from Cos cells we wanted to see whether CD4 degradation was rapid even in the absence of Vpu in Cos cells. Thus, we used the method of Willey *et al.* (*11, 12*) to measure intracellular CD4 levels in the presence and absence of Vpu. In brief, we cotransfected pWT (vpu^+) or pBG135 (vpu^-), along with pHIV-CD4 (*40*) and pHcenvSV (*41*), into Cos or HeLa cells. To measure steady-state CD4 levels we then performed immunoblot analysis on the cell lysates using polyclonal anti-CD4 antibodies to monitor CD4. The results of this experiment indicated that Vpu was required for rapid intracellular degradation of CD4 in both Cos and HeLa cells (data not shown). Thus, in contrast with particle release, the data indicate Vpu is able to induce CD4 degradation in Cos cells. This indicates that there is not a factor(s) in Cos cells that is the equivalent to Vpu. Moreover, this result is consistent with the notion that enhancement of particle release and induction of CD4 degradation are discrete, unconnected activities triggered by Vpu.

In HIV-1 particles, one of the major constituents of the viral core is the product of the *vpr* gene. Association of Vpr with particles is reliant on p6 (*27, 35*) so it is possible that the p6-mediated particle release from Cos cells involves the action of Vpr. To determine whether Vpr plays a role in p6-mediated release of particles from Cos cells we constructed a battery of vpr^- single, double, and triple mutants. Mutations were introduced into pWT, pMS168, pMS172, and pMS176 which create a stop codon after amino acid 11 of Vpr. These mutant DNAs are designated pMS178 ($p6^+pr^+vpr^-$), pMS177 ($p6^-pr^+vpr^-$), pMS179 ($p6^+pr^-vpr^-$), and pMS180 ($p6^-pr^-vpr^-$). We then transfected these DNAs into Cos cells (Fig. 3A) or HeLa cells (data not shown) and analyzed cytoplasmic protein expression and virus particle release by immunoblot analysis. All of the Vpr^- mutant DNAs expressed the Gag proteins anticipated from their genotype. Moreover, the absence of Vpr had no detectable effect in any of the alternative genetic backgrounds (Fig. 3A and data not shown). Thus, Vpr is unlikely to function in p6-mediated particle release.

Our results indicate that p6 is required for efficient virus particle release from Cos cells but that it is dispensible for this event in HeLa and Jurkat cells. These findings are in agreement with other studies which demonstrate a requirement for p6 for the efficient release of virus particle from Cos cells (*24*). However, our finding that p6 is not required for efficient virus particle release from HeLa cells differs from the results of a previous study (*26*). Huang *et al.* (*26*) found that p6 is required for efficient virus particle release from HeLa cells. One possible explanation for this discrepency is that in their study the viral proteins are produced in the context of a full-length, infectious HIV-1 molecular clone. Studies which indicate that p6 is not required express viral proteins from a system other than a full-length, infectious HIV-1 molecular clone (*27–31, 33, 34*). Our system ex-

presses viral proteins from a noninfectious HIV-1 molecular clone containing a deletion in the *env* gene.

Huang *et al.* (*26*) also demonstrated that virus containing mutations in the PTAP motif of p6 are efficiently released from HeLa cells when proteolytic processing of Gag does not occur. In our system we find that proteolytic processing of Gag does not significantly affect the phenotype of a p6 deletion in Cos cells. Since we do not see an altered phenotype for a p6 deletion in HeLa cells, it is difficult to draw conclusions regarding the role of proteolytic processing on a p6 deletion in HeLa cells. However, in Cos cells the lack of a significant effect of proteolytic processing on a p6 deletion in our system could be explained by the differences in the p6 mutations between these two studies. Huang *et al.* (*26*) reported the effect of proteolytic processing on the efficiency of release of virus particles containing mutations in the PTAP motif, while we studied the effect of proteolytic processing on the efficiency of release of virus particles containing a p6 deletion. Either the effect of proteolytic processing on the efficiency of release of particles containing p6 mutations from Cos cells is not significant, since we see only a modest increase in the efficiency of virus particle release from pMS176 (Figs. 3A and 3C), or the effect of proteolytic processing on p6 mutations is not manifested when the entire p6 domain is deleted.

We also observe an alteration in the extent of proteolytic processing of virus particles containing p6 deletions in both HeLa and Cos cells. This alteration is evident by the increased amount of p41 (MA-CA) and p25 (CA intermediate). In addition, virus particles containing a p6 deletion have a decreased p24/pr55 ratio (data not shown). This alteration in proteolytic processing could be due to a direct effect of p6 on HIV-1 PR or, more likely, the deletion or mutation of p6 could affect the overall conformation of pr55, resulting in the observed processing alteration.

The most straightforward explanation for our results is that there is a cell-specific difference that renders Vpu and p6 dispensable for particle release from Cos and HeLa (Jurkat) cells, respectively. There are two general models by which this could occur. First, there could be two distinct pathways that lead to release of HIV-1 particles from cells, one being Vpu-dependent and the other being p6-dependent. The dominant pathway would then be different in Cos and HeLa cells. We determined by the intracytoplasmic capsid formation assay that there is not a gross difference in the assembly of viral capsids between Cos and HeLa cells. It appears that virus assembly and budding occur at the plasma membrane, the hallmark of lenti- and type-C viruses in both cell lines. The second possibility is that Vpu and p6 function in distinct steps along a single pathway that leads to particle release. Cos cells could merely contain a factor that functionally substitutes for Vpu, while HeLa and Jurkat cells contain a factor that obviates the requirement for p6. A variation of this basic scheme is that the rate-limiting step in particle egress might vary in alternative cell lines. In HeLa and Jurkat cells, the Vpu-dependent step would be rate limiting in the absence of Vpu, while in Cos cells the p6-dependent step would be rate limiting when p6 is absent.

Our genetic data indicate that the activities of Vpu and p6 are not antagonistic and are consistent with the idea that both Vpu and p6 are positive factors that promote particle release. There are two relatively simple ways to account for the cell line difference in Vpu-dependent particle release. First, a specific cellular factor may be present in Cos cells that has a Vpu-like activity, thereby obviating the need for Vpu in particle release. A second possibility is that HeLa and Jurkat cells express a factor that negatively affects particle release and that Cos cells lack this factor. Vpu would then be required to counteract that negative factor in HeLa and Jurkat cells. Two complementary, and similar, possibilities can be invoked to account for the cell line difference in p6-dependent virus exit.

Vpu-mediated CD4 degradation occurs in both Cos and HeLa cells. If Vpu interacts with cellular factors to mediate CD4 degradation then those cellular factors must be common to both Cos and HeLa cells. These data also reinforce the idea that the two activities of Vpu are mechanistically separate (*42*).

Vpr is a protein found in particles that plays a role in viral replication by facilitating transport of the viral DNA–protein complex across the nuclear membrane (*40*). In addition, Vpr appears to affect the progression of cells through the cell cycle, an activity not clearly linked to replication (*43, 44*). Vpr is incorporated into particles by direct or indirect interaction with the p6 domain of Gag. On the other hand, our results suggest that Vpr plays no role in p6-mediated particle release. Thus, the two roles of the p6 domain of Gag (to augment particle release and to allow association of Vpr with particles) do not appear to be directly related.

Our initial experiments that examined particle release were predicated on the hypothesis that HIV-1 acquired a *vpu* gene to more successfully replicate in human cells. However, we do not yet know whether the cell line difference we observed with HeLa, Jurkat, and Cos cells is uniformly applicable to other simian and human cells. SIV can replicate in human cells. However, replication is accompanied by selection for variants that encode a truncated Env glycoprotein (*45*). Moreover, a recent report indicates that p6 is not absolutely required for HIV-1 particle release from African green monkey cells (BSC-40) (*21*). These observations suggest that the difference in particle release may not strictly correlate with the species from which the cells arise. More stringent challenge of our hypothesis will require analysis of particle release from a battery of additional cell lines.

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