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A Leucine Triplet Repeat Sequence $(LXX)_4$ in $p6^{gag}$ Is Important for Vpr Incorporation into Human Immunodeficiency Virus Type 1 Particles

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Incorporation of Vpr into human immunodeficiency virus type 1 (HIV-1) virions is mediated by the Gag protein, independently of other viral components. We have coexpressed Vpr and Gag constructs in a vaccinia virus expression system in order to map the region of Gag involved in Vpr packaging. Deletion of the carboxyl-terminal p6 region of Gag impaired the ability of Gag to package Vpr. To confirm the role of p6 in Vpr packaging, Rous sarcoma virus (RSV)-HIV chimeras containing HIV-1 p6 were constructed. Although RSV Gag does not package Vpr into virus particles, a chimera containing HIV-1 p6 is sufficient for Vpr incorporation. To map the region of p6 involved in Vpr packaging, a series of p6 point mutations and deletion mutations was analyzed. Mutations in the N-terminal p6 proline-rich domain, for which preliminary evidence shows a marked decrease in virion incorporated RNA, did not affect Vpr incorporation. Deletion of residues 1 to 31 of HIV-1 p6 did not affect Vpr packaging, but residues 35 to 47, including an $(LXX)_4$ domain, were required for Vpr incorporation into virus particles.

The human immunodeficiency virus type 1 (HIV-1) Gag polyprotein is synthesized on free ribosomes in a cytosolic compartment, and it is subsequently transported to the cytoplasmic face of the plasma membrane (44). The Gag proteins then interact to form particles which bud from the plasma membrane. During or after budding, a virus-encoded protease cleaves the Gag polyprotein into its mature products: MA (matrix, p17), CA (capsid, p24), NC (nucleocapsid, p9), and p6 (44). However, proteolytic processing is not a prerequisite for particle release. Moreover, no viral products other than the Gag polyprotein are required for budding.

vpr is one of six auxiliary or accessory genes of HIV-1 (46). It encodes a 96-amino-acid protein with a molecular mass of 14 kDa (31, 46) that is capable of forming oligomers (4, 49). HIV-1 *vpr* shares homology with both *vpr* and *vpx* of HIV-2 and simian immunodeficiency virus (HIV-2/SIV) (40).

Although *vpr* is dispensable for HIV-1 replication in many T-lymphoid cell lines, it promotes virus replication in quiescent cells, such as macrophages (1, 2, 7–9, 15, 32, 34, 43). To explain these effects, several biological functions of Vpr have been defined. HIV-1 Vpr is efficiently incorporated into virions, like Vpx incorporation into virions of HIV-2/SIV (5, 6, 17, 18, 23, 25–27, 34, 41, 47). Vpr is targeted to the nucleus (27, 48) and contributes to the transport of the viral preintegration complex from the cytoplasm to the nucleus in quiescent cells (16). The nuclear localization of Vpr may also relate to its weak nonspecific transcriptional activation properties (5), its ability to induce differentiation of rhabdomyosarcoma cells (24), its association with glucocorticoid receptors (35), and its ability to arrest cells in the G_2 stage of the cell cycle (28, 36). It remains to be determined how these activities relate to the possible pathogenic role of Vpr of SIV in a rhesus macaque model (11, 22).

It is perplexing that Vpr can be targeted to either the nucleus or the budding virus particle. The mechanism of these targeting functions, including Vpr incorporation into virus particles, is incompletely described. Virion RNA, envelope proteins, and *pol*-encoded proteins are not required for Vpr packaging into virus particles (23, 27, 34). However, a deletion of the carboxyl-terminal domain of the 55-kDa Gag precursor protein, including the p6 domain, abrogates Vpr particle incorporation (23, 27, 34). Although preliminary evidence suggests that p6 may have a role in virion RNA packaging (13) and a role in the final stages of particle release (14, 33), in some contexts it is dispensable for particle formation (19, 20, 34, 37, 38). The current study examines the role of p6 in Vpr particle incorporation, and it defines a sequence with a repeated leucine triplet sequence as critical for this activity.

MATERIALS AND METHODS

Cell lines and antisera. BSC40 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The anti-Vpr, anti-p24, and anti-Rous sarcoma virus (RSV) antisera were previously described (27, 42).

Construction of Vpr and Gag expression plasmids. Plasmids pTM3, pTM-VPR, and pTM-GAG(p55) were previously described (27). To construct pTM(p6−) (Fig. 1A), pTM-GAG(p55) was digested with *Xba*I and *Bgl*II, and the 2.5-kb fragment was isolated and ligated to pTM3 between the *Xba*I and *Bam*HI sites. pTM-GAG($p6_{19–52}$) was constructed by insertion of a PCR product with nucleotides 2180 to 3025 of *gag* from pTM-GAG(p55), obtained with the following primers: 5′-AGGTCTCGAGTAGAGACAACAACTCC and 5′-ATT GCTCGAGATCCTTTCCATCCCTGTGG. The reaction products were digested with *Xho*I and cloned into the *Xho*I site of pTM-GAG(p6−). This results in an insertion of the amino acid sequence proline-alanine-alanine-arginine between the HIV-1 Gag spacer region and the p6 sequences. pTM-GAG ($p6_{32–52}$) was constructed by a similar strategy with nucleotides 2226 to 3025, using the upstream primer 5′-AGCCTCGAGACAAGGAACTGTATCCT and the same downstream primer. This also results in an insertion of a proline-

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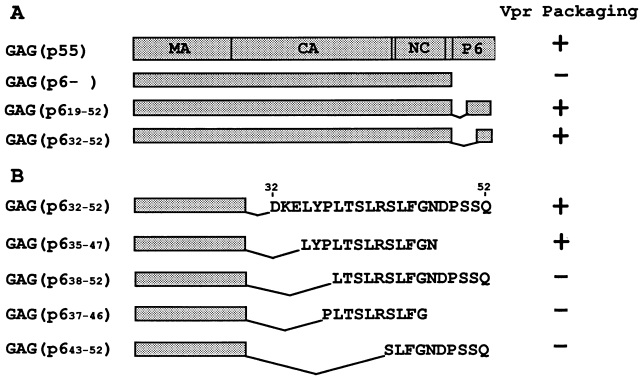

FIG. 1. HIV-1 Gag expression plasmids. (A) Domains of the p55 GAG polyprotein are shown at the top together with domains deleted in GAG(p6−), GAG($p6_{19–52}$), and GAG($p6_{32–52}$). (B) Residues from p6 attached to GAG(p6−), which include parts or all of residues 32 to 52, are indicated.

alanine-alanine-arginine sequence between the HIV-1 Gag spacer region and p6 sequences.

The other constructs with partial C-terminal sequences of p6 (Fig. 1B) were generated by linker ligation between the *Xho*I and *Sal*I sites of pTM-GAG(p6−). pTM-GAG($p6_{35–47}$) was constructed with 5′-TCGAGCTGTATCCTTTAACT TCCCTCAGGAGCCTCTTTGGCAACTAAG and 5′-TCGACTTAGTTGC CAAAGAGGCTCCTGAGGGAAGTTAAAGGATACAGC. pTM-GAG ($p6_{38–52}$) was constructed with 5′-TCGAGTTAACTTCCCTCAGGAGCCT CTTTGGCAACGACCCCTCGTCACAATAAG and 5′-TCGACTTATTGTG ACGAGGGGTCGTTGCCAAAGAGGCTCCTGAGGGAAGTTAAC. pTM-GAG($p6_{37–46}$) was constructed with 5′-TCGAGTTAACTTCCCTCAGGAGC CTCTTTGGCAACTAAG and 5′-TCGACTTAGTTGCCAAAGAGGCTCC TGAGGGAAGTTAAC. pTM-GAG($p6_{43–52}$) was constructed with 5′-TCGAG AGCCTCTTTGGCAACGACCCCTCGTCACAATAAG and 5′-TCGACTTA TTGTGACGAGGGGTCGTTGCCAAAGAGGCTC. The structure of each clone was confirmed by restriction enzyme and nucleotide sequence analysis.

RSV-HIV chimeric clones (3) were cloned into pTM3. pSV.Myr1.3h (42) encodes RSV Gag amino acid residues 1 to 577, a sequence which includes all of RSV *gag* except the protease-coding sequences. pSV.RHP6 contains the same RSV *gag* sequences as pSV.Myr1.3h, but with the HIV-1 p6-coding sequences attached to the 3′ end of RSV *gag*. pSV.RHE.D25S expresses a truncated RSV Gag product with amino acid residues 1 to 418 fused to the C terminus of HIV-1 Gag-Pol amino acid residues 241 to 731. The expression of HIV-1 protease was inactivated by changing the aspartic residue 25 to serine. To generate pTM-RSV-GAG (see Fig. 3A), pSV.Myr1.3h was digested with *Drd*I and *Xba*I; the fragment with nucleotides 336 to 2765 was isolated, blunt ended in the presence of deoxynucleoside triphosphates with the Klenow fragment of *Escherichia coli* DNA polymerase I, and cloned into the blunt-ended *Nco*I site of pTM3. pTM-RHP6 and pTM-RHE (see Fig. 3A) were generated from pSV.RHP6 and pSV.RHE.D25S, using the same strategy.

NL4-3 clones with mutations in the PTAPP domain, amino acid residues 7 to 11 of p6, were previously described (12, 13). The following oligonucleotides were used to introduce mutations changing PTAPP to PTALP (P10L) and PTAPP to PTNPP (A9N): 5′-CCAGAGCCAACAGCCCTACCAGAAGAGAGCTTC and 5′-AGACCAGAGCCAACAAACCCACCAGAAGAGAGC, respectively. These plasmids were digested with *Bcl*I, blunt ended with T4 DNA polymerase, and digested with *Pst*I. The fragment containing nucleotides 1415 to 2429 was cloned between the *Pst*I and *Stu*I sites of pTM-GAG(p55) to generate pTM-p6(P10L) and pTM-p6(A9N).

Infection-transfection protocol for vaccinia virus expression system and immunoprecipitation analysis. BSC40 cells were grown to 90% confluence on 60-mm-diameter plates, infected for 30 min at 37°C with vTF7-3 (10, 30) at a multiplicity of infection of 10, and transfected with pTM-based vectors with Lipofectin. Four hours after transfection, the cells were labeled for 20 h with 1.5 ml of methionine, cysteine, and leucine-free Dulbecco's modified Eagle medium containing 50 μCi of [4,5-^{3}H]leucine and 50 μCi of Tran[^{35}S]label. The culture supernatants were removed and centrifuged at 2,500 rpm for 15 min in a Beckman GS-6 rotor to remove cellular debris, and 0.1 volume of 10× radioimmunoprecipitation assay buffer (10% [vol/vol] Triton X-100, 5% [wt/vol] deoxycholate, 1% [wt/vol] sodium dodecyl sulfate [SDS], 2 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline [PBS]) was added. The cells were scraped in PBS, centrifuged at 1,000 rpm for 5 min in a Beckman GS-6 rotor, and then resuspended in 500 μl of 1× radioimmunoprecipitation assay buffer. The Gag and Vpr proteins were immunoprecipitated overnight at 4°C with the indicated antisera as described previously (27).

The amounts of Gag and Vpr immunoprecipitated from the culture supernatants were determined by densitometric analysis. The ratios of Vpr to Gag were determined and normalized to the ratio of Vpr to Gag (p55).

Sucrose density equilibrium gradients. BSC40 cells were infected, transfected, and labeled with [^{3}H]leucine and Tran[^{35}S]label as described above. Cellular debris was removed from the conditioned medium by centrifugation at 2,500 rpm for 15 min in a Beckman GS-6 rotor. The supernatant was layered on a linear 20 to 50% (g/g) sucrose gradient in PBS, and centrifugation was performed in a SW28.1 rotor at 20,000 rpm for 16 h at 4°C. Fractions were collected from the top of the tube.

RESULTS

p6 is required for Vpr particle incorporation. To examine Vpr packaging into virus-like particles, we used a vaccinia virus expression system that faithfully reproduces characteristics of HIV-1 assembly (27, 38). For this purpose, Vpr or Gag sequences were expressed from a pTM3-based plasmid which includes a T7 polymerase promoter (pTM-VPR or pTM-GAG). Plasmids were transfected into BSC40 cells infected with a vaccinia virus expressing T7 polymerase (vTF7-3), and the cells were metabolically labeled with [^{3}H]leucine and Tran[^{35}S]label for optimal detection of both Gag and Vpr proteins. Cell lysates were analyzed by immunoprecipitation to assess the level of Gag and Vpr expression, whereas virus-like particles present in cell supernatants were examined for Vpr incorporation.

To examine the role of p6 in Vpr packaging, pTM-GAG(p6−) (Fig. 1A) was constructed to express a truncated Gag polyprotein missing the entire p6 sequence [GAG(p6−)]. BSC40 cells were infected with vTF7-3, transfected with pTM-VPR and pTM-GAG(p55), expressing the full-length 55-kDa Gag polyprotein, or pTM-GAG(p6−), which is missing the entire p6 sequence. The cells were metabolically labeled with [^{3}H]leucine and Tran[^{35}S]label. Radiolabeled Vpr and Gag proteins were detected in cell lysates and cell supernatants by immunoprecipitation with either an anti-p24 or an anti-Vpr antiserum and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2).

Coexpression of pTM-GAG(p55) with pTM-VPR resulted in expression of both Gag (p55) and Vpr (p14) in cell lysates (Fig. 2, lanes 1 and 3) and export of Gag and Vpr into culture supernatants (Fig. 2, lanes 5 and 7). Gag and Vpr are both found in virus-like particles (27). In contrast, when pTM-VPR was expressed alone, no export of Vpr into culture supernatants could be detected (27). When pTM-VPR was expressed

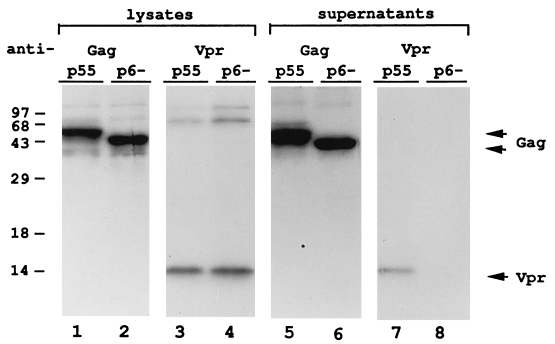

FIG. 2. p6 sequences are required for particle incorporation of Vpr. BSC40 cells were infected with vTF7-3, transfected with 10 μg of pTM-VPR and 20 μg of pTM-GAG(p55) (lanes 1, 3, 5, and 7) or pTM-GAG(p6−) (lanes 2, 4, 6, and 8), and metabolically labeled with Tran[^{35}S]label and [^{3}H]leucine. Radiolabeled Vpr and Gag proteins were detected in cell lysates and culture supernatants by immunoprecipitation with either an anti-p24 (lanes 1, 2, 5, and 6) or anti-Vpr (lanes 3, 4, 7, and 8) antiserum and then subjected to SDS-PAGE (13% polyacrylamide gel) analysis. Arrows to the right indicate the migration of Gag and Vpr proteins in the gel. Sizes are indicated in kilodaltons.

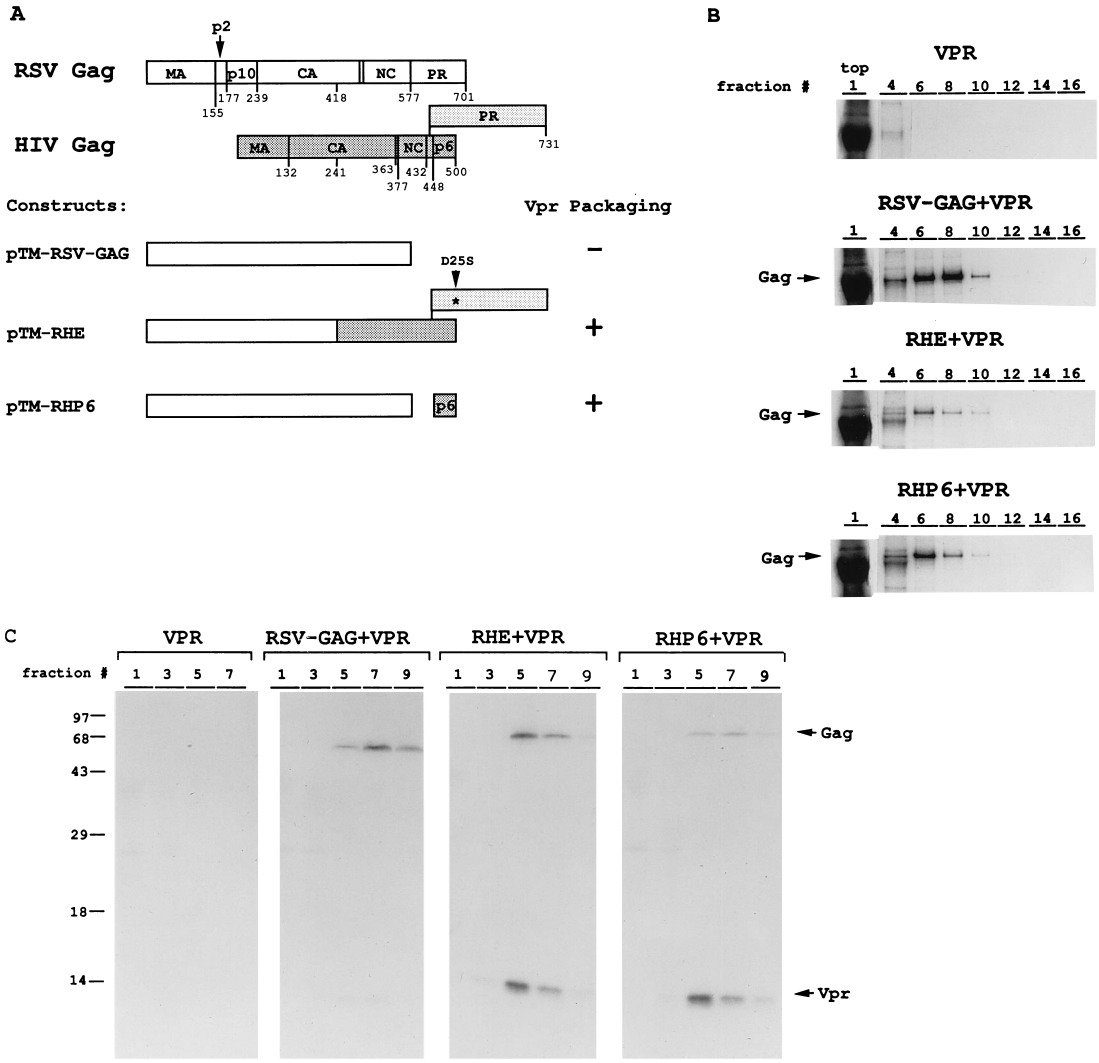

FIG. 3. Addition of HIV-1 p6 sequences to RSV Gag results in Vpr particle incorporation. (A) Schematic drawing of wild-type and chimeric Gag polyproteins. The Gag proteins of RSV (open boxes), HIV-1 Gag (darkly shaded boxes), and HIV-1 Pol products which include protease (PR) and a portion of reverse transcriptase (lightly shaded boxes) are illustrated at the top and are aligned at the sites in capsid (CA) used for making chimeras. The names of the Gag cleavage products are indicated, and the numbers refer to amino acid residues. The schematic diagrams of pTM-RSV-GAG and RSV-HIV chimeras (pTM-RHE and pTM-RHP6) are illustrated at the bottom and are aligned to the relative positions in RSV and HIV-1 Gag sequences. pTM-RSV-GAG includes RSV Gag residues 1 to 577. RHE includes RSV Gag residues 1 to 418 fused to HIV-1 Gag-Pol residues 241 to 731. The proteolytic activity of HIV-1 protease was inactivated by changing the Asp at residue 25 to Ser, a residue within the catalytic site of protease. RHP6 includes RSV Gag residues 1 to 577 fused to HIV-1 p6 sequences. The ability of each chimera to incorporate Vpr is indicated in the column to the right. (B) BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR alone or cotransfected with pTM-RSV-GAG, pTM-RHE, or pTM-RHP6 (as indicated). The cells were metabolically labeled with Tran[^{35}S]label and [^{3}H]leucine. The culture supernatants were harvested and centrifuged through 20 to 50% (g/g) sucrose gradients. The fractions were collected from the top of the gradients (1 ml per fraction). The presence of labeled proteins in each fraction was analyzed directly by SDS-PAGE (13% polyacrylamide gel) by loading 25 μl of sample from each fraction. Arrows to the left indicate the migration positions of Gag proteins. (C) The fractions including the peak of each gradient were analyzed by immunoprecipitation with rabbit anti-Vpr serum prior to SDS-PAGE. Arrows to the right indicate the migration positions of Vpr proteins which are specifically immunoprecipitated and Gag proteins which are nonspecifically immunoprecipitated. Sizes are indicated in kilodaltons. Numbers above the lanes in panels B and C indicate the gradient fractions; fraction 1 is from the top of the gradient, and fraction 16 is from the bottom of the gradient.

with pTM-GAG(p6−), both proteins were detected in cell lysates (Fig. 2, lanes 2 and 4), significant amounts of GAG (p6−) were found in the culture supernatant (Fig. 2, lane 6), but no Vpr was exported into the culture supernatant (Fig. 2, lane 8). The inability of pTM-GAG(p6−) to incorporate Vpr is not due to a defect in particle formation from this mutant Gag protein, since particles similar in density to those formed from pTM-GAG(p55) were found by sucrose density equilibrium gradient analyses (not shown). These data, from a loss-of-function assay, suggest that Gag p6 plays an important role in Vpr particle incorporation.

Fusion of HIV-1 p6 to RSV Gag allows Vpr particle incorporation. For further analysis of the mechanism of incorporation of Vpr into virions with a gain-of-function assay, chimeric Gag polyprotein expression constructs were generated by combining portions of the *gag* genes of RSV and HIV-1 (Fig. 3A). In the product of pTM-RHE (RHE), residues 1 to 418 of RSV Gag are fused with residues 241 to 500 of HIV-1 Gag, separated only by a single proline residue. Another chimeric protein, RHP6, expressed from pTM-RHP6, includes the HIV-1 p6 protein fused onto the end of an RSV Gag polyprotein which includes only the first seven residues of RSV protease.

RSV GAG, RHE, and RHP6 were expressed by using the vaccinia virus expression system. BSC40 cells were infected with vTF7-3, transfected with pTM-VPR alone or together with pTM-RSV-GAG, pTM-RHE, or pTM-RHP6, and labeled with [^{3}H]leucine and Tran[^{35}S]label. The cell lysates were harvested and immunoprecipitated with the anti-Vpr and anti-RSV antisera. No significant differences in Gag expression were found with RSV GAG, RHE, and RHP6, and similar amounts of Vpr were coexpressed in each case (not shown). For analysis of particle formation from these constructs, the culture supernatants were analyzed on sucrose density equilibrium gradients, and the presence of Gag proteins in the gradient fractions was directly analyzed by SDS-PAGE (Fig. 3B). The majority of labeled proteins was observed in the top fractions of the gradients in each case (Fig. 3B, fraction 1). In contrast, RSV GAG and both chimeric Gag proteins are found in fractions 4 to 10. The peak of each gradient was determined by analyzing the amount of Gag protein in each fraction by densitometric analysis of the autoradiograms (not shown). These data showed that RSV GAG, RHE, and RHP6 formed particles at a density of 1.12 to 1.13 g/ml, slightly lower than the density of a typical retrovirus particle (1.16 g/ml).

For the detection of Vpr in particles, the fractions including the peak of each gradient were analyzed by immunoprecipitation with the anti-Vpr antiserum and SDS-PAGE (12% polyacrylamide gel) (Fig. 3C). Expression of RSV GAG was insufficient for Vpr incorporation into particles. In contrast, expression of both chimeric Gag constructs, RHE and RHP6, allowed Vpr particle incorporation. The ability of RHP6 to incorporate Vpr into particles indicates that HIV-1 p6 sequences are sufficient for Vpr packaging in the context of another retrovirus Gag product. It should be noted that a small proportion of RSV GAG and both chimeric Gag proteins in the gradient fractions was immunoprecipitated nonspecifically as a result of the nonspecific binding of Gag proteins to protein A-Sepharose beads.

Mutation of the proline-rich region in p6 does not affect Vpr packaging. Preliminary evidence suggests that the only functional domain in p6 may be the PTAPP region, residues 7 to 11, at the N terminus (13, 14). Preliminary data show that the mutation of PTAPP to PTALP or PTNPP greatly decreases the function of this domain in viral RNA packaging (13). Therefore, it was of interest to determine if this domain is also involved in Vpr particle incorporation. To address this question, pTM-GAG expression plasmids were constructed with each PTAPP mutation, designated pTM-p6(P10L) and pTM-p6(A9N). BSC40 cells were infected with vTF7-3, transfected with pTM-VPR and pTM-GAG(p55), pTM-GAG(p6−), pTM-p6(P10L), or pTM-p6(A9N), and metabolically labeled, and lysates and culture supernatants were analyzed by immunoprecipitation with anti-Gag and anti-Vpr antisera and SDS-PAGE (Fig. 4). Both mutants were able to package Vpr in amounts comparable to those packaged by the wild-type construct (Table 1), indicating that this RNA packaging domain is not directly involved in Vpr packaging.

The N-terminal region of p6 is not involved in Vpr packaging. Since the PTAPP mutations had no effects on Vpr packaging, we next sought to determine how much of the N-terminal portion of p6 could be deleted without affecting Vpr particle incorporation. For this purpose, we constructed pTM-GAG($p6_{19–52}$), in which amino acid residues 1 to 18 of p6 were deleted, and pTM-GAG($p6_{32–52}$), in which amino acid residues 1 to 31 were deleted (Fig. 1A). These mutants were cotransfected with pTM-VPR into vTF7-3-infected BSC40 cells. After transfection, the cells were metabolically labeled with [^{3}H]leucine and Tran[^{35}S]label. Gag and Vpr proteins were

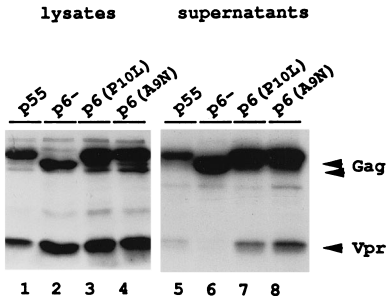

FIG. 4. PTAPP sequence in the N-terminal portion of p6 are not required for Vpr particle incorporation. BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR and Gag expression constructs with or without p6 [pTM-GAG(p55) and pTM-GAG(p6−)] or mutations in the p6 PTAPP sequence [pTM-p6(P10L) and pTM-p6(A9N)], as indicated. Metabolically labeled proteins from cell lysates (lanes 1 to 4) and cell supernatants (lanes 5 to 8) were immunoprecipitated with antisera to Vpr and p24 prior to SDS-PAGE. Arrows indicate the migration positions of Gag and Vpr in the gel.

immunoprecipitated from the cell lysates with the specific antisera, and similar amounts of the two proteins were found in each case (Fig. 5, lanes 1 to 4). The cell supernatants were analyzed on sucrose density equilibrium gradients. Both deletion constructs produced particles with a density of 1.16 g/ml (not shown). The peak fraction of each gradient was immunoprecipitated with anti-Gag and anti-Vpr antisera (Fig. 5, lanes 5 to 8). Both deletion mutants were able to package Vpr into particles with efficiency comparable to that of $Pr55^{gag}$ (Table 1), indicating that amino acid residues 1 to 31 of p6 are dispensable for Vpr particle incorporation. This finding also indicates that the C-terminal sequence of p6 is involved in Vpr packaging.

Identification of the Vpr packaging domain in the C-terminal region of p6. To map the Vpr packaging domain in the C terminus of p6, we generated a series of mutants containing different portions of amino acid residues 32 to 52 of p6. The sequence of the last 20 amino acids of p6 and those of the mutant constructs are shown in Fig. 1B. To characterize the ability of these constructs to incorporate Vpr into particles, BSC40 cells were infected with vTF7-3 and cotransfected with pTM-VPR and the pTM-p6 deletion mutants. The presence of Gag and Vpr in cell lysates and cell supernatants was assessed by immunoprecipitation and SDS-PAGE (Fig. 6A). Unexpectedly, we observed a lower level of expression for all of the Gag mutants containing partial p6 sequences (Fig. 6A, lanes 2 to 5). The efficiency of Vpr particle incorporation was determined by densitometry to measure the Vpr/Gag ratio in the culture

TABLE 1. Comparison of Vpr packaging efficiencies of the HIV-1 Gag p6 mutants

Gag protein	Vpr/Gag[a]
p55	1.00
p6(P10L)	0.80 ± 0.28
p6(A9N)	1.00 ± 0.29
$p6_{19–52}$	0.98 ± 0.14
$p6_{32–52}$	1.21 ± 0.24
$p6_{35–47}$	0.99 ± 0.03
$p6_{38–52}$	ND
$p6_{37–46}$	ND
$p6_{43–52}$	ND
p6−	ND

[a] Values were determined from two independent experiments. ND, not determined.

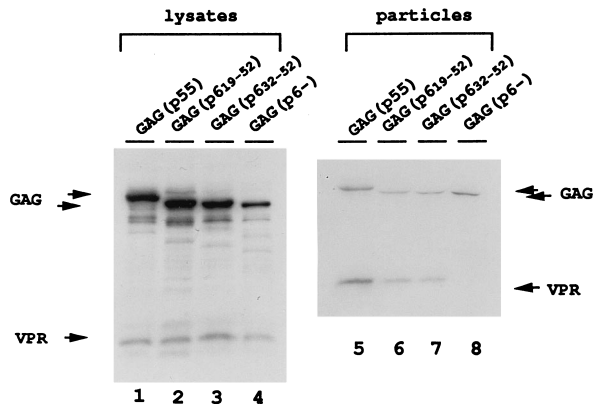

FIG. 5. The N-terminal region of p6 is dispensable for Vpr particle incorporation. BSC40 cells were transfected with pTM-VPR and Gag expression constructs with or without p6 [pTM-GAG(p55) and pTM-GAG(p6−)] or with amino acid residues 19 to 52 or 32 to 52 of p6 [pTM-GAG($p6_{19–52}$) and pTM-GAG($p6_{32–52}$)], as indicated. Metabolically labeled proteins from cell lysates were analyzed by immunoprecipitation with antisera to p24 and Vpr prior to SDS-PAGE (lanes 1 to 4). The supernatants from each transfection were centrifuged through 20 to 50% (g/g) sucrose gradients. The peak fraction was determined, and the labeled proteins in the peak fractions were analyzed by immunoprecipitation with antisera to p24 and Vpr prior to SDS-PAGE (lanes 5 to 8). Arrows indicate the locations of Gag and Vpr.

supernatant (Fig. 6A, lanes 7 to 12; Table 1). To make a more accurate comparison, lanes 8 to 11 in Fig. 6A were exposed for twice as long as lanes 9 and 12 in order to achieve more similar intensities for the bands with Gag proteins. The results showed that GAG($p6_{35–47}$) was able to incorporate Vpr with an efficiency similar to that of p55 (Fig. 6A, lanes 7 and 8; Table 1). In contrast, we were unable to detect any export of Vpr into supernatants with GAG($p6_{38–52}$), GAG($p6_{37–46}$), and GAG($p6_{43–52}$) (Fig. 6A, lanes 9 to 11).

To further confirm that Vpr was packaged into particles, sucrose density equilibrium gradient analysis was performed with the supernatants of cells cotransfected with pTM-VPR and pTM-GAG(p55) or pTM-GAG($p6_{35–47}$) (Fig. 6B). pTM-GAG($p6_{35–47}$) was able to form particles at a density of 1.16 g/ml, identical to that of particles with p55. Moreover, Vpr was present in the particles generated by pTM-GAG($p6_{35–47}$). This result indicates that in this context, the LYPLTSLRSLFG domain is sufficient for packaging Vpr.

DISCUSSION

The data presented here indicate that deletion of p6 impairs the ability of Pr55^{gag} to incorporate Vpr into virus particles. This result is in agreement with the previous findings of Paxton et al. (34). We have further demonstrated that p6 is sufficient to package Vpr in the context of a heterologous Gag polyprotein from RSV. The incorporation of Vpr into these chimeric Gag particles appeared to be mediated by the p6 region, since Vpr was not incorporated into RSV Gag particles lacking HIV-1 p6 sequences. RSV Gag has remarkably little sequence homology to HIV Gag, in spite of the apparent functional and organizational similarity between these proteins (33, 44). Therefore, the ability of these chimeric Gag proteins to package Vpr seems to be directly associated with the presence of the p6 sequences. Although the sucrose gradient analysis has shown that the RSV-HIV Gag chimeras produced with this vaccinia virus expression system form particles of a lower density than HIV-1 particles, Vpr was still packaged, indicating that these chimeric Gag polyproteins form a correct conformation to allow p6 to interact with Vpr directly or indirectly.

During the preparation of this article a report by Kondo et al. (21) which also demonstrated that p6 is sufficient for Vpr incorporation into virus particles was published. In that work, chimeric murine leukemia virus particles with HIV-1 p6 sequences were capable of Vpr incorporation. Furthermore, p6 residues 1 to 46 were sufficient for Vpr incorporation, whereas residues 1 to 41 were insufficient for that function. The current work confirms and extends these observations. Most notably, we have demonstrated that p6 residues 35 to 47 are critical for Vpr incorporation.

HIV-1 p6 has been shown to play a role in the late budding stage of particle assembly (14). Mutations of p6 prevented release of budded particles from the cell surface. The late budding function has been mapped to the proline-rich region at the N terminus of p6 (14, 33). However, the function of p6 in the late budding step seems to be specific to certain cell types, since some groups have not observed a reduction in particle release for p6 mutants (19, 20, 34, 37, 38). In the system used for the current study, we also did not observe a reduction in particle release with our p6 deletion mutant, p6− (Fig. 2, lane 6). Furthermore, no alteration in Vpr packaging was noted when the proline-rich domain, implicated in the budding function, was altered or deleted. Therefore, we conclude that the Vpr packaging domain of p6 does not overlap with the domain important for budding.

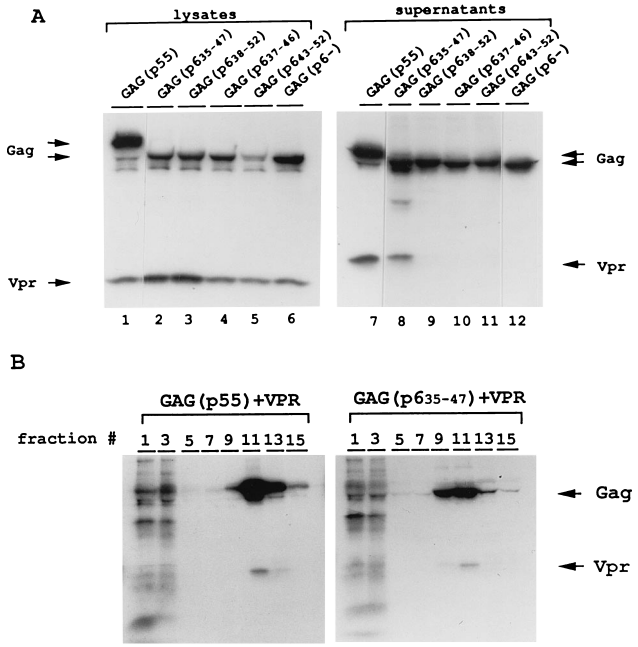

FIG. 6. A 13-residue domain of p6 is sufficient for Vpr particle incorporation. (A) BSC40 cells were infected with vTF7-3 and transfected with pTM-VPR and pTM-GAG(p55), pTM-GAG($p6_{35–47}$), pTM-GAG($p6_{38–52}$), pTM-GAG ($p6_{37–46}$), pTM-GAG($p6_{43–52}$), or pTM-GAG(p6−), as indicated. Metabolically labeled proteins from cell lysates (lanes 1 to 6) and cell supernatants (lanes 7 to 12) were immunoprecipitated with antisera to Vpr and p24 before SDS-PAGE. (B) BSC40 cells were infected with vTF7-3, transfected with pTM-VPR and pTM-GAG(p55) or pTM-GAG($p6_{35–47}$), and metabolically labeled. The culture supernatants were centrifuged through 20 to 50% (g/g) sucrose gradients. The presence of Gag and Vpr in each fraction was detected by immunoprecipitation with antisera to p24 and Vpr before SDS-PAGE. Numbers above the lanes indicate the gradient fractions; fraction 1 is from the top of the gradient, and fraction 15 is from the bottom of the gradient. Arrows indicate the locations of Gag and Vpr.

The mechanism of Vpr packaging may be due to direct interaction with p6. Mahalingham et al. (29) demonstrated an essential role for virion incorporation of a predicted amphipathic alpha-helical structure at the N terminus of Vpr, including amino acid residues 17 to 34. The current study demonstrates that the region of p6 involved in Vpr packaging contains an $(LXX)_4$ domain. It is tempting to speculate that these two domains might interact with one another. Interestingly, all four of the LXX motifs are required for Vpr packaging, since both GAG($p6_{38-52}$) and GAG($p6_{37-46}$), which each include only three LXX motifs, were unable to incorporate Vpr. It will be interesting to know whether increasing the number of LXX repeats will enhance Vpr packaging. Structural analysis by circular dichroism and nuclear magnetic resonance has suggested that p6 does not adopt a rigid conformation in solution (39), and Chou-Fasman and Garnier-Osguthorpe-Robson programs do not predict that the C-terminal domain of p6 forms a helical structure. Nevertheless, if such a helical structure forms within $Pr55^{gag}$, it would be predicted to be amphipathic.

The deletion of regions of Gag besides p6 also affects Vpr packaging. We have analyzed a Gag polyprotein with intact p6 sequences but a partial NC (p9) deletion or a partial MA (p17) deletion. The p9 mutant forms particles similar to wild-type Gag protein, but Vpr particle incorporation is impaired (data not shown). The MA deletion mutant forms intracellular particles (38), and no Vpr associates with these particles (data not shown). Therefore, it seems likely that the presence of other parts of the Gag protein contributes to Vpr packaging. It remains to be determined whether these effects are due to alteration of the conformation of p6 or occur via another mechanism.

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