Localization of the Vpx Packaging Signal within the C Terminus of the Human Immunodeficiency Virus Type 2 Gag Precursor Protein

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Viral protein X (Vpx) is a human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus accessory protein that is packaged into virions in molar amounts equivalent to Gag proteins. To delineate the processes of virus assembly that mediate Vpx packaging, we used a recombinant vaccinia virus-T7 RNA polymerase system to facilitate Gag protein expression, particle assembly, and extracellular release. HIV genes were placed under control of the bacteriophage T7 promoter and transfected into HeLa cells expressing T7 RNA polymerase. Western immunoblot analysis detected p55^{gag} and its cleavage products p39 and p27 in purified particles derived by expression of *gag* and *gag-pol*, respectively. *In trans* expression of *vpx* with either HIV-2 *gag* or *gag-pol* gave rise to virus-like particles that contained Vpx in amounts similar to that detected in HIV-2 virus produced from productively infected T cells. Using C-terminal deletion and truncation mutants of HIV-2 Gag, we mapped the p15 coding sequence for determinants of Vpx packaging. This analysis revealed a region (residues 439 to 497) downstream of the nucleocapsid protein (NC) required for incorporation of Vpx into virions. HIV-1/HIV-2 *gag* chimeras were constructed to further characterize the requirements for incorporation of Vpx into virions. Chimeric HIV-1/HIV-2 Gag particles consisting of HIV-1 p17 and p24 fused in frame at the C terminus with HIV-2 p15 effectively incorporate Vpx, while chimeric HIV-2/HIV-1 Gag particles consisting of HIV-2 p17 and p27 fused in frame at the C terminus with HIV-1 p15 do not. Expression of a 68-amino-acid sequence of HIV-2 containing residues 439 to 497 fused to the coding regions of HIV-1 p17 and p24 also produced virus-like particles capable of packaging Vpx in amounts similar to that of full-length HIV-2 Gag. Sucrose gradient analysis confirmed particle association of Vpx and Gag proteins. These results demonstrate that the HIV-2 Gag precursor (p55) regulates incorporation of Vpx into virions and indicates that the packaging signal is located within residues 439 to 497.

The process of primate lentivirus morphogenesis and maturation is complex. The basic structure of mature infectious virions includes an envelope derived from the infected cell plasma membrane containing protein products of the *env* gene; capsid (CA), matrix (MA), and nucleocapsid (NC) protein products of the *gag* gene; and enzymatic proteins encoded by the *pol* gene and the viral RNA associated with cellular tRNA primers (7, 12). Current evidence indicates that *gag* plays a fundamental role in the assembly of virions. Even in the absence of other viral proteins, Gag has the ability to assemble and form virus-like particles (6, 13, 26, 38). Human and simian immunodeficiency virus (HIV and SIV) Gag proteins are synthesized as polyprotein precursor molecules (p55). Gag is cotranslationally myristoylated and targets to the site of virus assembly, the cytoplasmic face of the plasma membrane. Subsequent to assembly, the Gag precursor is processed by a viral protease encoded by the *pol* gene. The fully processed HIV-1 Gag precursor protein gives rise to p17 (MA), p24 (CA), p7 (NC), and p6 (19). In addition to driving particle formation, Gag is also responsible for packaging of the viral genomic RNA. Tandem Cys-His motifs located within the NC protein facilitate RNA encapsidation through interaction with virus-specific *cis*-acting sequence (Ψ) located between the splice donor site and the *gag* initiation codon (1, 3, 14, 29).

In addition to *gag*, *pol*, and *env*, HIV and SIV contain

additional genes (*vpx* and *vpr*) which encode proteins packaged in virions in relative abundance (4, 18, 24, 42, 44). *vpx* and *vpr* are not found in all HIV and SIV genomes. *vpx* is unique to HIV-2, SIV_{MAC}, SIV_{SM}, and SIV_{AGM}, while *vpr* is contained in all HIV and SIV genomes except those of SIV_{AGM}. Striking amino acid sequence homology between Vpx and Vpr suggests that they arose by gene duplication and thus may share a common ancestor and have related biological functions (40, 41). Although *vpx* and *vpr* have been shown to augment virus infectivity (5, 15, 23, 32, 35), their precise function and the significance of their presence in the virus particle remain obscure.

Recent studies in our laboratory have demonstrated an ordered process for the intracellular transport and packaging of Vpx into HIV-2 virions (25). Immunofluorescence analysis has shown that Vpx localizes predominantly at the plasma membrane in cells infected with HIV-2, but when eukaryotically expressed in the absence of other viral proteins, it distributes within the cytoplasm and does not appear to localize on the cell surface (25). Using double-label immunofluorescence and Western blot (immunoblot) analysis, we have demonstrated that both surface localization and virion incorporation of eukaryotically expressed Vpx can be restored by *trans* complementation with *vpx* mutant HIV-2 proviral DNA (25). Additional data have suggested an important role of HIV-2 *gag* for regulating the subcellular localization and packaging of Vpx into virus: Vpx and Gag colocalize at the surface of infected cells, and Vpx is packaged in molar amounts comparable to Gag regardless of whether it is ex-

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pressed *in cis* or *in trans* (18, 25). Incorporation of HIV-1 Vpr into virions has recently been demonstrated to require interaction with Gag (28, 31, 36), and by analogy, Vpx may behave similarly. In this report, we show that interaction with the Gag precursor protein is required for Vpx packaging and describe use of *gag* deletion and truncation mutants, as well as HIV-1/HIV-2 *gag* chimeras, to systematically investigate the determinants that mediate incorporation of Vpx into virions. Our results demonstrate, for the first time, localization of a region within the C terminus of Gag that is sufficient to induce incorporation of Vpx into virions.

MATERIALS AND METHODS

Cell culture and viruses. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. Recombinant vaccinia virus, carrying the bacteriophage T7 polymerase gene (rVT7) under control of the 7.5K promoter (10, 11), was provided by Eric Hunter. HIV-2_{ST} (27) and HIV-2_{ROD} (16, 23) was propagated in SupT1 cells cultured with RPMI 1640 medium supplemented with 100 μ g of gentamicin per ml and 15% fetal bovine serum.

Monoclonal and polyclonal antibodies. Antibodies specific for Vpx included the monoclonal antibody 6D2.6 and the polyclonal rabbit antibody R4031 (24, 25). A monoclonal antibody reactive with HIV-2 Gag was kindly provided by A. A. Minassian (34). An antibody reactive to HIV-1 Gag (ATC1) was obtained from a hybridoma line purchased from American Tissue Culture Collection. Both HIV-1 and HIV-2 Gag monoclonal antibodies are reactive with Gag precursor and major capsid proteins.

Recombinant plasmids. The expression plasmid pGEM-3Z (Promega Corp.) was modified by ligation of the pET-11a (Novagen, Inc.) T7 transcriptional terminator (T7 ϕ) into the polylinker, generating pGT. The pGT polylinker and flanking T7, SP6, and T7 ϕ elements are illustrated in Fig. 1B. Plasmid pGT was used for expression of all viral genes under control of the T7 promoter. The *gag-pol* coding region of HIV-2_{ST} (pSXB1) was excised with *KpnI* (nucleotides 423 to 5303) and ligated into pGT, generating pGT-2gag.pol. DNA fragments encompassing HIV-2_{ST} *gag* (nucleotides 427 to 2116) and HIV-1_{HXB2D} *gag* (9, 37) (nucleotides 281 to 1839) were amplified by PCR while restriction enzyme sites for ligation into pGT were simultaneously introduced. HIV-2 and HIV-1 PCR products were digested with *EcoRI-KpnI* and *EcoRI-BamHI*, respectively, and subcloned into the polylinker of pGT, generating the recombinant plasmids pGT-2gag and pGT-1gag. Four additional recombinants were generated by modification of pGT-2gag: pGT-2gag Δ 148, pGT-2gag Δ 26, and pGT-2gag Δ 86 were generated by cleavage with *SmaI* and then separately with *NcoI*, *XhoI*, and *ApaI*, respectively, followed by treatment with Klenow enzyme and blunt-end ligation (see Fig. 4A). pGT-2gag Δ 65/p8 was prepared by removing a 195-bp fragment of pGT-2gag containing the entire NC coding region. This was accomplished by digestion with *NcoI* and *KpnI*, removing sequence downstream of p27, and then insertion of a *NcoI-KpnI* (p6-containing) DNA fragment amplified by PCR from pGT-2gag (see Fig. 4A and 5A). The 5' amplimeric primer was designed to introduce a *NcoI* cloning site and two cytosine bases for in-frame ligation of p6 to p2 at position 1663. *KpnI* was engineered into the 3' primer sequence at the C terminus of the *gag* reading frame for ligation into the pGT polylinker. Four different HIV-1/HIV-2 chimeric *gag* expression plasmids were constructed by PCR: pGTgag1-2, consisting of HIV-1 p17 and p24 fused upstream of HIV-2 p15; pGT-

FIG. 1. Eukaryotic expression of HIV-2 *gag* and *gag-pol* and extra-cellular release of virus-like particles. (A) Construction of HIV-2 *gag-pol*, *gag*, and *vpx* expression plasmids. Illustrated is the organization of the 5' end of the HIV-2 proviral genome, *gag*, *gag-pol*, and *vpx* reading frames, and subgenomic fragments (blackened bars) expressed in the rVT7 system. (B) Cloning sites and transcriptional promoters and termination elements of the pGT expression plasmid. (C and D) Expression of HIV-2 *gag-pol*, *gag*, and *vpx*. Cultures of rVT7-infected HeLa cells were independently transfected with pGT-2gag.pol, pGT-2gag, and pGT-vpx. Twenty-four hours later, culture supernatants were collected, centrifuged (125,000 $\times$ g, 2 h) over cushions of 20% sucrose, and subjected to Western blot analysis. Purified HIV-2_{ST} (pSXB1) virus was included as a control. Blots prepared from cell lysates (C) were probed separately with anti-Gag (α -gag; R1C7) and anti-Vpx (α -vpx; R4031) antibodies, and blots prepared from pellets (D) were probed with anti-Gag antibody R1C7.

*gag*2-1, consisting of HIV-2 p17 and p27 fused upstream of HIV-1 p15; pGTgag1-2^(Δ 26), consisting of HIV-1 p17 and p24 fused upstream of HIV-2 p15 modified by truncated at the *XhoI* site; and pGTgag1-2⁽⁶⁸⁾, consisting of 68 amino acids of HIV-2 *gag* fused to the C terminus of HIV-1 p24. pGTgag1-2 and pGTgag2-1 were constructed by ligating PCR-derived HIV-1 and HIV-2 DNA fragments (amplified from pGT-1gag and pGT-2gag) precisely at p24/p27-p2 junctions (see Fig. 5A). pGTgag1-2⁽⁶⁸⁾ was constructed by ligating an *NheI-XhoI* (p6-containing) DNA fragment (nucleotides 1640 to 2033) amplified from pGT-2gag Δ 65/p8 into *NheI-SalI*-cut pGTgag1-2. pGTgag1-2^(Δ 26) was generated by *XhoI-SalI* cleavage of pGTgag1-2 and subsequent religation, resulting in truncation of 26 C-terminal amino acid residues. The pGT-vpx expression

vector was constructed by ligating an *EcoRI-SalI* (*vpx*-containing) DNA fragment excised from the functional simian virus 40-based pSR α -*vpx* expression plasmid (25) into pGT. This fragment was originally derived from the HIV-2_{ST} proviral genome, nucleotides 5300 to 5879 (Fig. 1A). The pGT-*vpr* expression plasmid was constructed by ligation of a PCR-amplified *vpr*-containing DNA fragment (nucleotides 5107 to 5400) into pGT. For this construction, the HIV-1_{YU2} (30) proviral genome, which contains a full-length 97 residue *vpr* coding region, was used.

Western immunoblot analysis. Viral proteins were characterized by Western blot analysis as previously described (2). Viral and cellular proteins were solubilized with electrophoresis buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol), boiled, and separated by electrophoresis in 15% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose (0.2- μ m pore size; Schleicher & Schuell) by electroblotting, incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in phosphate-buffered saline [PBS]), and then incubated overnight at 4°C with virus protein-specific antibodies diluted in blocking buffer. Viral proteins were visualized by using horseradish peroxidase-conjugated secondary antibody and 3,3'-diaminobenzidine as the substrate.

Metabolic labeling and radioimmunoprecipitation. HeLa cells were infected with rVT7 at a multiplicity of infection of 10 and 2 h later transfected by using Lipofectin (Bio-Rad Laboratories). Six hours after transfection, cells were washed and incubated in methionine- and cysteine-free Eagle's medium for 30 min at 37°C and then in methionine- and cysteine-free medium supplemented with [³⁵S]methionine and [³⁵S]cysteine (Tran³⁵S-Label; 50 μ Ci/ml; ICN Biomedicals). For analysis of transfection-derived virions, culture supernatants were subjected to ultracentrifugation (125,000 $\times$ g, 2 h) over 20% sucrose cushions. For radioimmunoprecipitation, pellets and cells were solubilized in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100), clarified, and incubated for 16 h at 4°C with virus-specific antibody bound to protein A-Sepharose CL-4B (Pharmacia). Precipitates were washed three times with RIPA buffer, boiled for 3 min in Laemmli sample buffer, and analyzed on SDS-15% polyacrylamide gels. Labeled polypeptides were detected by autoradiography of dried gels on Kodak X-Omat XAR-5 film.

Immunofluorescence analysis. Indirect immunofluorescence was performed as described previously (39). Briefly, HeLa cells were plated in 35-mm-diameter wells on glass coverslips, grown to 50 to 70% confluency, infected with rVT7 (multiplicity of infection of 10), and transfected by using Lipofectin. Twenty-four hours posttransfection, coverslip preparations were washed with PBS, fixed with methanol-acetone (1:1) at -20°C for 5 min, and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Coverslips were then incubated with PBS containing 1% bovine serum albumin (BSA) for 15 min at 37°C. Coverslip preparations were incubated for 30 min at 37°C with either an anti-Vpx or anti-Gag monoclonal antibody diluted 1:10 in PBS containing 1% BSA. Cells were then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Tago Inc.) for 30 min at 37°C, 1:25 dilution. Coverslip preparations were mounted in 1:9 PBS-glycerol containing 0.1% phenylenediamine (Fisher Scientific) and examined with a fluorescence microscope.

RESULTS

Expression of HIV-2 *gag*, *gag-pol*, and *vpx*. To investigate the role of Gag in the assembly of virions containing Vpx, we adapted a recombinant vaccinia virus-T7 (rVT7) RNA polymerase system in which viral genes were expressed under control of the bacteriophage T7 promoter (8). Figure 1A depicts the 5' half of the HIV-2 proviral genome and indicates *gag*, *gag-pol*, and *vpx* DNA fragments used for expression in this system. HeLa cells were transfected with pGT-2*gag*, pGT-2*gag-pol*, and pGT-*vpx*. Twenty-four hours later, cells and supernatants were solubilized and examined by Western blot analysis. Gag monoclonal antibody R1C7 detected proteins with molecular masses of 55, 39, and 27 kDa in cells transfected with pGT-2*gag-pol* and of 55 kDa in cells transfected with pGT-2*gag* (Fig. 1C). Proteolytic cleavage of Gag was a consistent finding in cells expressing *gag-pol* and is possibly the result of high intracellular levels of protease. Vpx was detected with anti-Vpx polyclonal antibody R4031. The ability of *gag* gene products to self-assemble following expression in mammalian cells has been demonstrated for a number of retroviruses, including HIV (13, 26) and SIV (6). Western blot analysis of pellets prepared by ultracentrifugation (125,000 $\times$ g, 2 h) of culture supernatants shows that pGT-2*gag-pol* produced particles containing p55, p39, and p27 Gag and that pGT-2*gag* produced particles containing unprocessed p55 Gag (Fig. 1D). For reference, purified HIV-2_{ST} virus (pSXB1) (27) was included and shows comigration of p27 with that derived from pGT-2*gag-pol* transfected cells. Thin-section electron microscopy and density gradient analysis confirmed budding and release of particles from transfected HeLa cells with characteristics similar to those of virions derived from productively infected T cells; peak concentrations of p27 antigen were detected at a density of 1.17 g/ml (data not shown). These data demonstrate that expression of HIV-2 *gag* and *gag-pol* results in assembly and release of virus-like particles. In addition, they demonstrated proteolytic processing of Gag by the viral protease encoded by expression of pGT-2*gag-pol*.

In trans complementation of *vpx* with HIV-2 *gag* and *gag-pol*. Earlier studies conducted in our laboratory demonstrated that localization of Vpx on the cell surface and its incorporation into virions require additional HIV-2-specific determinants (25). The effects of *gag* expression were examined by comparing the subcellular distribution of Vpx when expressed alone and with *gag*. By immunofluorescence microscopy, Vpx was observed to localize at the cell surface when coexpressed with Gag (Fig. 2A), exhibiting a staining pattern similar to that observed for Gag (Fig. 2B). Identical results were found when *vpx* was coexpressed with *gag-pol* (data not shown). When pGT-*vpx* was transfected alone, a very different staining pattern was observed: Vpx did not localize on the cell surface but stained predominantly in the perinuclear space (Fig. 2C). These observations indicate that expression of only *gag* or *gag-pol* is sufficient to regulate the targeting of Vpx to the site of virus assembly, the cytoplasmic face of the plasma membrane.

To determine whether *trans* complementation with *gag* and/or *gag-pol* was sufficient to induce incorporation of Vpx into virions, supernatants of cotransfected (pGT-2*gag*/pGT-*vpx* and pGT-2*gag-pol*/pGT-*vpx*) HeLa cells were collected, centrifuged through cushions of 20% sucrose, and analyzed by immunoblotting. The results demonstrate Vpx in pellets derived from coexpression of pGT-*vpx* with both Gag-Pol and Gag (Fig. 3). Blots of HIV-2_{ST} (pSXB1) were included as controls. Comparison of the amounts of Vpx relative to Gag indicates that virus-like particles (Gag and Gag-pol) incorpo-

FIG. 2. Subcellular localization of Vpx. HeLa cells infected with rVT7 were either cotransfected with pGT-vpx and pGT-2gag (A and B) or transfected with pGT-vpx alone (C). Twenty-four hours after transfection, cells were analyzed by indirect immunofluorescence microscopy using monoclonal antibodies specific for Vpx (A and C) and Gag (B). Bound monoclonal antibodies were visualized with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin.

rate Vpx protein at least as efficiently as virus. However, the relative amounts of Vpx per virion or virus-like particle cannot be determined from this analysis. The efficiency of Vpx incorporation into virus-like particles by using more quantitative

FIG. 3. Incorporation of Vpx into HIV-2 virus-like particles. rVT7-infected HeLa cells were transfected with pGT (cell cont.) and pGT-vpx (vpx cont.) alone and in combination with pGT-2gag.pol and pGT-2gag. Forty hours after transfection, culture supernatants were ultracentrifuged ($125,000 \times g$, 2 h) over 20% sucrose cushions, and pellets were examined by Western blot analysis. Sucrose-purified HIV-2 virus (pSXB1) was included for reference. Blots were probed with a Gag monoclonal antibody (A) and Vpx-specific antiserum (B).

methods for analysis is discussed later in this report. These data indicate that assembly and packaging of Vpx are mediated by the HIV-2 Gag protein and that proteolytic processing of the precursor protein is not required.

Assembly and packaging of Vpx require amino acids located within the C terminus of Gag. To begin to address the question of whether specific determinants of Gag regulate incorporation of Vpx into virions, plasmid pGT-2gag Δ 148 was constructed by truncation of the *gag* open reading frame at the 5' *Nco*I site (Fig. 4A). Since this severely diminished the capability to produce virus-like particles, cells cultures cotransfected with pGT-vpx and pGT-2gag Δ 148 were examined by immunofluorescence microscopy. Although Gag retained surface localization properties, Vpx was restricted to the cytoplasm and perinuclear region, suggesting that determinants downstream of p27 are required for surface localization and possibly virion incorporation (data not shown). To more clearly identify sequences important for virion incorporation, three additional C-terminal modifications were made to the *gag* reading frame. Plasmids pGT-2gag Δ 86 and pGT-2gag Δ 26, respectively, truncated synthesis of 86 and 26 C-terminal amino acid residues, and pGT-2gag Δ 65/p8 deleted 65 residues that encompassed the entire p8 coding region (Fig. 4A). Sequence deleted by pGT-2gag Δ 65/p8 overlapped that truncated by pGT-2gag Δ 86. Wild-type and mutant Gag expression plasmids were cotransfected with pGT-vpx into HeLa cells to compare their abilities to form particles containing Vpx protein. Twenty-four hours after transfection, pellets were prepared from culture supernatants, solubilized in electrophoresis buffer, and characterized by Western blot analysis. By using Gag-specific antibodies, all three mutant expression plasmids were demonstrated to facilitate the production of Gag virus-like particles with an efficiency similar to that of the wild-type expression plasmid, pGT-2gag (Fig. 4B). Western analysis also confirmed that the mutants expressed Gag proteins with reduced molecular weights corresponding to the respective truncations or deletion of the reading frame. Analysis of the same preparations with Vpx-specific antibody R4031 revealed association of Vpx with Gag particles derived from pGT-2gag Δ 26 and pGT-2gag Δ 65/p8 expression (Fig. 4C). Gag particles derived from cells cotransfected with pGT-2gag Δ 86 and pGT-vpx, however, were found to be devoid of detectable amounts of Vpx.

FIG. 4. Mutational analysis of Gag-mediated Vpx packaging. (A) Mutagenesis of the HIV-2 *gag* coding sequence. Illustrated is the Gag precursor polyprotein and C-terminal modifications prepared to analyze sequence-specific requirements of Vpx packaging. Plasmid pGT-29agΔ148 was constructed by truncating *gag* at the 5' *NcoI* site (position 1662). Truncations of 26 and 86 amino acid residues were generated by deleting sequences downstream of the *XhoI* and *ApaI* sites, generating pGT-29agΔ26 and pGT-29agΔ86, respectively. pGT-29agΔ65/p8 was generated by deleting a 195-bp region between the *NcoI* sites, eliminating translation of 65 amino acids. (B to D) Western blot analysis of purified virus-like particles. Cultures of HeLa cells were independently cotransfected with pGT-vpx plus pGT-29ag, pGT-29agΔ26, pGT-29agΔ86, and pGT-29agΔ65/p8. Twenty-four hours later, particles were concentrated from cell culture supernatants by ultracentrifugation through 20% sucrose cushions, solubilized in electrophoresis buffer, and analyzed for protein content by Western blot analysis using Gag (B)- and Vpx (C)-specific antibodies. Lysates of transfected cells prepared 24 h posttransfection were also analyzed for Vpx protein content (D).

Analysis of cell lysates confirmed expression of Vpx and reproducibly demonstrated its intracellular accumulation compared with cells from which nondefective Vpx packaging virions were produced (Fig. 4D). Together, these mutants involved the entire linear sequence of p15^{gag}. Neither deletion of 65 amino acid residues between the *NcoI* sites (pGT-29agΔ65/p8) nor truncation of 26 residues from the C terminus (pGT-29agΔ26) appeared to reduce the efficiency of Vpx packaging, which suggests that the 59 residues between the *ApaI* and *XhoI* sites mediate incorporation of Vpx into virions.

Localization of the Gag Vpx packaging signal. Although the analysis presented above demonstrates that the C terminus of *gag* is important for Vpx packaging, it is also possible that this region represents only part of a larger discontinuous structure or that truncation of *gag* at the *ApaI* site alters protein confirmation at a distant site in a manner that effects Vpx packaging. Since HIV-1 does not effectively incorporate detectable amounts of Vpx into virions and since our data demonstrate that this is also true for virus-like particles derived from expression of HIV-1 *gag* (Fig. 5), we constructed HIV-1/HIV-2 *gag* chimeras in order to further characterize the role of HIV-2 p15 sequences in mediating Vpx packaging. Four different chimeric *gag* expression plasmids were constructed:

pGTgag1-2, pGTgag2-1, pGTgag1-2^(Δ26), and pGTgag1-2⁽⁶⁸⁾ (Fig. 5A). These plasmids were cotransfected into HeLa cells expressing T7 RNA polymerase independently with pGT-vpx. As controls, pGT-vpx was transfected alone and in combination with pGT-1gag, pGT-2gag, and pGT-2gag^(Δ86). After 24 h, culture supernatants were collected, clarified, and centrifuged over 20% sucrose cushions. Pellets and transfected cells were solubilized in electrophoresis buffer and examined by Western blot analysis (Fig. 5B). In comparison with wild-type (pGT-2gag), similar amounts of Vpx were detected in pGTgag1-2-derived particles. Vpx was not detected in particles derived from pGTgag2-1. As an internal control, an HIV-1 *vpr* expression plasmid was also independently cotransfected with pGTgag2-1 and demonstrated particle formation and efficient packaging of Vpr (data not shown). The *gag* chimera pGTgag1-2⁽⁶⁸⁾ also produced particles that contained Vpx. Repeated analysis consistently demonstrated production of virus-like particles and associated Vpx protein in amounts similar to that for wild-type HIV-2 *gag* (pGT-2gag). Vpx was not detected when expressed alone (pGT-vpx) or when expressed with either pGT-1gag or pGT-2gag^(Δ86). Blots prepared from cell lysates and probed with an anti-Vpx antibody show similar intracellular levels of Vpx for all transfections (Fig. 5B, mid-

FIG. 5. Localization of the Vpx packaging signal to residues within the C terminus of HIV-2 Gag. (A) HIV-1/HIV-2 gag gene fusions. Shaded bars represent HIV-1 sequence, and white bars represent HIV-2 sequence. Natural proteolytic cleavage sites and corresponding protein products of the Gag precursor polyprotein are shown (18, 19). Amino acid and nucleotide sequences at gene fusion junctions are also illustrated. Restriction enzyme sites used in the constructions are indicated, and nucleotides introduced by PCR to generate *NheI* cloning sites are underlined; original nucleotides are shown above underlined sequences. Cytosines (C) introduced by PCR primer design adjacent to the *NcoI* site for in-frame fusion of the *NcoI*-*XhoI* fragment to form pGTgag1-2(68) are indicated by asterisks. (B) Western blot analysis of recombinant virus-like particles. HeLa cells were transfected with pGT-vpx alone and in combination with the gag expression plasmids indicated. Twenty-four hours after transfection, cells were solubilized in electrophoresis buffer, and supernatants were collected and centrifuged over cushions of 20% sucrose (125,000 × g, 2 h). Cells and pellets were examined by Western blot analysis. Replica blots prepared from pellets were probed with an anti-Vpx antibody (top) and an anti-Gag monoclonal antibody (bottom). Blots prepared from cell lysates were probed with an anti-Vpx antibody (middle).

dle). Analysis with a Gag monoclonal antibody confirmed gag expression and extracellular release of virus-like particles from transfection of all chimeras (Fig. 5B, bottom).

To confirm incorporation of Vpx into virus-like particles derived from pGTgag1-2(68), sucrose gradient analysis was performed. Culture supernatants of cells cotransfected with pGT-vpx/pGTgag1-2(68) were collected and pelleted through cushions of 20% sucrose. Pellets were resuspended in PBS and then centrifuged for 18 h over linear gradients of 20 to 65% sucrose. Fractions were collected, diluted 1:3 in PBS, and again precipitated by ultracentrifugation. Pellets were solubilized in electrophoresis buffer and examined by Western blot analysis (Fig. 6A). Replica blots were probed separately with anti-Gag (top panel) and anti-Vpx (bottom panel) antibodies. The results demonstrate cosedimentation of Vpx and Gag; peak concentrations of both were detected in fractions 10 to 12. Refractometric analysis of the sucrose fraction demonstrated peak concentrations of Gag at 1.15 to 1.17 g/ml. These data suggest that the virus type-specific determinants of Vpx packaging are located within the 68 amino acid residues of HIV-2_{ST} p15.

Semiquantitative analysis of Vpx virion incorporation. To ensure that our data showing differences in the capabilities of HIV-1 and HIV-2 Gag for inducing incorporation of Vpx into virus-like particles were not due to larger quantitative differences in detection of respective Gag proteins by using virus type-specific Gag antibodies, we analyzed purified HIV-1,

HIV-2, and chimeric virus-like particles by semiquantitative methods for Vpx and Gag content. pGT-1gag, pGT-2gag, pGTgag2-1, pGTgag1-2, and pGTgag1-2(68) were transfected alone and in combination with pGT-vpx into HeLa cells and pulse-labeled with [³⁵S]methionine and [³⁵S]cysteine. Virus-like particles were purified from culture supernatants by ultracentrifugation through 20% sucrose cushions, solubilized in RIPA buffer, and analyzed by radioimmunoprecipitation (Fig. 7). Similar to the results shown above by Western blot analysis, virus-like particles produced by cotransfection of pGT-vpx with pGT-2gag, pGTgag1-2, and pGTgag1-2(68) were shown to contain Vpx protein. Radioanalytic imaging analysis (AMBIS) performed on dried gels revealed, after adjustment for differences in the number of potential metabolic labeling sites (methionines and cytosines), nearly equal ratios of Gag and Vpx, 1:1 to 1:2 (Fig. 7). These results are similar to those calculated by directly comparing molar amounts of Vpx and Gag purified from virus particles (20). HIV-1 Gag and Gag chimeras containing HIV-1 p17 and p24 [pGT-1gag, pGTgag1-2, and pGTgag1-2(68)] were detected in amounts similar to that of HIV-2 Gag. Cotransfection of pGTgag2-1 and pGT-1gag with pGT-vpx gave rise to similar levels of virus-like particles without detectable Vpx protein. Prolonged exposures of dried gels revealed minor amounts of Vpx associated with pellets derived from pGTgag2-1 and pGT-1gag transfection (data not shown). Cells transfected with pGT-vpx alone did not reveal Vpx protein pelletable from culture supernatants. Anal-

FIG. 6. Sucrose gradient analysis. (A) Pellets prepared from culture supernatants of HeLa cell cotransfected with pGTgag1-2⁽⁶⁸⁾ and pGT-vpx were resuspended in PBS, layered over a 20 to 65% linear gradient of sucrose, and centrifuged for 18 hs at 125,000 $\times$ g. Fractions (0.5 ml) were collected from the bottom of the tube, diluted in PBS, precipitated, and solubilized in electrophoresis buffer for Western blot analysis. Replica blots were probed with anti-Gag (top) and anti-Vpx (bottom) antibodies. Fraction 1 represents the first collection from the bottom of the gradient, and fraction 19 represents the last collection. (B) Sucrose gradient fractions were analyzed for density by using a refractometer. Results are graphed for fractions 1 to 19.

ysis of cell lysates demonstrated high-level expression of *vpx* in all cultures transfected with pGT-vpx (data not shown). These data support the view that the packaging signal for Vpx is located in the HIV-2 *gag* C terminus, within residues 439 to 497.

DISCUSSION

In this study, we present novel data which demonstrate the role of HIV-2 Gag in the assembly of virions containing Vpx. Our results show that HIV-2 Gag is alone sufficient to induce packaging of Vpx into virus-like particles. By coexpressing *vpx* with a panel of *gag* deletion and truncation mutants and HIV-1/HIV-2 *gag* chimeras, we localized a region of 59 amino acids positioned near the C terminus of Gag that mediates efficient incorporation of Vpx into virions. Moreover, virion incorporation was linked to subcellular targeting and localization of Vpx on the cytoplasmic face of the plasma membrane.

Since virus particles produced by *gag* and *gag-pol* recombinant baculovirus and vaccinia virus expression systems encapsidate viral RNA (17, 22, 38) and since Vpx has been shown to exhibit RNA binding properties (20), we cannot exclude entirely the possibility that some Vpx is incorporated into virions through an interaction with RNA. Our data do, however, suggest that RNA is not required for Vpx packaging. Neither deletion of *gag* sequences encoding the NC protein nor deletion of the RNA encapsidation signal (Ψ), by reconstruction of the pGT-2gag expression plasmid (data not shown), altered the efficiency of Vpx packaging. Moreover, virus-like particles produced from the expression plasmid pGT-2gag Δ 86, which truncated 86 C-terminal amino acids yet contained the

FIG. 7. Semiquantitative analysis of Vpx packaging. HeLa cells expressing T7 RNA polymerase were transfected with pGT-2gag, pGT-1gag, pGTgag1-2, pGTgag2-1, and pGTgag1-2⁽⁶⁸⁾ alone and in combination with pGT-vpx. Cell monolayers were pulse-labeled for 20 h with [³⁵S]methionine and [³⁵S]cysteine. Particles were concentrated from culture supernatants by ultracentrifugation through cushions of 20% sucrose and solubilized in RIPA buffer. Particles derived from transfections involving plasmids containing HIV-1 p24 sequence [pGT-1gag, pGTgag1-2, and pGTgag1-2⁽⁶⁸⁾] were immunoprecipitated with HIV-1 Gag- and Vpx-specific antibodies bound to protein A-Sepharose. Particles derived from transfections involving plasmids containing HIV-2 p24 sequence (pGT-2gag and pGTgag2-1) were immunoprecipitated with HIV-2 Gag- and Vpx-specific antibodies bound to protein A-Sepharose. Supernatants from cells transfected with pGT-vpx and pGT (control) were included and immunoprecipitated with an anti-Vpx antibody. By using radioanalytical imaging (AMBIS), particle-associated Vpx and Gag content was measured from dried gels. The ratio of Gag to Vpx was calculated and is shown. U indicates immunoprecipitation with undetectable levels of Vpx.

NC coding region as well as the Ψ signal, were found to be devoid of detectable Vpx protein (Fig. 4C). The demonstration that most Vpx is located outside the virus core (43) may also suggest that its packaging is not the result of an interaction with viral nucleic acids.

Recently, several groups have reported results that indicate an important role of the HIV-1 Gag protein for mediating incorporation of Vpr into virions. Through expression of proviral DNA, mutated to truncate *env* and *gag* sequences, p55^{gag} was demonstrated to be required for incorporation of Vpr into HIV-1 virus particles (28, 36). Using a vaccinia virus T7 expression system, Lu et al. demonstrated that coexpression of Vpr with HIV-1 Gag resulted in incorporation of Vpr into Gag virus-like particles (31). In both systems, consecutive truncations of the *gag* reading frame were made to study the requirements of Vpr packaging. These studies indicate that p6 is required for Vpr virion incorporation (31, 36). In studies of HIV-2, our results (Fig. 3) and those of others (31) demonstrate that the HIV-2 Gag precursor protein mediates incorporation of Vpx into virus-like particles. Furthermore, our studies demonstrate an important role of sequence downstream of NC; pGT-2gag Δ 86 truncated 86 amino acids of the *gag* coding sequence at the *Apal* site and cannot mediate incorporation of Vpx in virus-like particles (Fig. 4C). Expression of plasmids that either deleted NC (pGT-2gag Δ 65/p8) or

truncated 26 C-terminal amino acids (pGT-2gag Δ 26) did not alter the efficiency of Vpx packaging (Fig. 4C). These results suggest that HIV-2_{ST} Gag amino acids 439 to 497 contain determinants that play an important role for regulating incorporation of Vpx into virions. Together, these data indicate that the C-terminal region of HIV Gag is required for incorporation of Vpx into virions. These analyses alone, however, cannot exclude the possibility that mutagenesis induced conformational changes that abrogate interaction of Vpx with Gag at a different site(s). It may also be possible that residues within p6 alone are not sufficient for Vpx packaging and represent only part of a larger discontinuous structure.

Using *trans*-complementation experiments, our earlier data and those reported recently by Paxton et al. indicated that Vpx was not capable of incorporation into HIV-1 virions in detectable amounts (25, 36). In contrast to these findings, Matsuda et al. reported substitution of the HIV-1 *vpr* gene with the SIV_{MAC} *vpx* gene and indicated that expression *in cis* with the HIV-1 proviral genome resulted in the production of virions containing Vpx (33). In the transient expression system (rVT7) described above, Western blot analysis failed to demonstrate packaging of Vpx into HIV-1 Gag particles (Fig. 5). Using radioimmunoprecipitation analysis, we quantified the amount of Vpx and Gag in viral particles. This analysis demonstrated a relationship of approximately 1:1 to 1:2 (Gag/Vpx) for HIV-2 Gag and Vpx (Fig. 7). Long exposures of autoradiograms also revealed minor amounts of Vpx protein associated with HIV-1 virions (not shown). Such virus type-specific packaging and use of the vaccinia virus expression system provided the opportunity to use HIV-1/HIV-2 chimeras to define the role of the Gag C terminus in Vpx packaging. Cotransfection of pGT-vpx with pGTgag1-2 demonstrated efficient incorporation of Vpx into HIV Gag recombinants containing HIV-2 p15 (p8 and p6), while cotransfection with pGTgag2-1, containing HIV-2 p17 and p27, was not capable of incorporating detectable amounts of Vpx (Fig. 6 and 7). This result indicates that the Vpx packaging signal is localized within p15.

Deletion and truncation studies suggest that the C-terminal region, between residues 439 and 497, is required for Vpx packaging. To further define the role of HIV-2 p15 in Vpx packaging, we constructed pGTgag1-2 Δ 26, deleted of 26 C-terminal amino acids of p15, and pGTgag1-2⁽⁶⁸⁾, consisting of HIV-1 p17 and p24 fused in frame with 68 amino acids of HIV-2 p15 containing residues 439 to 497. Western blot analysis demonstrated that pGTgag1-2 Δ 26 and pGTgag1-2⁽⁶⁸⁾ induced Vpx packaging in amounts similar to those induced by pGTgag1-2 and pGT-2gag (Fig. 6). Continuous gradient sucrose analysis confirmed that Vpx was associated with pGTgag1-2⁽⁶⁸⁾-derived particles (Fig. 7). Together, these results indicate that a 68-amino-acid sequence of HIV-2 p15, containing residues 439 to 497, is sufficient to mediate incorporation of Vpx into virions. To more clearly understand the nature of virus type-specific Vpx packaging and differences in the capabilities of HIV chimeric Gag particles to mediate Vpx packaging, purified virus-like particles were analyzed by semiquantitative methods to compare Vpx and Gag content. Using ³⁵S labeling and radioanalytic imaging analysis, we confirmed our Western blot data and demonstrated that HIV-2 wild-type (pGT-2gag) and chimeric expression plasmids containing HIV-2 p15 residues 439 to 497 [pGTgag1-2 and pGTgag1-2⁽⁶⁸⁾] incorporate similar relative amounts of Gag and Vpx into virus-like particles, approximately 1:1 to 1:2 (Gag/Vpx).

While these data identify a sequence within HIV-2 Gag that mediates Vpx packaging, the exact mechanism by which this occurs is not clear. Unpublished data from our laboratory and data reported by Horton et al. (21) may suggest that packaging

is regulated by direct interaction of Vpx with the Gag precursor polyprotein. Horton et al. (21) reported that in purified mature virus-like particles, an anti-Vpx antibody coprecipitates p27^{gag}. Although this finding suggests association of Vpx and p27^{gag} in virions, the significance and relevance to Vpx packaging are not clear. Our findings demonstrate that HIV-2 p27 does not itself directly induce Vpx packaging but reveal an essential role of amino acids localized near the C terminus of Gag. Together, these results may suggest that incorporation of Vpx into virions is mediated by an interaction with amino acids positioned in the C terminus of the Gag precursor and that subsequent to assembly and proteolytic processing, Vpx associates with CA. It may also be possible that an association with CA does not occur directly but involves p6 or other viral and/or cellular proteins. The loss of Vpx from purified virus cores, observed after removal of the virus envelope with detergent (33), may also suggest that Vpx does not bind directly to CA.

Recent reports on *vpr* demonstrated that packaging into the HIV-1 virion is also mediated by *gag* and is independent of *pol* and *env* expression (28, 31, 36). Truncational studies demonstrated that p6 is required for Vpr packaging (31, 36). Our laboratory has also found that p6 truncation abrogates Vpr packaging. Using pGTgag2-1, we demonstrated further that the determinants of Vpr packaging are localized within the C-terminal p15 region of Gag (unpublished data). These data show, once again, striking similarities between *vpx* and *vpr*. The common involvement of p6 Gag in Vpx and Vpr packaging may underlie some of the amino acid conservation between these two proteins. The role of p6 for regulating accessory protein packaging may also indicate a function that has only recently been recognized. More detailed analyses are necessary to elucidate the mechanisms that mediate interaction of Vpx and Vpr with Gag and their incorporation into virions.

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