Incorporation of Vpr into Human Immunodeficiency Virus Type 1 Requires a Direct Interaction with the p6 Domain of the p55 Gag Precursor

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The 96-amino acid Vpr protein is the major virion-associated accessory protein of the human immunodeficiency virus type 1 (HIV-1). As Vpr is not part of the p55 Gag polyprotein precursor (Pr55\textsuperscript{gag}), its incorporation requires an anchor to associate with the assembling viral particles. Although the molecular mechanism is presently unclear, the C-terminal region of the Pr55\textsuperscript{gag} corresponding to the p6 domain appears to constitute such an anchor essential for the incorporation of the Vpr protein. In order to clarify the mechanism by which the Vpr accessory protein is trans-incorporated into progeny virion particles, we tested whether HIV-1 Vpr interacted with the Pr55\textsuperscript{gag} using the yeast two-hybrid system and the maltose-binding protein pull-down assay. The present study provides genetic and biochemical evidence indicating that the Pr55\textsuperscript{gag} can physically interact with the Vpr protein. Furthermore, point mutations affecting the integrity of the conserved L-X-S-L-F-G motif of p6\textsuperscript{gag} completely abolish the interaction between Vpr and the Pr55\textsuperscript{gag} and, as a consequence, prevent Vpr virion incorporation. In contrast to other studies, mutations affecting the integrity of the NCP7 zinc fingers impaired neither Vpr virion incorporation nor the binding between Vpr and the Pr55\textsuperscript{gag}. Conversely, amino acid substitutions in Vpr demonstrate that an intact N-terminal α-helical structure is essential for the Vpr-Pr55\textsuperscript{gag} interaction. Vpr and the Pr55\textsuperscript{gag} demonstrate a strong interaction in vitro at salt concentrations as high as 900 mM could not disrupt the interaction. Finally, the interaction is efficiently competed using anti-Vpr sera. Together, these results strongly suggest that Vpr trans-incorporation into HIV-1 particles requires a direct interaction between its N-terminal region and the C-terminal region of p6\textsuperscript{gag}. The development of Pr55\textsuperscript{gag}-Vpr interaction assays may allow the screening of molecules that can prevent the incorporation of the Vpr accessory protein into HIV-1 virions, and thus inhibit its early functions.

Unlike simple retroviruses, HIV-1\textsuperscript{1} encodes for regulatory genes 

\begin{description}
\item[tat and rev] as well as for accessory (vpr, vpu, vif, and nef) genes, together referred to as the auxiliary genes. The tat and rev regulatory genes have been shown to be absolutely essential for viral replication \textit{in vitro} (1). Recently, an increasing number of studies demonstrate that mutations affecting the other auxiliary genes, called the accessory proteins, cause significant phenotypic defects in HIV-1 replication, suggesting that these accessory genes may play pivotal roles during \textit{in vivo} infection and pathogenesis (2).
\end{description}

The Vpr accessory gene product encodes a 14-kDa, 96-amino acid nuclear protein that is expressed late during viral replication in a Rev-dependent manner (3). This protein is highly conserved between HIV-1, HIV-2, and simian immunodeficiency virus (SIV). In addition, HIV-2 and SIVs encode for a protein, Vpx, that has been shown to possess many structural as well as functional similarities with the Vpr protein (4). Functionally, the HIV-1 Vpr protein harbors two main biological activities. First, early during infection of nondividing cells, Vpr is implicated in the nuclear translocation of the preintegration complex (5, 6). The mechanism by which Vpr influences the transport of the preintegration complex remains unclear. Although no classical nuclear localization signal have been clearly demonstrated in Vpr, it is likely that Vpr acts through interactions with cellular proteins involved in the nuclear import of macromolecules. In fact, it was recently demonstrated that Vpr could associate with importin-α and the nucleoporin Nsp1, and thus possibly play the role of an importin-β-like protein (7–9). Consistent with its involvement in the nuclear targeting of the preintegration complex, Vpr was shown to be required for efficient replication in nondividing cells such as monocytes and macrophages (6, 10, 11). The ability of Vpr to arrest the cell cycle constitutes the second biological activity associated with this protein (12, 13). The cytostatic effect of Vpr was shown to result in a specific block in the G2 phase of the cell cycle, which was correlated with the inactivation of the Cdc2 kinase (14, 15). The functional role of Vpr-mediated cell cycle arrest in proliferating and nondividing HIV target cells is still unclear. However, Stewart \textit{et al.} (16) recently demonstrated that Vpr could also induce apoptosis following cell cycle arrest, suggesting a contribution to CD4 cell depletion during HIV-1 disease. As well, the cell cycle arrest action of Vpr was shown to increase viral expression in dividing T cells as well as in macrophages (11, 17, 18).

An important feature of the HIV-1 Vpr and the HIV-2/SIV Vpx proteins is that they are selectively incorporated into the virus particles, which indeed suggests an early function for

\begin{itemize}
\item $\alpha$-nitrophenyl-β-D-galactopyranoside
\item MBP, maltose-binding protein
\item Glc, glucose
\item Gal/Raf, galactose and raffinose
\item RT, reverse transcriptase
\item PCR, polymerase chain reaction
\item PBS, phosphate-buffered saline
\item X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
\end{itemize}

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these two proteins during the viral life cycle (19, 20). The localization of Vpr and VpX within virions is still unclear. Immunoelectron microscopic studies suggested that the HIV-1 Vpr protein localized beneath the viral envelope, co-localizing with the Gag p24 core structures (21). However, a more recent analysis by Kewalramani and Emerman (22) placed the VpX protein within HIV-2 cores. As Vpr and VpX are not synthesized as part of the Gag or Gag-Pol polyprotein precursors, they must utilize a distinct mechanism in order to be incorporated into virion particles. Lavallée et al. (23) reported that Vpr could be specifically incorporated in trans within virus-like particles originating only from the expression of the Pr55^Gag. The C-terminal p6 domain of the Pr55^Gag was subsequently demonstrated to be essential for the incorporation of Vpr into virus particles (24, 25). Furthermore, the integrity of a very conserved motif in the C-terminal region of the p6 domain, L-X-S-L-F-G, was shown to be critical for Vpr virion incorporation (26, 27). Previous studies clearly established that the predicted amphipathic α-helical structure located within the N-terminal region of Vpr was important for its packaging into virions (28–30). Based on the data accumulated so far, the mechanism of Vpr incorporation into virion particles suggests a direct interaction between the p6 domain of the p55 Gag precursor and Vpr. Nonetheless, Vpr was also shown to associate with other domains from the Pr55^Gag. The zinc fingers of NCp7 have recently been suggested to be important for the virion incorporation of Vpr (31, 32). In addition, evidence suggesting an association between Vpr and the MAP17 has also been obtained (33).

In order to clarify the mechanism by which the HIV-1 Vpr protein is trans-incorporated into virion particles, we used protein-to-protein interaction assays to investigate Vpr-Gag interactions. The present work provides genetic (yeast two-hybrid) and biochemical evidence indicating that the incorporation of Vpr into HIV-1 particles involves a direct association between Vpr and the p55 Gag polypeptide precursor. We demonstrate that the region of Vpr is necessary and sufficient for this interaction. The direct binding of Vpr to the p55 precursor may constitute a target for the development of molecules that could prevent Vpr virion incorporation, and thus, Vpr early functions.

EXPERIMENTAL PROCEDURES

Bacterial and Yeast Strains—Manipulations of bacterial strains and of DNA were performed by standard methods (34) unless otherwise noted. Escherichia coli AG1-competent cells were used for routine DNA manipulations. Yeast strain EGY48 (MATa, trp1, ura3, his3, leu2:plexAop6-leu2) was used as a host strain for all two-hybrid experiments and was obtained from the laboratory of Dr. Roger Brent (Massachusetts General Hospital, Boston, MA).

Construction of Plasmids—To construct the bait plasmids LexA-Vpr, LexA-Vpu, and LexA-P55^Gag, the vpr and gag genes from the HXB2 provirus plasmid (35) and the vpu gene from the HxBH10 (36) provirus plasmid were amplified by polymerase chain reaction (PCR) using the 5′ primer 5′-GGCCTAAGGCTGGTACGCTAAA-3′ and the 3′ primer 5′-GACCTTTCAAGAATCTAATA-3′ for Vpr, the 5′ primer 5′-GGAGGAGGCACTGCTGCCGAG-3′ and the 3′ primer 5′-GAGGGAGGCGCCATGCGAGG-3′ for Gag, the 5′ primer 5′-GTCATGACATGGG-ATCCACCACTATAA-3′ and 3′ primer 5′-TCTTCTGGATCCTAGGACCCATTACA-3′ for Vpu, and the Hxb10 His^+ reporter plasmid (37). The PCR fragments were then cloned in this latter restriction site restriction site in translational frame with the codons of the LexA DNA binding domain of the pEG202 vector (37). These latter bait fusion proteins were produced constitutively from pEG202, a 2-μm HIS3 plasmid under the control of the ADH1 promoter and encoded 1.5% of total cellular protein in LexA C-terminal deletion region, which contributes to the operator occupancy by LexA derivatives (37). To construct LexA-Vpr mutants, such as LexA-VprE25K, LexA-VprA30F, LexA-VprG65E, LexA-VprSR79–80ID, and LexA-VprR80A, the similar Vpr PCR fragments amplified from HXB2 harboring the different Vpr mutants, E25K, A30F, G65E, SR79–80ID, and R80A, were fused in frame with the LexA DNA binding domain of the pEG202 vector. The design and construction of these mutants have been described elsewhere (18, 29). The Gag BamHI fragment was also cloned in the pET-21c expression vector (Novagen), which was used to produce in vitro labeled Pr55^Gag.

The prey plasmids B42-Vpr, B42-P55^Gag, and B42-Vpu were constructed by digesting the Vpr, P55^Gag, and Vpu cDNAs from pEG202 with EcoRI and XhoI. These EcoRI-XhoI fragments were placed in pJG4–5, a 2-μm TRP1 plasmid (38), in translational frame with the codons for the simian virus 40 large T nuclear localization signal, the B42 transactivation domain, and the hemagglutinin epitope tag. Because the pJG4–5 vector is under the control of the GAL1 promoter, the expression of the prey fusion proteins was inducible in yeast grown on minimal medium containing 2% galactose and 1% raffinose (Gal/Raf) but not in yeast grown on 2% glucose (Glu).

Construction of the Moloney murine leukemia virus (MLV) Gag/ HIV-1 p6 chimeric construct and the HIV-1 Pr55^Gag (pL444/F458) p6 double mutant were described previously (25, 39). As well, the design and construction of the NCp7 mutants were described elsewhere: H23C (31), C28S/C49S (25), and K14-T50 (40). The MLV gag, MLV gag/ HIV-1 p6, HIV-1 Pr55^Gag (pL444/F458) p6 double mutant, HIV-1 Pr55^Gag (pL7 H23C), HIV-1 Pr55^Gag (pL7 C28S/C49S), and HIV-1 Pr55^Gag (pL7 K14-T50) were amplified by PCR using the following primer sets: MLV gag: 5′ primer 5′-GGGCGGCTGACAATCTGTTCAACCTC-3′; 3′ primer 5′-GCAAGGATCTCCTAGTCTAGGTCAGGAGGA-3′. MLV gag/ HIV-1 p6: 5′ primer 5′-GGGCGGCTGACAATCTGTTCAACCTC-3′; 3′ primer 5′-GCAAGGATCTCCTAGTCTAGGTCAGGAGGA-3′. HIV-1 Pr55^Gag (pL444/F458) p6 double mutant: HIV-1 Pr55^Gag (pL H23C), HIV-1 Pr55^Gag (pL7 C28S/C49S), and HIV-1 Pr55^Gag (pL7 K14-T50) were produced by PCR using the following primer sets: MLV gag: 5′ primer 5′-GGGCGGCTGACAATCTGTTCAACCTC-3′; 3′ primer 5′-GCAAGGATCTCCTAGTCTAGGTCAGGAGGA-3′.

To generate Vpr and Vpu expression plasmids, the vpr and vpu genes from the HIV-1 ELI isolate were amplified by PCR using the 5′ primer 5′-AGACTGACGAACACAGCAGCAGAC-3′ and the 3′ primer 5′-GGCGGCTGACATGTCGACCCAGAC-3′ and the 3′ primer 5′-GGCGGCTGACATGTCGACCCAGAC-3′. HIV-1 Pr55^Gag (pL444/F458) mutant: the HIV-1 Pr55^Gag (pL H23C), (pL7 C28S/C49S), (pL7 K14-T50) mutants: 5′ primer GCGCGGCTGACATGTCGACCCAGAC-3′. HIV-1 K14-T50 (pL7 K14-T50) was amplified by PCR using the following primer sets: MLV gag: 5′ primer 5′-GGGCGGCTGACAATCTGTTCAACCTC-3′; 3′ primer 5′-GCAAGGATCTCCTAGTCTAGGTCAGGAGGA-3′.

Transformation of Strain with Reporter, Bait, and Prey Plasmids—The selection strain were made by transforming the EGY48 yeast strain with a URA3 lacI (β-galactosidase) reporter plasmid and the different HIS3 bait plasmids by the lithium acetate method (34). The yeast selection strain harboring the bait and reporter plasmids were transformed with different prey plasmids, and transformation phenotype was used (in addition to His and Ura markers for bait and LacZ reporter plasmids, respectively) for selection of transformants with the prey plasmids.

Determination of Bait-Prey Interaction—Five independent transformants containing the appropriate bait and prey plasmids were streaked on Gal/Raf-Ura His Trp medium for amplification. Two days later, transformants were restreaked on plates containing Glc Ura− His− Trp− 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) medium or Gal/Raf Ura− His− Trp− X-Gal medium to assess transcriptional activation of the lacZ reporter gene.

β-Galactosidase Activity in Liquid Cultures of Yeast—Cells were assayed for β-galactosidase activity by the o-nitrophenyl-β-D-galactopyranoside (ONPG) method (34).

In Vitro Binding Studies—To produce MBP, MBP-Vpu, and MBP-Vpr proteins, E. coli XL1-BLUE cells (New England Biologs) transformed with pMAL-c2, pMAL-c2Vpu, or pMAL-c2Vpr plasmids were cultured in M9 medium (0.2 mM NaCl, 10 mM MgSO4, 0.1 mM CaCl2, 0.5% casamino acids, 0.5% glucose, 0.2 mM thiamine, and 0.1 mg/ml ampicillin). Protein expression was then induced by adding isopropyl-1-β-D-thiogalactopyranoside (IPTG) at 3 h at 37°C. Then, bacteria were harvested, resuspended in 35 ml of ice-cold PBS, and broken by sonication (five 30-s pulses at 100 watts, Sonics & Materials, Inc.). The resulting lysates were centrifuged for 30 min at 4000 × g and used for binding to amylase resin (New England Biologs).

To investigate Vpr-Gag interaction, we first prepared MBP, MBP-Vpu, and MBP-Vpr-bound amylase resin by incubating equivalent lev-
els of MBF, MBP-Vpu, and MBP-Vpr proteins with amylose resin (50% v/v) for 60 min at 4°C. Then, equal amounts of in vitro synthesized [35S]methionine-labeled Pr55<sup>gag</sup> (TNT coupled reticulocyte lysate system; Promega) diluted in Column Buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA) were incubated with MBF, MBP-Vpu, or MBP-Vpr-amylose resin for 2 h at 4°C. These complexes were then washed several times with Column Buffer, and bound proteins were eluted with 10 mM maltose, loaded onto a 12.5% SDS-PAGE for autoradiography or Western blot analysis.

**Immunoprecipitation and Western Blot—**Yeast EGY48 cells expressing B42-Pr55<sup>gag</sup> and LexA-Vpr, which was used in yeast two-hybrid assay, were lysed in 500 μl of RIPA lysate buffer (41) by beating with glass beads five times for 2 min each. After being removed from beads and cell debris by centrifugation (10,000 × g) at 4°C, the specific proteins in the cell lysates were immunoprecipitated with either a rabbit anti-Vpr serum (23) or a mouse anti-p24 antibody (ATCC HB-9725). Immunoprecipitated proteins were then subjected to SDS-PAGE and subsequently blotted on nitrocellulose (0.45 mm; Schleicher & Schuell). LexA-Vpr and B42-Pr55<sup>gag</sup> fusion proteins were identified by the rabbit anti-Vpr serum, or a rabbit anti-p24 antibody (NIH 384), respectively, with highly sensitive ECL chemiluminescence detection system as recommended by the manufacturer (Amer sham Pharmac ia Biotech).

**Radioimmunoprecipitation and Virion Incorporation Assay—**Virion incorporation assay was performed as described previously with slight modifications (29). Briefly, for the HIV-1 Pr55<sup>gag</sup>-Vpu (p644/L45S) mutant, MT-4 cells (5 × 10<sup>5</sup>) were transfected using the DEAE-dextran method with either 10 μg of wild-type provirus constructs (pNL4.3) or provirus harboring the Pr55<sup>gag</sup> (p644/L45S) mutation. 48 h after transfection, cells were metabolically labeled with 200 μCi of [35S]methionine for 12 h. Radiolabeled cells and 20% sucrose cushion-pelleted virions were lysed in RIPA buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 1% (v/v) Triton, 0.2% (w/v) phenylmethylsulfonyl fluoride) and immunoprecipitated with a mix of rabbit anti-Vpr and HIV-1-seropositive human serum, as described previously (29). The immunoprecipitated complexes were loaded on a 12.5% (w/v) SDS gel and analyzed by autoradiography. To test virion incorporation of Vpr for the HIV-1 p7 H23C and HIV-1 p7 ΔK14-T50 mutants, COS-7 cells (1 × 10<sup>6</sup>) were transfected with either 10 μg of wild-type pNL4.3 constructs or provirus harboring the p7 H23C or p7 ΔK14-T50 mutations using a standard calcium phosphate method. 36 h after transfection, COS-7 cells were labeled with 200 μCi of [35S]methionine for 12 h. Immunoprecipitation procedure was exactly the same as described above for the HIV-1 Pr55<sup>gag</sup>-Vpu (p644/L45S) mutant. A ratio system previously used to assess levels of Vpr virion incorporation relative to the levels found in the cell using other virion proteins as standards (p6ERT) was used (29). Densitometric analysis of autoradiograms was performed with a Molecular Dynamics personal densitometer using an ImageQuant<sup>TM</sup> software version 3.22.

**RESULTS**

**Genetic Evidence That Vpr and the Pr55<sup>gag</sup> Physically Interact—**The experimental system described by Golemis et al. (37) was used to investigate the interaction between Vpr and the Pr55<sup>gag</sup> of HIV-1. In order to do so, the Vpr and p55 proteins were fused at the C terminus of the LexA DNA binding domain (bait) and the B42 bacterial transactivator (prey). To monitor the specificity of interactions, the native plasmids containing only the LexA DNA binding domain or the B42 bacterial transactivator domain, or fused with another HIV-1 accessory protein, Vpu, which is unlikely to interact with HIV-1 Gag protein (42), were used as controls of specificity. The host strain (EGY48) contains the LEU2 and the lacZ reporter genes, both carrying LexA operators instead of native upstream regulatory sequences. A EGY48 yeast cell containing a bait (LexA-fusion) plasmid and reporters (LEU2 and lacZ) remains inactive for the expression of leucine utilization unless a prey vector that expresses an interacting protein as a fusion molecule to the B42 acid box transactivator domain (43). In the above described system (37), the expression of the B42-fusion proteins is conditional on the presence of galactose (Gal/Raff) in the culture medium since the expression is directed by the GAL1 promoter.

Essentially, the yeast strain EGY48 was transformed with different combinations of bait and prey plasmid constructions and selected on Glc Ura<sup>+</sup> His<sup>+</sup> Trp<sup>-</sup> medium. Five independent transformants were then cultured on selective galactose media containing X-Gal and assessed for their ability to interact together. B, a clone shown to be positive for Vpr-Gag interaction was grown in galactose selective media. Yeast cells were lysed and immunoprecipitated with either rabbit anti-Vpr or mouse anti-p24 sera. Samples were then electrophoresed on either a 8.5% (for B42-Pr55<sup>gag</sup>) or 12.5% (for LexA-Vpr) SDS-PAGE, transferred on nitrocellulose, and immunoblotted with either anti-Vpr or anti-p24 antibodies.

**FIG. 1. Specificity of interaction between Vpr and the p55 Gag precursor in the yeast two-hybrid system.** A, the EGY48 reporter strain containing LexA, LexA-Vpr, or LexA-Pr55<sup>gag</sup> was transformed with B42, B42-Vpu, B42-Vpr, or B42-Pr55<sup>gag</sup>. The yeast cells (five independent transformants) were then cultured on selective galactose media containing X-Gal and assessed for their ability to interact together. B, a clone shown to be positive for Vpr-Gag interaction was grown in galactose selective media. Yeast cells were lysed and immunoprecipitated with either rabbit anti-Vpr or mouse anti-p24 sera. Samples were then electrophoresed on either a 8.5% (for B42-Pr55<sup>gag</sup>) or 12.5% (for LexA-Vpr) SDS-PAGE, transferred on nitrocellulose, and immunoblotted with either anti-Vpr or anti-p24 antibodies.
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**Fig. 2.** The p6 domain from the Pr55<sup>gag</sup> is necessary and sufficient for the Vpr-Pr55<sup>gag</sup> interaction. **A,** shown are the different constructs used to assay binding in the two-hybrid system. MLVgag represents the full-length p65 Gag precursor from the MLV, MLVgag/HIV-p6 represents the HIV-1 p6 domain fused in-frame to the C-terminal region of the p6 domain. The L- and X-NC are underlined. L44P and F45S are indicated in bold.

**B,** Yeast strains containing the LexA-Vpr fusion, strong β-galactosidase expression was detected by the observation of blue colonies (lane 7). This suggests that Vpr and the p65 Gag precursor are directly interacting in vivo in the yeast cell nucleus. Interestingly, the interaction between Vpr and Pr55<sup>gag</sup> is observed only in one direction, that is, when Vpr is fused to LexA sequences and the Pr55<sup>gag</sup> to the B42 transactivation domain. When the two fusions are switched, the interaction is not observed (lane 4). In addition, evidence of Vpr homo-oligomerization was observed using the yeast two-hybrid system (lane 8), as previously shown by others (44). When B42-Vpu (lanes 2 and 9), LexA (lanes 5 and 10) or B42 (lanes 1 and 6) protein alone were used, growth on X-Gal medium in the presence of galactose did not result in the appearance of blue colonies. As well, controls in which LexA-Vpu was introduced in yeast containing either B42-Vpr or B42-Pr55<sup>gag</sup> did not show any sign of β-galactosidase expression (data not shown). These interaction experiments were also confirmed using the ONPG colorimetric assay (Fig. 2B). The results presented in Fig. 1A suggest direct and specific binding of Vpr to the Pr55<sup>gag</sup>.

Finally, Western blot analysis was performed to confirm that the LexA-Vpr bait and the B42-Pr55<sup>gag</sup> prey expressed the expected fusion proteins (Fig. 1B). A 36-kDa LexA-Vpr fusion protein was detected using a rabbit polyclonal anti-Vpr serum, while the B42-Pr55<sup>gag</sup> fusion corresponded to a 68-kDa protein using a rabbit anti-p24 serum. Since the B42-Pr55<sup>gag</sup> is a large fusion protein, the other bands detected by Western blot analysis are likely to be degradation products generated during yeast protein extraction.

**Regions within the Pr55<sup>gag</sup> and Vpr Important for the Interaction**—Previous reports clearly established that the p6 domain of the Pr55<sup>gag</sup> was necessary and sufficient for Vpr virion incorporation (24, 25, 27). In order to determine the domains of importance for the Vpr-Pr55<sup>gag</sup> direct interaction, we first tested different Gag constructs in the two-hybrid system (Fig. 2A). Kondo et al. (25) showed that the addition of the p6 domain to the C terminus of the MLV p65 Gag precursor was sufficient to mediate Vpr incorporation, while the wild-type MLV Gag precursor was incapable of incorporating Vpr (Fig. 2A). In order to determine if this specific incorporation of Vpr into MLV/HIV p6 chimeric virus was based on a direct interaction between the chimeric Gag gene product and Vpr, we quantitatively measured β-galactosidase activity in the yeast two-hybrid system using the ONPG colorimetric assay. As shown in Fig. 2B, the β-galactosidase activity detected for the Pr55<sup>gag</sup>-Pr55<sup>gag</sup> interaction is roughly 2 times the one detected for the association between Vpr and the Pr55<sup>gag</sup>. The β-galactosidase activity detected for the Vpr-MLV/HIVp6 interaction is similar to the one observed for the Vpr-Pr55<sup>gag</sup> interaction (Fig. 2B). As expected, no interaction was observed with the wild-type p65 MLV Gag precursor. Since the only difference between these two constructs is the addition of the p6 domain, this result suggests that Vpr can specifically associate with the p6 domain of HIV-1, and that p6<sup>mut</sup> is necessary and sufficient for the association with Vpr. In order to pinpoint the region of the p6 domain that may act as a potential Vpr binding site, 2 out of the 52 amino acids from the p6 domain were mutated in the Pr55<sup>gag</sup>. Leu<sup>44</sup>

mutations perturbed the highly conserved L-X-S-L-F-G motif located in the C-terminal region of the p6 domain. The L-X-S-L-F-G motif is underlined. L44P and F45S are indicated in bold. MA, matrix (p17); CA, capsid (p24); NC, nucleocapsid (p7).
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...was changed for a Pro and Phe<sup>45</sup> for a Ser (Fig. 2A). These two substitutions altered the conserved L-X-S-L-F-G motif of the p6 domain shown to be important for Vpr incorporation (26, 27, 39). As demonstrated in Fig. 2B, this mutation rendered the HIV-1 Pr55<sup>wt</sup> completely incapable of associating with Vpr. Interestingly, this mutation in the context of a proviral construct failed to incorporate Vpr (Fig. 5A). In order to confirm that the lack of β-galactosidase activity detected with the B42-MLV Gag and the B42-HIV-1 Pr55<sup>wt</sup> (p6 L44P/F45S) fusions was not due to stability problems or the absence of protein expression, we performed Western blot analysis to detect these fusion proteins. Western blot clearly demonstrated that the presence of NCp7 mutants on the Vpr-Pr55<sup>wt</sup> interaction. Fig. 2A demonstrates the structure of the NCp7 zinc fingers. Different mutants affecting the zinc binding domains (H22C is a substitution of His<sup>22</sup> for Cys; C28S/C49S contains substitutions of Cys<sup>28</sup> and Cys<sup>49</sup> for Ser; ΔK14-T50 is a deletion of both zinc fingers) were fused in translational frame with the B42 transactivator and assayed for β-galactosidase activity using the ONPG colorimetric method. As shown on Fig. 3B, the E25K and the A30F mutants were unable to interact with both the HIV-1 Pr55<sup>wt</sup> and the MLV Gag/HIV p6 fusion, while the other Vpr mutants were not affected. Similar expression of all LexA-Vpr mutants was confirmed by Western blot analysis (data not shown). Together, these results suggest that the mechanism by which Vpr is transincorporated into HIV-1 particles involves a direct physical interaction with the p6 domain of the Pr55<sup>wt</sup>.

de Rocquigny et al. (31) recently reported that the integrity of the zinc finger structures in the NCp7 was important for Vpr virion incorporation. Consequently, we investigated the effect of NCp7 mutants on the Vpr-Pr55<sup>wt</sup> interaction. Fig. 2A demonstrates the structure of the NCp7 zinc fingers. Different mutants affecting the zinc binding domains (H22C is a substitution of His<sup>22</sup> for Cys; C28S/C49S contains substitutions of Cys<sup>28</sup> and Cys<sup>49</sup> for Ser; ΔK14-T50 is a deletion of both zinc fingers) were fused in translational frame with the B42 transactivator and assayed for β-galactosidase activity using the ONPG colorimetric method. As shown on Fig. 3B, the E25K and the A30F mutants were unable to interact with both the HIV-1 Pr55<sup>wt</sup> and the MLV Gag/HIV p6 fusion, while the other Vpr mutants were not affected. Similar expression of all LexA-Vpr mutants was confirmed by Western blot analysis (data not shown). Together, these results suggest that the integrity of the predicted N-terminal α-helical structure in Vpr is essential for the physical association with the Pr55<sup>wt</sup>. As well, this result confirms the relevance of the binding assay used in this study since the two mutants shown not to interact with the Pr55<sup>wt</sup> were shown not to incorporate virion particles (29).

**Direct In Vitro Interaction between Vpr and the p55 Gag Precursor**—In order to confirm whether the association between Vpr and the Pr55<sup>wt</sup> observed in yeast could be reproduced using another approach, we took advantage of an in vitro binding assay using recombinant fusion proteins. First, the vpr gene was introduced into the pMAL-c2 vector in fusion with the maltose-binding protein (MBP). MBP, MBP-Vpu, and MBP-Vpr fusion proteins were then produced in bacteria and purified as described under “Experimental Procedures.” Then, the entire Gag open reading frame was used to generate in vitro labeled protein. The in vitro translated Pr55<sup>wt</sup> was incubated with MBP, MBP-Vpu, or MBP-Vpr fusion proteins, that were previously immobilized on amylene resin. Following a 2-h incubation, the complexes were washed several times, eluted, and then analyzed on a 12.5% SDS-PAGE. As shown in Fig. 4A, the Pr55<sup>ag</sup> was able to specifically interact with MBP-Vpr, and...
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We next attempted to analyze the strength of the Vpr-Gag interaction by studying the effect of increasing salt concentration on the recovery of the Pr55<sup>gag</sup> by the MBP-Vpr proteins. This technique was previously used to determine the strength of the interaction between the cyclophilins A and B and the Gag protein (48). As demonstrated in Fig. 4B, the Vpr-Pr55<sup>gag</sup> interaction could sustain 900 mM NaCl. Coomassie Blue staining of the gel demonstrated that equal amounts of MBP-Vpr were still present after washing with these different salt concentrations (data not shown). Together, these results demonstrate biochemical evidence for a direct binding between the Vpr protein and the Pr55<sup>gag</sup>, and confirm the data demonstrated genetically using the yeast two-hybrid system (Fig. 1). Furthermore, we conclude that the association between the Pr55<sup>gag</sup> and Vpr is a strong interaction in vitro, sustaining salt concentrations of 900 mM NaCl.

We next wanted to determine if we could interfere with the interaction by specifically blocking the accessibility of the Pr55<sup>gag</sup> to the MBP-Vpr fusion protein. In order to address this question, we used two different anti-Vpr antibodies: a polyclonal rabbit antiserum generated against recombinant Vpr protein (23) and a rabbit polyclonal anti-peptide serum that recognizes the first 19 amino acids of Vpr (48). As controls, anti-Vpu and anti-Myc antibodies were used for the experiments. These different antibodies were incubated for 3 h in the presence of immobilized MBP-Vpr, washed several times to remove unbound and nonspecifically attached antibodies, and then presented to equal amounts of [35S]methionine-labeled Pr55<sup>gag</sup>. Although the anti-Vpu and anti-Myc antibodies demonstrated slight nonspecific competition with the MBP-Vpr-Pr55<sup>gag</sup> interaction, this nonspecific effect never reached the levels observed with the anti-Vpr antibodies in several experiments. Interestingly, the anti-Vpr antibody directed against the first 19 amino acids of Vpr (lane 3) interfered more efficiently with the MBP-Vpr-Pr55<sup>gag</sup> interaction than the polyclonal anti-Vpr (lane 2). The result presented in Fig. 4C suggests that the binding between Vpr and p55 can be affected and that the potential use of other molecules could be used to affect Vpr virion incorporation.

**Mutations That Affect the Binding between Vpr and the Pr55<sup>gag</sup> Also Affect Vpr Virion Incorporation**—In order to confirm our hypothesis that direct binding between Vpr and the Pr55<sup>gag</sup> is required for Vpr virion incorporation, we investigated whether our mutants in the Pr55<sup>gag</sup> were still capable of incorporating the Vpr accessory protein into virions. To analyze the HIV-1 Pr55<sup>gag</sup> (p644/5/45S) mutant for Vpr virion incorporation, MT-4 cells were transfected with an infectious proviral clone of HIV-1 (pNL4.3) expressing in cis either the wild-type or the Pr55<sup>gag</sup> (p644/5F/45S) protein. Following transfection, the cells were radiolabeled and the ability of Vpr to be incorporated into virion was monitored by immunoprecipitating both cell and virion-associated viral proteins. We

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**Fig. 4. In vitro interaction between Vpr and the Pr55<sup>gag</sup>.** A, equal amounts of MBP (lane 3), MBP-Vpu (lane 2), and MBP-Vpr (lane 1) were affinity-purified on amylose resin and incubated with 5 μl of in vitro translated [35S]methionine-labeled Pr55<sup>gag</sup>. Following a 2-h incubation at 4 °C, the samples were washed five times, eluted, loaded on a 12.5% SDS-PAGE, and subjected to autoradiography. The input lane was loaded with one-fifth of the amount of Pr55<sup>gag</sup> used in the binding reactions (lane 4). B, to test the strength of the Vpr-Gag interaction, MBP-Vpr-Pr55<sup>gag</sup> bound complexes, were washed four times with column buffer containing 200 mM NaCl, and subsequently washed with 200, 300, 500, or 900 mM NaCl-containing buffer. After these different washes, the different samples were submitted to 10 mM maltose for elution of MBP-bound complexes and analyzed on a 10% SDS-PAGE and by autoradiography. C, equal quantity of MBP-Vpr fusion protein affinity-purified by amylose resin were incubated for 3 h (4 °C) with four different antibodies: a polyclonal rabbit serum directed against recombinant Vpr (lane 2), a rabbit serum generated against a peptide corresponding to the first 19 amino acids of Vpr (lane 3), anti-Vpu (lane 4), anti-Myc (lane 5), and with no antibody (lane 6). After incubation, unbound and nonspecifically attached antibodies were washed twice. Then, 5 μl of [35S]methionine-labeled, in vitro translated Pr55<sup>gag</sup> were added to the different samples, followed by a 2-h incubation at 4 °C. Samples were then washed, eluted, and submitted to electrophoresis on a 12.5% SDS-PAGE and to autoradiography.
have previously described a ratio system to assess the amount of Vpr found in the virions as a proportion of the total amount found in the cell using other virion proteins as standards (RT) (29). Essentially, the virion-associated Vpr value is calculated based on the level of internal control p66 (RT) present in the virions as well as the proportion of the total Vpr found in the cell as determined by densitometric scanning of autoradiograms. This value is then compared with wild-type virion-associated Vpr value, which is set at 100%. As can be observed in Fig. 5A, substantial amounts of Vpr could be detected when proteins from wild-type virions were immunoprecipitated (lane 8). However, no detectable amount of Vpr protein was observed in virions generated from the proviral construct harboring the p6 L44P/F45S mutation (Fig. 5A, lane 7), even though Vpr was expressed in transfected MT-4 cells (Fig. 5A, lane 3). This observation is consistent with our result (Fig. 2B) that this mutant in the context of the Pr55\textsuperscript{gag} is incapable of binding Vpr, thus suggesting that a direct interaction between Vpr and the p6 domain of the Pr55\textsuperscript{gag} is required for Vpr virion incorporation.

We previously demonstrated that proviral constructs harboring the HIV-1 p7 C28S/C49S mutations in the nucleocapsid region of Gag did not extensively affect Vpr virion incorporation (23). This is consistent with our binding analysis (Fig. 2B), suggesting that the integrity of the zinc binding motifs of the NCp7 is not critical for the interaction between Vpr and the Pr55\textsuperscript{gag}. In order to further confirm this, we decided to analyze the ability of Vpr to incorporate in viruses harboring either the HIV-1 p7 H23C or the HIV-1 p7 ΔK14-T50 mutations. Because these viruses have been shown to be highly affected in their ability to package HIV-1 RNA (40), these viruses are not replication-competent. Consequently, we decided to use COS-7 cells for viral particle production as described previously (23). Using the same antisera as for the HIV-1 p6 L44P/F45S mutations, viral proteins from lysed cells and sucrose cushion-pelleted virions were immunoprecipitated for both the HIV-1 p7 H23C and the HIV-1 p7 ΔK14-T50 proviral constructs. Fig. 5B shows the virion-associated proteins from one of two independent experiments. As can be seen, both virion particles generated from the HIV-1 p7 H23C and the HIV-1 p7 ΔK14-T50 proviral constructs were still competent in incorporating Vpr (Fig. 5B, lanes 11 and 12, respectively). Moreover, quantification (Fig. 5C) using densitometric analysis revealed that Vpr is trans-incorporated to levels similar to wild-type virus into the HIV-1 p7 H23C mutant virions. Because the HIV-1 p7 ΔK14-T50 virion particles are highly affected in their Gag processing (no detectable p66RT band, very low p24/p25 Gag; Fig. 5B, lane 11), we did not quantify Vpr virion incorporation (Fig. 5C). Nonetheless, Fig. 5B (lane 11) shows that Vpr is still incorporated in substantial amounts in the HIV-1 p7 ΔK14-T50 virion particles.

**DISCUSSION**

HIV-1 Vpr is the major virion-associated accessory protein. As Vpr is not synthesized as part of the Gag polyprotein precursor, it must utilize a distinct mechanism in order to be...
incorporated into virion particles. It has been clearly demonstrated through Gag deletion analysis that the virion incorporation of the Vpr protein requires the p6 domain from the p55 precursor (24, 25, 27). However, the association between Vpr virion incorporation and Vpr-Pr55\textsuperscript{gag} binding was still missing. Several groups (26, 27) demonstrated that single amino acid substitutions or deletions of either Leu\textsuperscript{44} or Phe\textsuperscript{45} in the p6 domain abolished the ability of Vpr to be incorporated in the context of MLV/HIV p6 or Rous sarcoma virus/HIV p6 chimeric viruses. In addition, the inability of our HIV-1 Pr55\textsuperscript{gag} (p6 L44P/F45S) double mutant to incorporate Vpr was confirmed in the context of an infectious proviral clone of HIV (Fig. 5A). Consequently, the inability of Vpr to interact with the HIV-1 Pr55\textsuperscript{gag} (p6 L44P/F45S) double mutant (Fig. 2B) brings a direct correlation between the lack of direct binding to the Pr55\textsuperscript{gag} and the incapacity of Vpr to be incorporated into virion particles.

Evidence of direct interaction between Vpr and other Gag domains has been recently reported. de Rocquigny et al. (31) reported that the zinc fingers of the nucleocapsid protein (NC) were important for Vpr virion association. Moreover, using chemically synthesized peptides, they demonstrated that Vpr could directly interact with NCp7, but not with p6 in vitro. However, our result demonstrated that only mutants in the p6 domain (LF-PS) resulted in the loss of binding between Vpr and the Pr55\textsuperscript{gag}, while mutations or the complete deletion of the p7 zinc fingers did not affect the interaction (Fig. 2B) and Vpr incorporation (Fig. 5B). It is possible that the zinc fingers of the NCp7 are important for Vpr binding in the context of the mature NC. However, our results indicate that in the context of the Pr55\textsuperscript{gag}, the zinc finger motifs are less critical for the Vpr-Pr55\textsuperscript{gag} interaction. Moreover, in contrast to de Rocquigny et al. (31), who demonstrated significant defects in Vpr virion incorporation using NC mutants, in particular with the HIV-1 p7 H23C mutant, our result did not reveal extensive impairment of Vpr virion-incorporating ability (Fig. 5, B and C). It is possible that this discrepancy results from experimental differences. Indeed, the basis for quantification of Vpr encapsidation are different, de Rocquigny’s group used Western blot while we used radioimmunoprecipitation. Consequently, the importance of NCp7 in HIV-1 Vpr incorporation still needs to be demonstrated. In fact, Wu et al. (49) deleted the complete NCp8 sequence in HIV-2 and did not affect the ability of Vpxp to be incorporated into virions, while removal of p6 sequences resulted in loss of Vpxp incorporation.

The structural domains within Vpr important for incorporation have also been studied. The extensive mutagenic analysis of several groups agrees that the amphipathic α-helical region located between amino acids 18 and 34 in the N terminus of Vpr is important for the incorporation of this accessory protein (28–30, 50, 51). Our binding studies using five Vpr mutants, E25K, A30F, Q65E, S179-80D, and R80A, showed that the two mutants that were unable to interact with the Pr55\textsuperscript{gag} were the mutants that lost their ability to be incorporated (29). This result also correlates the loss of incorporation of Vpr to its incapacity to directly interact with the Pr55\textsuperscript{gag}. The observation that Vpr could only interact with the Pr55\textsuperscript{gag} when fused to the C terminus of the LexA DNA binding domain (Fig. 1A, lane 7) but not when fused to the B42 transactivator (Fig. 1A, lane 4) suggests that this interaction requires proper conformation. It is likely that either the B42-Vpr or the LexA-Pr55\textsuperscript{gag} fusion, or both, are not presented in the proper structure to expose their respective binding domain. Interestingly, we suspect that the p6-binding motif of Vpr requires more than just the predicted N-terminal α-helical domain since attempts to compete the in vitro Vpr-Pr55\textsuperscript{gag} association with a series of Vpr peptides were unsuccessful. Peptides harboring Vpr amino acids 1–19, 19–35, or 23–37 could not compete the interaction between Vpr and the Pr55\textsuperscript{gag}. Moreover, Yao et al. (52) demonstrated that fusion proteins containing amino acids 1–62, which harbor the predicted N-terminal α-helical moiety of Vpr, are incapable of incorporating virion particles, while polypeptides fused to amino acids 1–80 of Vpr are efficiently incorporated. These results suggest that the presence of the leucine/isoleucine-rich domain of Vpr (amino acids 60–80) might be important for correct folding or exposition of the predicted N-terminal α-helical region. Similar results have also been observed by Sato et al. (52). It is noteworthy that our anti-Vpr peptide serum directed against the N-terminal region of Vpr (amino acids 1–19) was more effective in affecting the interaction with the Pr55\textsuperscript{gag} than the antibody directed against recombinant Vpr (Fig. 4C), which principally recognizes epitopes lying between amino acids 19 and 72. It is possible that, even though the leucine/isoleucine-rich region of Vpr might be important, the critical region for the Vpr-Pr55\textsuperscript{gag} interaction is the predicted N-terminal α-helical structure of Vpr.

From our and other groups’ results, we present a model for the molecular mechanism by which the Vpr protein is trans-incorporated into progeny virions. We suggest that HIV-1 Vpr can only associate with p6 in the context of the Pr55\textsuperscript{gag}. In this context, p6 would have a proper conformation to directly associate with Vpr, and subsequently pull it within forming virions. Then, upon activation of the viral protease, the Pr55\textsuperscript{gag} would be processed and mature p6 would be release from the Pr55\textsuperscript{gag}. This release of p6 would result in its dissociation from Vpr. Subsequently, Vpr could associate with other virion proteins such as NC in the viral core in order to fulfill its functional role in the context of the preintegration complex early in infection.

In summary, the results presented here demonstrate direct interaction between Vpr and Gag in the context of the p55 precursor. Furthermore, our results suggest that Vpr trans-incorporation requires a direct binding to the p6 domain of the Pr55\textsuperscript{gag}. The development of an assay that demonstrates this critical interaction may allow the screening of molecules that prevent Vpr virion association and thus, Vpr early function, which could ultimately impair HIV-1 infection.

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Addendum—During review of this work, a paper appeared (Selig, L., Pages, J.-C., Tanchou, V., Préveral, S., Berlioz-Torrent, C., Liu, L. X., Erdtmann, L., Darlix, J.-L., Beuarous, R., and Benichou, S. (1999) J. Virol. 73, 592–600) in which a direct interaction between Vpr and the Pr55\textsuperscript{gag} of HIV-1 was reported.

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