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Mutagenesis of the putative α-helical domain of the Vpr protein of human immunodeficiency virus type 1: Effect on stability and virion incorporation

(secondary structure/immunoprecipitation)

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ABSTRACT ***vpr* is one of the auxiliary genes of human immunodeficiency virus type 1 (HIV-1) and is conserved in the related HIV-2/simian immunodeficiency virus lentiviruses. The unique feature of Vpr is that it is the only nonstructural protein incorporated into the virus particle. Secondary structural analysis predicted an amphipathic α-helical domain in the amino terminus of Vpr (residues 17–34) which contains five acidic and four leucine residues. To evaluate the role of specific residues of the helical domain for virion incorporation, mutagenesis of this domain was carried out. Substitution of proline for any of the individual acidic residues (Asp-17 and Glu-21, -24, -25, and -29) eliminated the virion incorporation of Vpr and also altered the stability of Vpr in cells. Conservative replacement of glutamic residues of the helical domain with aspartic residues resulted in Vpr characteristic of wild type both in stability and virion incorporation, as did substitution of glutamine for the acidic residues. In contrast, replacement of leucine residues of the helical domain (residues 20, 22, 23, and 26) by alanine eliminated virion incorporation function of Vpr. These data indicate that acidic and hydrophobic residues and the helical structure in this region are critical for the stability of Vpr and its efficient incorporation into virus-like particles.**

Human and simian immunodeficiency viruses (HIV and SIV) encode several accessory or auxiliary proteins which characterize them as "complex" retroviruses. The conserved nature of the auxiliary genes in lentiviruses, in general, is likely to contribute to the ability of HIV and SIV to establish and maintain infection (1, 2). Work from several laboratories has suggested that the accessory gene products may function at various steps of the viral life cycle to collectively regulate replication, cytopathicity, and latency (3). Four of these accessory genes (*vif, tat, rev,* and *nef*) are conserved among all HIV and SIV isolates, while three others (*vpu, vpr,* and *vpx*) are present only in certain HIV/SIV subgroups (4, 5). Of the accessory genes, *tat* and *rev* are absolutely essential for viral replication (3, 6). Although the other accessory genes are dispensable for viral growth *in vitro*, depending on the target cells (7), studies carried out with rhesus macaques infected with SIV indicated the importance of *nef* and *vpr* for the development of disease (8, 9). These results suggest a critical role for the accessory genes in HIV pathogenesis.

HIV-1 Vpr is a protein of 96 aa (5, 10). The subgenomic mRNA representing *vpr* is a singly spliced species, and *vpr* expression is dependent on Rev (11, 12). Proteins with homology to HIV-1 Vpr have also been noted in HIV-2 and SIV (13–15). Based on a comparison of several Vpr and Vpx proteins, Tristem *et al.* (16) have postulated that HIV-2 *vpx* arose by duplication of an ancestral *vpr* gene. An important feature of HIV-1 Vpr is that it is associated with the virus particle (17, 18). During assembly Vpr is selectively transduced through interaction with structural proteins of the virus (19–21), and Vpr requires the p6 domain of the Gag protein for incorporation (21). Investigation by two groups showed that Vpr is localized in the nucleus, though there is disagreement with regard to the sequences responsible for nuclear localization (20, 22). Another observation is that Vpr functions as a weak transcriptional activator of the HIV-1 long terminal repeat and several heterologous promoters (23). The ability of Vpr to induce differentiation pathway in a tumor cell line was also reported (24). Vpr has been shown to be a positive regulator of virus replication (23, 25, 26). This effect was minimal at high multiplicity of infection, and moderate effect was observed at low multiplicity of infection in T cells and peripheral blood lymphocytes (26, 27). A strikingly different picture emerged from studies with macrophages. Utilizing a chimeric proviral genome which has the ability to infect macrophages. Westervelt *et al.* (28) reported that *vpr* and *vpu* were required for productive infection in macrophages, and Balotta *et al.* (29) showed that antisense oligonucleotides directed against *vpr* suppressed HIV-1 replication in macrophages. Hattori *et al.* (30) showed the requirement of *vpr* for HIV-2 infection of macrophages. Studies carried out with a macrophage-tropic HIV-1 molecular clone in one of our laboratories showed that Vpr plays a vital role in the productive infection of macrophages (31, 32). Further, Heinzinger *et al.* (33) reported that Vpr influences nuclear localization of viral nucleic acids in nondividing host cells.

The mechanism by which Vpr is incorporated into virus particles is not known. We recently demonstrated that proline substitutions in a predicted amphipathic helix in the amino-terminal region eliminated virion incorporation, whereas proline insertion elsewhere in the protein did not (34). This suggested that the helical domain may play a role in Vpr incorporation. In an effort to understand the contribution of specific residues of the putative helical domain for the virion incorporation function of Vpr, we have carried out mutagenesis of the acidic and hydrophobic residues present in this amphipathic helical region. The results reported here establish the importance of this region of Vpr for both stability and virion incorporation.

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Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus.

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MATERIALS AND METHODS

Cells, Virus, and Expression Plasmids. The recombinant vaccinia virus vTF7-3, which synthesizes T7 RNA polymerase in infected cells (35), was used for the expression studies. The genes encoding HIV-1 Gag polyprotein and Vpr were cloned downstream of the T7 promoter in pCDNA3 (Invitrogen) to generate pCDGag and pCDVpr expression plasmids, respectively. Overlap extension polymerase chain reaction (PCR) (36) was used to introduce the site-specific mutations into *vpr* of the macrophage-tropic molecular clone HIV-1 89.6 (32, 37). The null mutant, VprΔ, was generated as described (34). HeLa cells were used for transfection experiments and were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum.

Infection, Transfection, Metabolic Labeling, and Immunoprecipitation. HeLa cells (10^6) in 35-mm dishes were infected with vTF7-3 at a multiplicity of infection of 10 for 1 hr. DNA transfection was carried out with Lipofectin (BRL). Transfected HeLa cells were washed with phosphate-buffered saline, starved for 1 hr in DMEM lacking serum, methionine, and cysteine; and then labeled with ^{35}S protein-labeling mix (DuPont/NEN) at 200 μCi/ml (1.2 Ci/mmol; 1 Ci = 37 GBq). Cells were labeled for 2–5 hr or, for pulse–chase experiments, pulse-labeled for 30 min and chased by incubation for various periods in the presence of excess unlabeled methionine and cysteine. Immunoprecipitation was carried out and incorporation of Vpr into the virus-like particles directed by HIV-1 Gag was determined as described (34).

RESULTS

Structural Features of HIV-1 Vpr. The predicted Vpr amino acid sequence of the macrophage-tropic HIV-1 strain 89.6 is presented in Fig. 1*A*. Secondary structural analysis using the Chou–Fasman algorithm (39) predicts an α-helical structure at the amino terminus (residues 17–34) (40), while residues 36–86 comprise a β-sheet structure (39). Hydrophobic-moment calculations with the Genetics Computer Group software package also strongly suggest an α-helical structure in the amino-terminal region, with a value of 0.64, and projection of the residues 17–34 of Vpr onto the helical wheel further shows the strong amphipathic character (Fig. 1*B*). Based on the parameters used for the classification of amphipathic helices, the putative helix noted in Vpr may belong to class H (40). In addition, the carboxyl terminus of Vpr is rich in basic residues (arginine, lysine, and histidine), and a cluster of leucine and isoleucine residues is noted in a region comprising residues 60–80. Sequences with similar structural features have also been noted in Vpr and Vpx of HIV-2 and SIV (15). The putative helical domain of HIV-1 Vpr contains five negatively charged residues (positions 17, 21, 24, 25, and 29) and four leucine residues (positions 20, 22, 23, and 26).

Mutagenesis of Acidic Residues of the Helical Domain of HIV-1 Vpr. Previous studies showed that residues in the amino-terminal putative helical domain are essential for the incorporation of Vpr into virus-like particles (34). Introduction of proline in place of glutamic acid at 21 and 24, to disrupt the helical structure, completely eliminated the virion incorporation, whereas proline substitution elsewhere in the protein did not. To further address the contribution of specific residues of the putative helical domain to virion incorporation and to characterize their role in the stability of Vpr, we have carried out extensive mutagenesis. To evaluate individual acidic residues of the helical domain, we substituted helix-disrupting proline at 17, 21, 24, 25, and 29 individually and in combination (Fig. 2*A*). To evaluate the effect of conservative changes, Vpr mutants were generated in which aspartic acid was substituted for all glutamic residues in the helical domain (Vpr αE→D). Further, glutamine was also substituted for all acidic residues

A

MEQAPEDQGPQREPYNDWTLELLEELKNEAVRHFPRIWLHSLGQHIY
ETYGDTWTGVEALIRILQQLLFIHFRIGCRHSRIGIIQHRRTRNGASKS

B

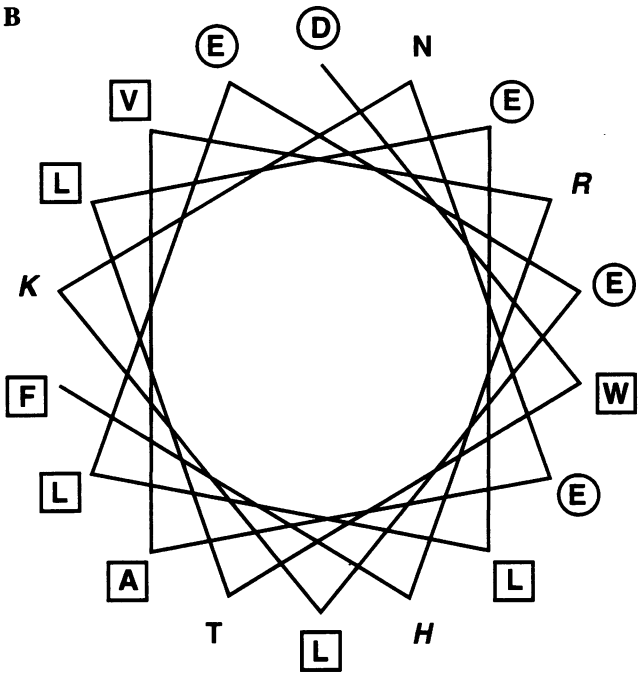

FIG. 1. Secondary structure analysis of the Vpr amino acid sequence of macrophage-tropic HIV-1 strain 89.6. (*A*) The predicted amino acid sequence of HIV-1 Vpr. Underlined sequence represents the putative α-helical domain. (*B*) Helical wheel diagram (38) of the putative amphipathic helix of residues 17–34 of Vpr. Positively charged amino acids are shown in italics, negatively charged amino acids are circled, and hydrophobic residues are boxed.

in the helical domain (Vpr αD,E→Q) to evaluate the contribution of negatively charged residues to the virion incorporation of Vpr. The resultant plasmid clones were confirmed by DNA sequence analysis.

Effect of Mutations in the Helical Domain on Vpr Expression. We employed a vaccinia virus–T7 RNA polymerase expression system (vTF7-3) to study the effect of mutations in the amphipathic helix on the expression of Vpr in cells and its incorporation into virus-like particles directed by the HIV-1 *gag* gene. vTF7-3-infected HeLa cells were transfected with wild-type or mutant *vpr* expression plasmids by the Lipofectin method. Cells were labeled for 2–5 hr, lysed, immunoprecipitated with anti-Vpr antiserum, and analyzed by SDS/12% PAGE. As expected, the cells transfected with *vpr* expression plasmid produced a 14-kDa protein, whereas the mock-transfected and pCDVprΔ-transfected cells did not (Fig. 2*B*). Two additional bands of approximately 12 and 16 kDa were seen in most cell lysates, including mock and pCDVprΔ, indicating that they were nonspecific. Transfection with each of the mutants resulted in detectable levels of Vpr in cell lysate, although the 17-D → P mutant consistently produced extremely low levels (Fig. 2*B*). In addition, another mutant was tested in which each of the four leucine residues of the helix was replaced with alanine (L20, 22, 23, 26→A), which also produced detectable levels of Vpr in lysates (data not shown). Most of the mutations (except for αD, E→Q) resulted in proteins with slightly slower migration than wild type (Fig. 2*B*), although these differences were generally modest and probably related to conformational changes.

Effect of Mutations on the Stability of Vpr. The different levels of Vpr detected in transfected cells (Fig. 2*B*) prompted us to analyze the effect of individual mutations on the stability

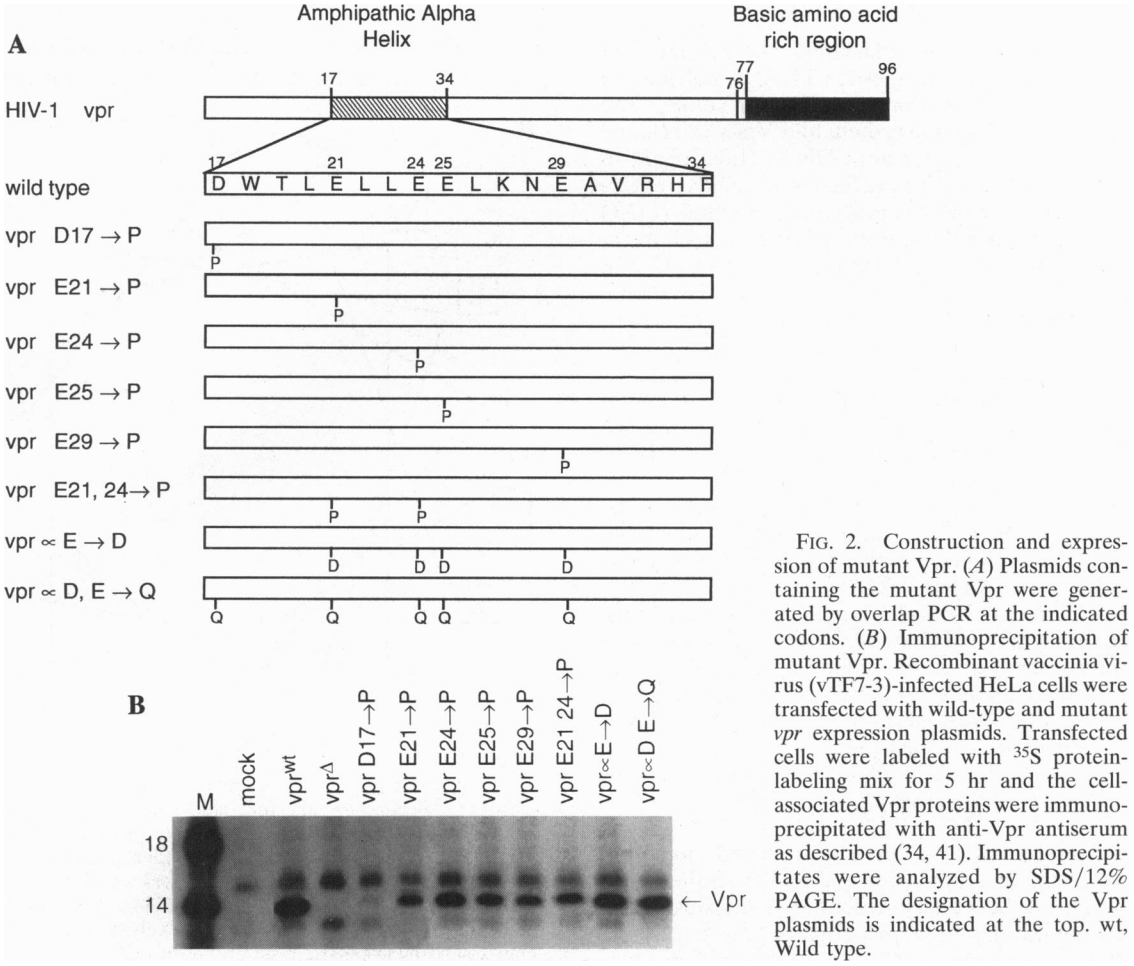

FIG. 2. Construction and expression of mutant Vpr. (*A*) Plasmids containing the mutant Vpr were generated by overlap PCR at the indicated codons. (*B*) Immunoprecipitation of mutant Vpr. Recombinant vaccinia virus (vTF7-3)-infected HeLa cells were transfected with wild-type and mutant *vpr* expression plasmids. Transfected cells were labeled with ^{35}S protein-labeling mix for 5 hr and the cell-associated Vpr proteins were immunoprecipitated with anti-Vpr antiserum as described (34, 41). Immunoprecipitates were analyzed by SDS/12% PAGE. The designation of the Vpr plasmids is indicated at the top. wt, Wild type.

of the protein. Plasmids encoding wild-type or mutant Vpr were transfected into vaccinia virus-infected HeLa cells and the cells were pulse-labeled with ^{35}S for 30 min. After various chase periods, lysates were immunoprecipitated with anti-Vpr antibody and subjected to SDS/12% PAGE. During the 10-hr chase period the levels of Vpr detected were gradually reduced in several mutants in comparison to wild type (Fig. 3). After a 5-hr chase, little or no Vpr was present in cells transfected with E21→P, E24→P, E25→P, or E29→P mutants, while the 21, 24E→P mutant showed slightly decreased Vpr stability. Notably, the D17→P mutant could not be detected even immediately after the 30-min labeling period, even though low levels could be seen in lysates after longer (2–5 hr) labeling periods (Fig. 2*B*). In contrast, neither aspartic acid substitution for glutamic acid (αE→D) nor glutamine substitution for aspartic acid and glutamic acid (αD, E→Q) in the amphipathic helix had a significant effect on the stability of Vpr (Fig. 3). These results suggest that proline disruption of the amphipathic helix destabilizes Vpr in the transfected cells.

Incorporation of Vpr into Virus-Like Particles. To address the role of specific amino acids in the putative amino-terminal amphipathic helix on the incorporation of Vpr into virus-like particles directed by HIV-1 Gag, we transfected the vTF7-3-infected HeLa cells with *gag* expression vector pCDGag in combination with wild-type or mutant *vpr* expression plasmids. Immunoprecipitation of Vpr and Gag was performed both in cell lysates and in culture media with anti-Vpr antiserum and HIV-1 antiserum after 5 hr of continuous labeling (Fig. 4). When cell lysates were examined, Vpr was found in cells transfected with wild-type and all mutant *vpr* plasmids, with or without *gag* cotransfection (data not shown). When culture media were tested, expression of Vpr alone did not result in the export of Vpr. Expression of Gag resulted in a 55-kDa product in the cell culture supernatant and, as expected, coexpression of Vpr and Gag resulted in the export of Vpr into the culture medium in association with virus-like particles (Fig. 4). Aspartic acid substitution for glutamic residues (αE→D) resulted in a mutant Vpr that was incorporated into the virus-like particles and transported into the culture medium similarly to wild type (Fig. 4). Glutamine substitution for aspartic and glutamic acid (Asp-17; Glu-21, -24, -25, and -29) also resulted in Vpr incorporation (Fig. 4), although the intensity of the Vpr band was consistently less than wild type. In contrast, no Vpr could be detected in the supernatant of cells transfected with proline substitution mutants (Fig. 4), despite detectable levels expressed in cells. These data support a role for the putative helix in the amino-terminal region in Vpr incorporation into virus-like particles.

DISCUSSION

Vpr is unique among the HIV-1 accessory proteins because of its association with virus particles (17, 18). Several studies have shown that the protein encoded by *gag* is sufficient for incorporation of Vpr into virus particles (19–21), and results obtained with the use of deletion mutants have indicated that the p6 domain of the precursor protein is the critical Gag element for Vpr incorporation (21). The experiments presented in this paper shed light on a domain of Vpr that is essential for incorporation.

To delineate the domains of Vpr involved in virion incorporation, we carried out site-specific mutagenesis of *vpr*. Earlier studies compared the primary nucleotide sequences of *vpr* of several HIV-1 isolates and identified conserved residues

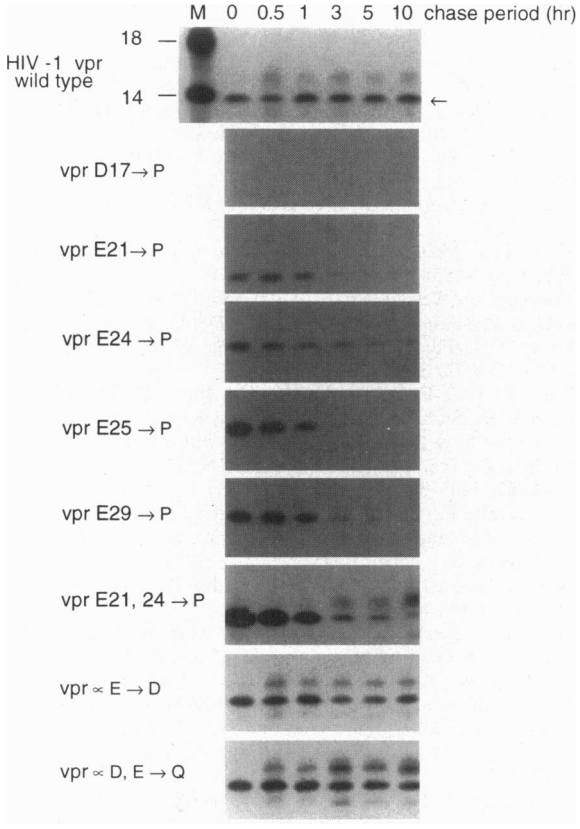

FIG. 3. Stability of mutant Vpr proteins. Transfected cells were pulse-labeled for 30 min with 200 μCi of ^{35}S protein-labeling mix and chased for different periods. Cells were then lysed and cell-associated Vpr protein was immunoprecipitated with anti-Vpr antiserum and analyzed by SDS/12% PAGE. Chase periods are indicated at the top. Lane M, size markers (18 and 14 kDa).

in the predicted amino acid sequences (16). Secondary structural analysis carried out by the Chou–Fasman (39) prediction method revealed the presence of a putative α-helical domain with an amphipathic structure in the amino terminus of Vpr which is highly conserved among HIV-1 isolates (5, 16). Our previous work showed that several amino acid substitutions which were predicted to disrupt the helical domain eliminated the incorporation of Vpr into virus-like particles, while mutations elsewhere did not (34). Therefore, in this analysis we carried out extensive mutagenesis of the helical domain to test for effects on expression, stability, and virion incorporation function of Vpr.

In the first set of mutants each of the acidic residues of this region—Asp-17, Glu-21, Glu-24, Glu-25, and Glu-29—was individually replaced with proline. Each of these mutants resulted in detectable Vpr in cells, except for the 17D→P mutant, which was often only barely detectable. Further experiments involving pulse–chase analysis showed that stability of these mutants was lower than wild type. Paxton *et al.* (21) failed to detect Vpr in cells when alanine was substituted for Cys-76. This was thought to result from decreased stability, although we recently found that this same mutation in the context of the HIV-1 89.6 Vpr did not affect expression (34). Zhao *et al.* (22) reported that Vpr mutants containing substitution mutations in the leucine-rich domain (aa 60–80) or deletion of residues 43–96 resulted in Vpr that was highly unstable, although those studies did not analyze the stability by a pulse–chase method. Our results, together with data from other laboratories, suggest that multiple domains of Vpr contribute to its stability in cells.

Most notable, however, was that incorporation experiments showed that each of these proline insertion mutants was not incorporated into virus-like particles. The helix-destabilizing properties of proline insertion are well documented (39, 42, 43). Substitution of proline for aspartic acid or glutamic acid in the amphipathic helix abrogated Vpr export into the cell culture supernatant in association with virus-like particles, demonstrating that Vpr incorporation into virus-like particles directed by HIV-1 Gag requires the putative amino-terminal amphipathic helical domain. Aspartic acid substitution for glutamic acid (αE→D) had no effect on the incorporation of Vpr, suggesting that acidic substitution retains virion incorporation of Vpr. Interestingly, replacement of aspartic and glutamic residues by glutamine in the αD,E→Q mutant did not eliminate incorporation, although the Vpr band intensity was less. Chau–Fasman analysis of this mutant protein suggests that these residues have the capacity to maintain helical the structure. Further, substitution of alanine for the four leucine residues in the helical domain resulted in a Vpr which was not incorporated into virus-like particles, despite detectable ex-

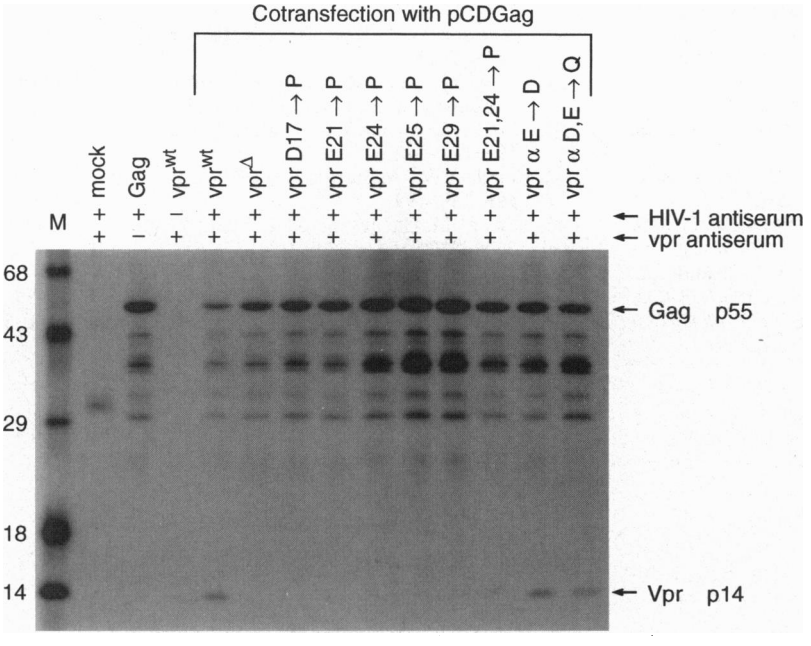

FIG. 4. Incorporation of Vpr into virus-like particles directed by Gag. Cotransfection of pCDGag and pCD-Vpr wild-type and mutant plasmids was carried out with vTF7-3-infected HeLa cells as described (34). After 5 hr of labeling, virus-like particles were immunoprecipitated with anti-HIV and anti-Vpr antiserum and analyzed by SDS/12% PAGE. The electrophoretic positions of Gag and Vpr are shown at right. Lane M, size markers (68, 43, 29, 18, and 14 kDa).

pression in cells (data not shown); this observation supports a critical role for the helical domain in virion incorporation.

The mechanism by which Gag and Vpr interact to allow for Vpr incorporation into the virus particle remains unknown. A survey of viral and cellular proteins (44, 45) has revealed that nearly 10% of viral proteins contain amino-terminal amphipathic helices. An analysis of the protein helical motifs, correlated with specific biological functions, suggests that amino acid sequences in the amphipathic helix are essential for normal processing and stability by ensuring proper conformation. In accordance with these observations, the putative helical domain present in Vpr plays an important role both in its stability and in its incorporation into virus particles. As the p6 domain of Gag has been shown to be essential for Vpr incorporation, it is possible that Vpr interacts directly with Gag in the cytoplasm (46, 47) or that an interaction between these proteins occurs at the membrane before budding. Alternatively, since Vpr may interact with a cellular protein (22), the association of Vpr and Gag may be mediated by a cellular factor(s). In addition, Vpr has been shown to dimerize in eukaryotic cells (21, 48), but it is not known whether multimerization is essential for protein stability and particle incorporation.

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