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Mutational Analysis of Cell Cycle Arrest, Nuclear Localization, and Virion Packaging of Human Immunodeficiency Virus Type 1 Vpr

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Human immunodeficiency virus type 1 Vpr is a virion-associated, regulatory protein that is required for efficient viral replication in monocytes/macrophages. The protein is believed to act in conjunction with the Gag matrix protein to allow import of the viral preintegration complex in nondividing cells. In cells, Vpr localizes to the nucleus. Recently, we showed that Vpr prevents the activation of $p34^{cdc2}$-cyclin B. This results in arrest of Vpr-expressing cells in the G_2/M phase of the cell cycle. Here, we use a panel of expression vectors encoding Vpr molecules mutated in the amino-terminal alpha-helical region, the central hydrophobic region, or the carboxy-terminal basic region to define the functional domains of the protein. The results showed cell cycle arrest was largely controlled by the carboxy-terminal basic domain of the protein. In contrast, the amino-terminal alpha-helical region of Vpr was required for nuclear localization and packaging into virions. The carboxy terminus appeared to be unnecessary for nuclear localization. In the alpha-helical region, mutation of Ala-30 to Pro resulted in a protein that localized to the cytoplasm. Surprisingly, fusion of Vpr to luciferase resulted in a molecule that failed to localize to the nucleus. In addition, we show that simian immunodeficiency virus Vpr, but not Vpx, induces G_2 arrest. We speculate that Vpr has two sites for interaction with cellular factors: one in the alpha-helical region that specifies nuclear localization and one in the carboxy-terminal domain that is required for Cdc2 inhibition.

The human immunodeficiency virus type 1 (HIV-1) accessory gene *vpr*, while dispensable for viral replication in T-cell lines and in primary activated peripheral blood mononuclear cells (1, 2, 7, 10, 26), is required for efficient replication in primary monocytes/macrophages. The role of Vpr in AIDS pathogenesis is not well understood but has been recently addressed in studies of simian immunodeficiency virus (SIV)-infected macaques. In one study, macaques infected with SIV_{mac239} containing a mutation of the Vpr methionine initiation codon (17) progressed to AIDS at a slower rate than those infected with a control virus. In some of the infected animals, the mutation reverted, resulting in an increased viral burden and a more rapid progression to disease. In another study, *vpr*-mutant SIV_{mac239} retained full pathogenicity, but in conjunction with mutation of *vpx* it replicated to low levels and was nonpathogenic in infected macaques (11).

Packaging of Vpr and Vpx into virions is a specific and efficient process. Vpr and Vpx can be present in virions at quantities equimolar to that of Gag (6, 15, 35). Their packaging into virions is controlled by the region of the Gag precursor polyprotein $Pr55^{gag}$ corresponding to the carboxy-terminal cleavage product, p6 (20, 29, 34). The specificity of packaging is suggested by the finding that HIV-1 Vpr is incorporated into HIV-1 capsids but not into those of other retroviruses (29). Furthermore, a chimeric virus consisting of HIV-1 with the p6 region of HIV-2 packaged HIV-2 accessory proteins but failed to package HIV-1 Vpr (34). Recently, it has been shown that a putative alpha-helical region near the amino terminus plays a role in the packaging of Vpr into virions and in maintaining the stability of the protein (21, 22).

Several possible roles for Vpr in HIV-1 replication have been suggested. Heinzinger et al. (14) showed that shortly after viral entry, Vpr, in conjunction with a nuclear localization sequence present in the Gag MA protein (MA-NLS), allows import of the viral preintegration complex to the nuclei of nondividing cells. Such a role is consistent with the presence of Vpr in virions and with its nuclear localization in transfected cells (20). Also consistent with a nuclear import role is the finding that Vpr is dispensable in dividing cells, such as activated peripheral blood mononuclear cells, but is required for replication in macrophages, which do not divide (3, 8).

A second role for Vpr in HIV-1 replication was recently suggested by Rogel et al. (32), who showed that, in vitro, Vpr prevents the establishment of chronically infected HIV-1 producer cell lines. This property appeared to be due to the ability of Vpr to block cell division by blocking the mitotic cell cycle in the G_2 phase. Similarly, cells infected with *vpr*-positive HIV-1 arrest in G_2, while those infected with *vpr*-negative virus continue to proliferate (13). The cell cycle arrest induced by Vpr may be the basis of earlier findings by Levy et al. (19), who showed that Vpr expression in rhabdomyosarcoma cells prevented their replication and induced their differentiation. Thus, Vpr may have two roles, one involving nuclear import and a second involving cell cycle arrest. Consistent with this, we previously provided evidence supporting a post-nuclear import role for Vpr in monocyte/macrophage infection. In that study, we showed that virion-packaged Vpr could not fully account for the effect of Vpr in increasing viral infectivity in monocytes (8). This finding suggested a role for newly synthesized Vpr in infected monocytes. While the selective advantage provided to the virus by arresting cells in G_2 remains unknown, we specu-

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lated that it may play a role either in blocking HIV-1-induced apoptosis or in increasing the resistance of infected cells to cytolytic-T-lymphocyte-induced cytolysis (13).

We recently reported the results of our studies on the biochemical mechanism by which Vpr arrests the cell cycle in G_2 (13). In cycling cells, the transition from G_2 to M is regulated by the complex of the cyclin-dependent kinase $p34^{cdc2}$ and cyclin B (reviewed in references 23–25). Late in G_2, $p34^{cdc2}$ is dephosphorylated at two inhibitory amino acid residues, Thr-14 and Tyr-15. This activates the complex which then phosphorylates several cellular substrates involved in the intricate events of M phase. Vpr inhibits the activation of the $p34^{cdc2}$-cyclin B complex, resulting in arrest in G_2 (13). This inhibition appeared to result from increased phosphorylation of $p34^{cdc2}$ at the inhibitory sites (13). Whether Vpr acts directly or indirectly on Cdc2-cyclin B is not known.

Here, we use a panel of expression vectors encoding mutated Vpr molecules to define the functional domains of Vpr. We tested the mutated Vpr molecules for their ability to arrest the cell cycle, to localize to the nucleus, and to be packaged into HIV-1 virions. The results show that the amino-terminal alpha-helical region of Vpr controls nuclear localization and virion packaging, while the carboxy-terminal basic region is required for cell cycle arrest. In addition, we show that fusion of Vpr to another protein prevents its accumulation in the nucleus.

MATERIALS AND METHODS

Plasmids. HIV-1 Vpr expression vector pc-5′-tag-Vpr, previously referred to as pcDNA-tag-Vpr (29), is based on pcDNA1/amp (Invitrogen) and contains the *vpr* gene of NL4-3. Mutations in pc-5′-tag-Vpr were generated by oligonucleotide-directed mutagenesis, using an altered-sites mutagenesis kit (Stratagene). pc-tag-Vpr(SIV) and pc-tag-Vpx(SIV) express SIV Vpr and Vpx, respectively, and were constructed by PCR amplifying the two genes from SIV_{mac239} (4). SIV Vpr was amplified by using a 5′ primer containing a *Bam*HI site and a 3′ primer containing an *Eco*RI site (primers GCGCAGGATCCACCATGTACCCATAC GATGTTCCAGATTACGCTGAAGAAAACCTCCAGAAAAT and CGCG GATCCTTATAGCATGCTTCTAGAGGGCGG). SIV Vpx was amplified by using a 5′ primer containing a *Hin*dIII site and a 3′ primer containing a *Bam*HI site (primers CGCGAAGCTTATGTACCCATACGATGTTCCAGATTAC GCTTCAGATCCCAGGGAGA and CGCGGATCCTTCTTCCATTTATGC TAG). PCR products were cleaved with the appropriate restriction enzymes and ligated to similarly cleaved pcDNA1/amp. pNL4-3-R^-E^- consists of an NL4-3 provirus containing mutations in *vpr* and *env* and has been previously described (29). pc-CD4 consists of pcDNA1/amp containing a cDNA for human CD4 and has been previously described (18).

Transfection. 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. Cells (2×10^6) seeded the previous day in 10-cm-diameter culture dishes were transfected with 20 μg of plasmid DNA by calcium phosphate precipitation as described previously (5). For virion packaging studies, the cells were transfected with 15 μg of pc-tag-Vpr and 5 μg of pNL4-3-R^-E^-. For cell cycle analysis, 293 cells were typically transfected with 2 μg of pc-CD4 and 18 μg of Vpr or Vpx expression vector.

Preparation of virions and cell lysate. Culture supernatants were collected 48 h posttransfection and clarified by filtering through a 0.45-μm-pore-size filter. Virions were pelleted by ultracentrifugation for 1 h at 100,000 × *g* and then solubilized in 100 μl of lysis buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100). Virus lysates were quantitated by measuring reverse transcriptase activity as previously described (12). Cell lysates were prepared by removing the cells from the tissue culture dish with 4 ml of phosphate-buffered saline (PBS) containing 5 mM EDTA and centrifuging at 1,500 rpm for 10 min in a Sorvall RT6000B centrifuge. The pellet was resuspended in 200 μl of ice-cold lysis buffer. The lysate was incubated on ice for 1 min and clarified by centrifuging for 1 min at 16,000 × *g* in a microcentrifuge. The supernatant was transferred to a fresh tube, and the protein concentration was measured (Bio-Rad). Cell and virus lysates were stored at −80°C.

Cell cycle analysis. Cells were harvested 3 days posttransfection and were tested simultaneously for cell surface CD4 and DNA content by flow cytometry, as previously described (33). Briefly, 10^6 cells were incubated in 0.1 ml of PBS–1% fetal calf serum containing 1 μg of Leu3a for 30 min on ice. The cells were then washed with 2 ml of PBS and incubated with 0.5 μg of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin in 0.1 ml of PBS–1% fetal calf serum for 30 min on ice. The cells were washed in 2 ml of PBS and fixed in 0.25% paraformaldehyde–PBS for 1 h on ice. The fixed cells were permeabilized with 0.2% Triton X-100–PBS for 15 min at 37°C and then treated with 10 μg of propidium iodide-PBS per ml and RNase-A for 1 h at 37°C. Fluorescence was analyzed on a FACSCAN (Becton Dickinson) and also by using CellFit software (Becton Dickinson). Data on 10,000 cells were collected for each analysis and are presented after removing $CD4^-$ cells (typically 50% of the total) from the analysis. The blocking percentile was defined in each experiment by setting the $G_2/M:G_1$ ratio for cells expressing wild-type Vpr [$G_2/M:G_1$(Vpr)] to 1 and the $G_2/M:G_1$ ratio of cells transfected with pcDNA1/amp [$G_2/M:G_1$(pcDNA1/amp)] to 0. The blocking percentile of each mutant was then calculated by determining the position of each experimental $G_2/M:G_1$ ratio [$G_2/M:G_1$(mutant)] within these two values and multiplying the result by 100. This calculation is represented by the formula $\{[G_2/M:G_1(\text{mutant})] - [G_2/M:G_1(\text{pcDNA1/amp})]\}/\{[G_2/M:G_1(\text{Vpr})] - [G_2/M:G_1(\text{pcDNA1/amp})]\} \times 100$.

Immunoblot analysis. Protein from cell lysates (100 μg) and from virions (10 U of reverse transcriptase) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as described previously (29). The proteins were transferred to an Immobilon filter (Millipore Corp.) and probed with 12CA5 monoclonal antibody (Babco), diluted 1:10,000, and then with horseradish peroxidase-labeled goat anti-mouse immunoglobulin, diluted 1:10,000. The filter was developed by using the enhanced chemiluminescence method (ECL; Amersham).

Immunofluorescence. Cells were harvested 24 h posttransfection and plated on poly-L-lysine-coated glass coverslips (5.0×10^4). The next day, the cells were fixed for 20 min in 4% paraformaldehyde–PBS, permeabilized for 15 min in PBS containing 0.2% Triton X-100, and blocked by incubating for 30 min in 10% PBS–fetal calf serum. The coverslips were incubated for 1 h with 12CA5 monoclonal antibody (Babco), diluted 1:500. After extensive washing with PBS, the coverslips were incubated for 30 min with FITC-conjugated goat anti-mouse immunoglobulin, diluted 1:250. The coverslips were washed several times with PBS and mounted on glass slides in Fluoromount-G (Southern Biotech). Immunofluorescence was visualized with a Leica TCS 40 confocal microscope.

RESULTS

Vpr arrests cells in G_2 over a wide range of expression levels. We (13) and others (32) have previously shown that 293 cells transiently transfected with expression vectors encoding Vpr become arrested in the G_2 phase of the cell cycle. Vpr-induced cell cycle arrest results in a dramatic increase in the proportion of cells in the population in the G_2 phase of the cell cycle. This increase can be quantitated by determining the ratio of the number of cells containing a 2 N DNA content (cells in G_1) to those containing a 4 N DNA content (cells in G_2/M). On the basis of this finding, we developed a rapid assay for Vpr-induced cell cycle arrest that is useful for testing various properties of this activity. In the assay, 293 cells were transfected with Vpr expression vector and a small amount of CD4 expression vector, pc-CD4. Three days later, the cells were stained with anti-CD4 monoclonal antibody Leu3a and FITC-conjugated secondary antibody and propidium iodide. The cell cycle distribution of the transfected cells was then assessed by flow cytometry. By gating on the cells expressing CD4, we were able to restrict the analysis to the Vpr-expressing cells in the population, thus increasing the sensitivity of the analysis by removing the background, nontransfected cells. To remove from the analysis the cells in the population not expressing Vpr, we gated on the CD4 expressing cells. We have previously demonstrated the effectiveness of this procedure (13).

To test the range in expression level over which we could detect G_2 arrest with this assay, we transfected 293 cells with decreasing amounts of pc-5′-tag-Vpr and a fixed amount (2 μg) of pc-CD4. The results showed that, consistent with our previous findings, transfection of 293 cells with 18 μg of pc-5′-tag-Vpr dramatically increased the proportion of cells in the G_2/M phase of the cell cycle ($G_2/M:G_1$ ratio of 9.5, compared with 0.6 for cells transfected with pcDNA1/amp; Fig. 1, graphs). Reducing the amount of Vpr expression vector in the transfection to as low as 2 μg did not substantially alter the $G_2/M:G_1$ ratio of the population (Fig. 1, graphs). Immunoblot analysis of similarly transfected 293 cells showed that decreasing the amount of Vpr expression vector in the transfection

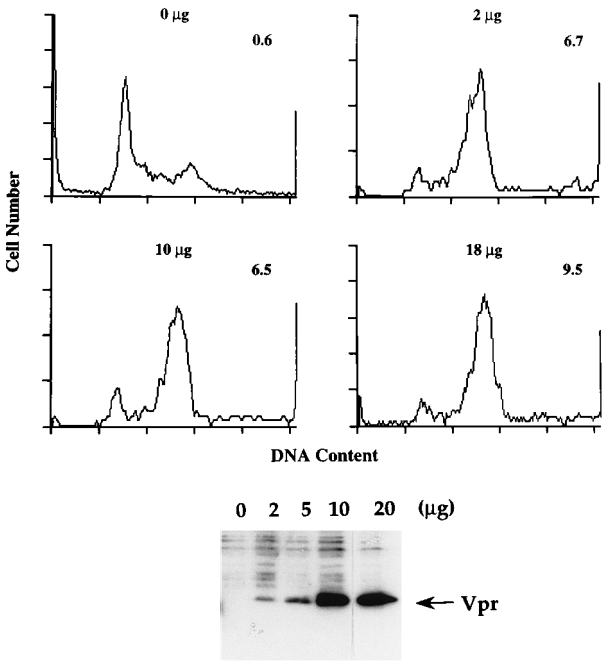

FIG. 1. G_2 arrest induced by different amounts of HIV-1 Vpr. (Graphs) 293 cells were transfected with the indicated amounts of pc-5′-tag-Vpr and CD4 expression vector pc-CD4 (2.0 μg). The cells were stained with propidium iodide and with Leu3a and FITC-conjugated rabbit anti-mouse immunoglobulin. The cells were analyzed by a fluorescence-activated cell sorter for DNA content and CD4 surface expression. Cells not expressing CD4 were gated out. G_2/M:G_1 ratios are indicated in the upper right of each graph. (Gel) 293 cells were transfected with the indicated amounts of pc-5′-tag-Vpr, and lysates (100 μg of protein) were analyzed by immunoblotting with anti-HA monoclonal antibody 12CA5.

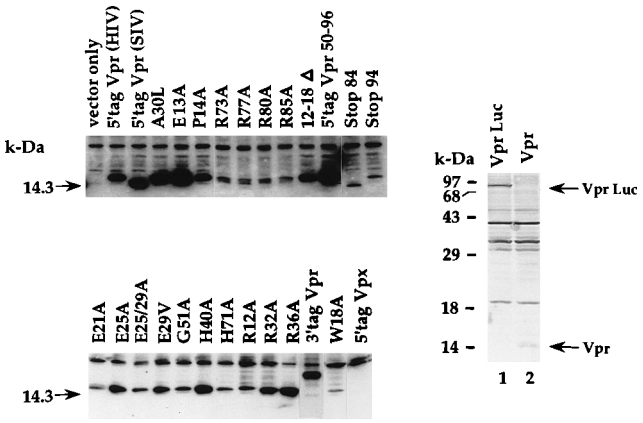

FIG. 2. Immunoblot analysis of wild-type and mutant tagged Vpr molecules. 293 cells were transfected with vectors encoding HA-tagged wild-type or mutant HIV-1 Vpr (top panel), HIV-1 Vpr (right panel), HA-tagged SIV Vpx (top panel), or Vpr-luciferase fusion protein (right panel), as indicated. Lysates (100 μg of protein) were analyzed by immunoblotting with anti-HA antibody 12CA5 (top and bottom panels) or rabbit anti-Vpr serum (right panel). (Right panel) 293 cells were transfected with vectors encoding the Vpr-luciferase fusion protein (lane 1) or wild-type Vpr (lane 2), and lysates were analyzed with rabbit anti-Vpr serum.

resulted in a concomitant decrease in the amount of Vpr present in the cells (Fig. 1, gel). Since most of the cells remained arrested in G_2 under these conditions, the decrease in the amount of Vpr produced was not due to a decrease in the number of cells transfected but was due to a decrease in the amount of Vpr expressed in each cell. Thus, expression of barely detectable amounts of Vpr was sufficient to arrest the cells in G_2. These findings cannot be explained by diffusion of Vpr from the transfected cells to bystander cells, since gating on the $CD4^-$ cells in the population showed that they were not arrested in G_2 (data not shown). These results suggested that the assay is sensitive to relatively small amounts of Vpr. This finding is important, since several of the Vpr mutants analyzed below were expressed at levels lower than that of the wild-type protein.

Construction and characterization of mutant Vpr molecules. As shown in Fig. 6, Vpr can be divided into three domains on the basis of its primary sequence: an amino-terminal, negatively charged region that is predicted to form an amphipathic helix (amino acids 17 to 34) (21, 22); a central hydrophobic domain (amino acids 35 to 75); and a carboxy-terminal, positively charged region (amino acids 80 to 96). To determine which of these regions is important for inducing cell cycle arrest, we constructed expression vectors encoding wild-type Vpr containing an amino- or carboxy-terminal influenza virus hemagglutinin (HA) epitope tag (pc-5′-tag-Vpr and pc-3′-tag-Vpr, respectively). We then changed specific nucleotides of pc-5′-tag-Vpr by site-directed mutagenesis to generate a panel of expression vectors encoding amino-terminal HA-tagged Vpr molecules containing single amino acid substitutions (indicated in Fig. 2). For mutagenesis, we targeted conserved residues in each of the domains of the protein, changing them to Ala, with the exception of Ala-30 and Glu-29, which we changed to Leu and Val, respectively. These included mutations in the amino terminus (R12A [an R-to-A mutation at position 12], E13A, and P14A), the alpha-helical region (W18A, E21A, E25A, E25/29A, E29V, A30L, and R32A), the central hydrophobic domain (R36A, H40A, G51A, and H71A), and the carboxy-terminal basic domain (R73A, R77A, R80A, and R85A). In addition, we constructed vectors encoding an HA-tagged carboxy-terminal Vpr fragment [pc-5′-tag-Vpr(50-96)], a Vpr molecule with deletions of amino acids 12 to 18 [pc-5′-tag-Vpr(12-18Δ)], and carboxy-terminal truncations of Vpr lacking twelve or two amino acids [pc-5′-tag-Vpr(stop84) and pc-5′-tag-Vpr(stop94), respectively]. We also constructed a vector encoding a luciferase-Vpr fusion protein, pc-Vpr.Luc. In addition, to test the cell cycle arrest properties of the SIV accessory gene products, we constructed expression vectors encoding HA-tagged SIV Vpr and SIV Vpx [pc-5′-tag-Vpr(SIV) and pc-5′-tag-Vpx(SIV), respectively].

We first tested whether each of the vectors would encode stable mutant Vpr molecules. To do this, we transfected 293 cells with each vector and detected the Vpr by immunoblot analysis with monoclonal antibody 12CA5, which recognizes the HA tag. The results showed that each protein was expressed at detectable levels in the transfected cells (Fig. 2). Some mutants were expressed at levels significantly lower than those of the wild type, presumably because of decreased stability. Molecules that were expressed at low levels include R73A, R77A, R80A, R85A, W18A, stop84, and stop94. Of all the Vpr mutants, W18A, stop84, and stop94 were expressed at the lowest levels. As is shown below, stop94 and W18A were as efficient as wild-type Vpr in the cell cycle arrest assay. This finding is consistent with the results described above showing that only small amounts of Vpr are required to arrest the cell cycle. Thus, the lack of ability of any of the mutants to arrest the cell cycle cannot be ascribed to an insufficient amount of protein in the transfected cells.

Amino acid residues of Vpr required for G_2 arrest. To determine the amino acid residues of Vpr required for cell cycle

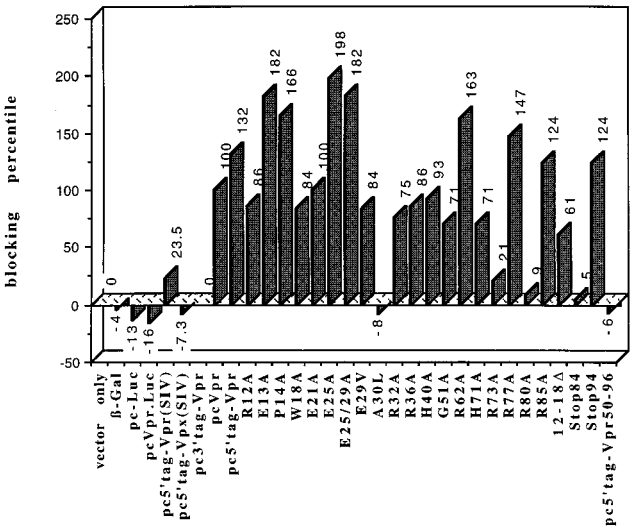

FIG. 3. Cell cycle blocking activity of mutated Vpr molecules. 293 cells were transfected with pSV-CD4 (2 μg) and pc-5′-tag-Vpr mutants (18 μg). After 3 days, the cell cycle distribution of the cells was determined as described in Materials and Methods. The cell cycle blocking ability of each mutant is expressed relative to $G_2M/:G_1$ ratios determined in parallel transfections with a vector expressing the wild-type amino-terminally tagged molecule, pc-5′-tag-Vpr, and with vector-alone controls (normalized to 100 and 0%, respectively), as described in Materials and Methods. Proteins that arrest the cell cycle more efficiently than the wild type have a blocking percentile greater than 100, while some that are inactive have a negative blocking percentile due to experimental variability. The data presented are representative of two to three independent analyses for each mutant.

arrest, we measured the relative cell cycle blocking activity of each of the mutant Vpr molecules in the transient transfection assay. In independent repetitions of these experiments we noticed some experimental variability (±20%) in the $G_2/M:G_1$ ratio of cells transfected with wild-type Vpr. To increase the accuracy of the data, we performed parallel transfections with pcDNA1/amp and with pc-5′-tag-Vpr in each repetition of the experiment. We then expressed the activity of each mutant protein in terms of its blocking percentile. The blocking percentile, calculated as described in Materials and Methods, reflects the $G_2/M:G_1$ ratio of the mutant Vpr molecule relative to that of wild-type Vpr and control vector-transfected cells.

The results of this analysis showed that the majority of mutants tested maintained wild-type-level or higher blocking activity (Fig. 3). These included Vpr molecules with mutations in the amino terminus (R12A, E13A, P14A, and 12-18Δ), the alpha-helical region (W18A, E21A, E25A, E25A/29A, E29V, and R32A), the central hydrophobic domain (R36A, H40A, G51A, R62A, and H71A), and the basic carboxyl-terminal region (R77A, R85A, and stop94). Only two missense mutants, A30L, in the amino-terminal alpha-helical region, and R80A, in the basic carboxyl-terminal region, failed to arrest the cell cycle. The importance of the carboxy-terminal region in mediating cell cycle arrest is supported by results with additional mutants. The cell cycle arrest activity of Vpr R73A, in which a basic residue close to R-80 has been substituted, was significantly reduced. In addition, truncation of the carboxy-terminal 12 amino acids of Vpr (stop84) inactivated the protein. Furthermore, Vpr tagged at the carboxy terminus (pc-3′-tag-Vpr) was inactive in cell cycle arrest. This finding is not likely to be due to improper folding of the protein, since this molecule is stably expressed, localizes to the nucleus, and is incorporated into virions. Of the mutations in the alpha-helical region, only A30L was inactive in cell cycle arrest (Fig. 3). It is unlikely that this indicates a direct requirement for the alpha-helical region; it is more likely that the mutation alters the overall conformation of the protein. This interpretation is supported by the inability of this molecular to be packaged into virions (described below). Several of the mutants tested (E13A, P14A, E25A, E25/29A, R62A, and R77A) exhibited blocking percentiles substantially greater than 100. However, it is not clear whether this reflects an increased ability to arrest the cell cycle or experimental variability. In cells arrested in G_2, the number of cells that form the G_1 peak is quite small and therefore subject to statistical variability that can substantially increase the calculated $G_2/M:G_1$ ratio. Taken together, these results suggest that the carboxy terminus of Vpr is required to mediate cell cycle arrest.

The fusion protein Vpr-Luc, consisting of luciferase linked to the amino terminus of Vpr, was inactive in the cell cycle arrest assay (Fig. 3). Failure to arrest the cell cycle was not likely to be due to improper folding, since the protein exhibited a high level of luciferase activity ($>10^5$ cpm/10^4 cells) and was efficiently incorporated into virions (data not shown). As described below, this protein fails to localize to the nucleus. Its inability to arrest the cell cycle may be due to its cytoplasmic localization.

We also tested the ability of SIV Vpr and Vpx to block the cell cycle. SIV Vpr showed low but significant levels of blocking activity, while SIV Vpx showed no detectable activity (Fig. 3). These data suggest that SIV Vpx does not arrest the cell cycle and therefore has probably evolved a different function in viral replication.

Nuclear localization of mutated Vpr molecules. While it is clear that Vpr localizes to the nucleus (20), the amino acid residues responsible for this property have not yet been identified. The protein does not contain a canonical nuclear localization sequence but does have a cluster of 6 Arg residues at the carboxy terminus which could be a candidate nuclear localization sequence. Lu et al. reported that this region of the protein was both necessary and sufficient to direct Vpr to the nucleus (20). To further define the amino acids of Vpr required for nuclear localization, we determined the cellular localization of the panel of mutant Vpr molecules. To do this, we transfected 293 cells with each expression vector and then visualized the HA-tagged proteins by immunofluorescence with monoclonal antibody 12CA5. As expected, wild-type, amino-terminal HA-tagged Vpr was clearly localized in the nucleus, as was SIV Vpx (Fig. 4). In contrast, HA-tagged β-galactosidase localized primarily to the cytoplasm, indicating that the observed nuclear localization of HA-tagged Vpr was not an artifact induced by the HA tag or by fixation conditions. We reproducibly observed one or two dark spots within the stained nuclei, presumably nucleoli, from which Vpr was excluded. This pattern contrasts with that reported for Tat and Rev, the two other HIV-1 nuclear regulatory proteins (9, 30). These proteins are believed to be concentrated in the nucleoli as the result of shuttle proteins that transport them to this region of the nucleus (16, 31). Presumably, Vpr does not share this pathway into the nucleus.

We next tested the cellular localization of each of the mutated Vpr molecules indicated in Fig. 2. Of the Vpr missense mutants, with the exception of A30P (shown in Fig. 4), each of the mutated molecules retained its ability to localize to the nucleus. We also tested carboxy-terminal truncations of Vpr lacking 18 or 33 amino acids (stop80 and stop65). Both of these localized to the nucleus as efficiently as the full-length protein. In contrast, a carboxyl-terminal fragment of Vpr, Vpr(50-96), localized predominantly to the cytoplasm (Fig. 4). Taken to-

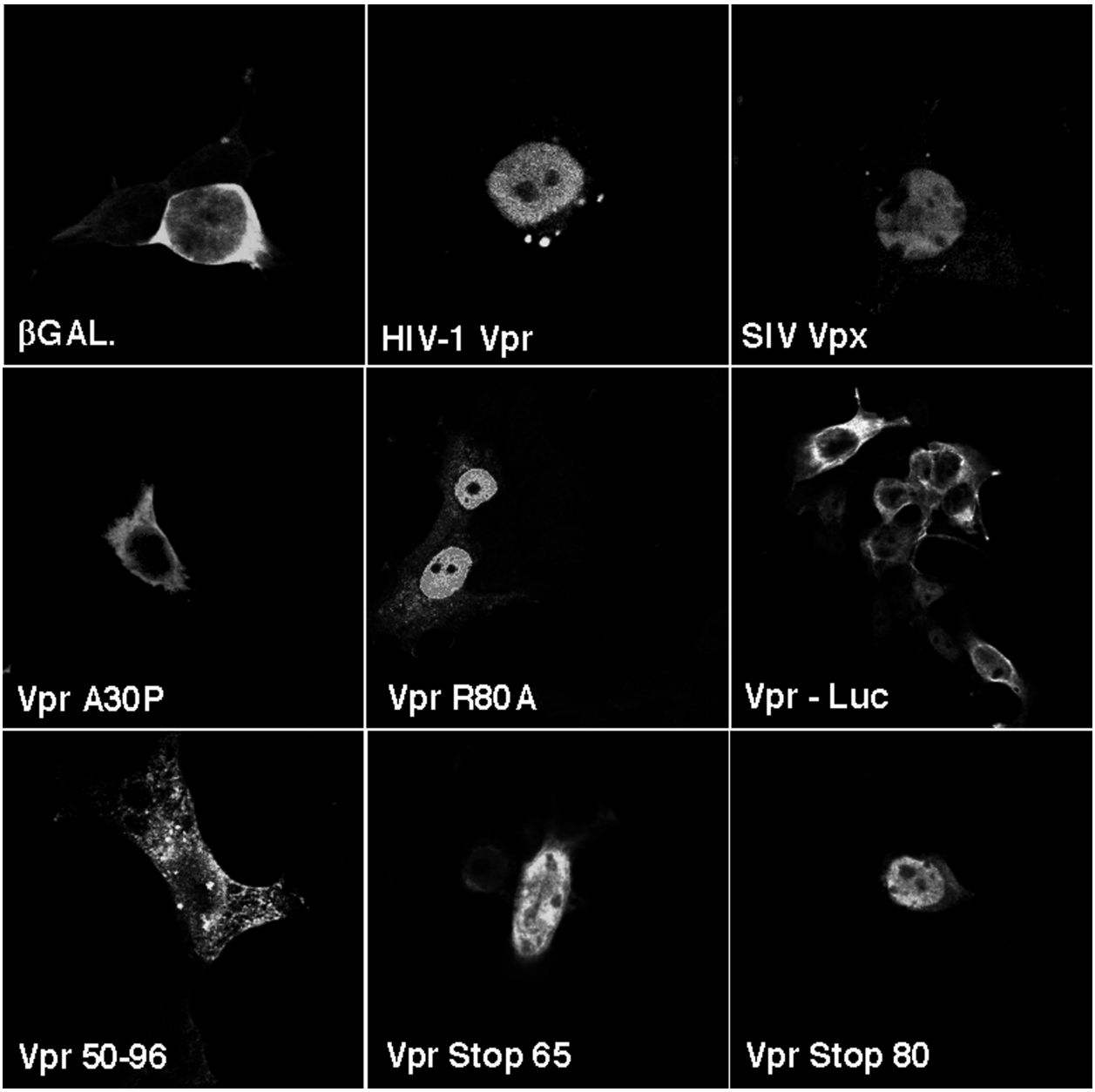

FIG. 4. Immunofluorescence of mutant tagged Vpr in 293 cells. 293 cells were transfected with vectors expressing mutant HA-tagged Vpr or HA-tagged β-galactosidase. After 1 day, the cells (10^5) were transferred onto coverslips and incubated with monoclonal anti-HA antibody 12CA5 and FITC-conjugated goat anti-mouse immunoglobulin. The stained cells were visualized by confocal microscopy, and cells representative of the population were photographed.

gether, these data suggest that the carboxy terminus of Vpr is neither necessary nor sufficient for nuclear localization. Therefore, the nuclear localization sequence of Vpr is likely to reside in the amino-terminal half of the protein. The importance of the amino-terminal region of Vpr in nuclear localization is further supported by the finding that Vpr A30P failed to localize to the nucleus. Mutation of amino acid 30 to Leu, which is not predicted to disrupt the amino-terminal alpha helix, did not affect nuclear localization. This finding suggests that the presence of an alpha helix in this region is required for nuclear import.

Transfection of 293 cells with pc-tag-Vpr-Luc, which encodes a fusion protein consisting of luciferase fused to the amino terminus of Vpr, resulted in a molecule that failed to localize to the nucleus (Fig. 4). This is not likely to be due to improper folding of either of the fusion partners, since the fusion protein is efficiently incorporated into virions and is highly active in luciferase assays. In addition, we constructed and tested a fusion protein in which luciferase was linked to the carboxy terminus of Vpr and one in which β-galactosidase was fused to the amino terminus of Vpr. Both of these proteins were stably expressed in cells, but neither localized to the nucleus by immunofluorescence (data not shown). Given the role played by Vpr in actively transporting the viral preintegration complex into the nucleus, these results were unexpected. Our results are consistent with a mechanism of nuclear localization in which Vpr passively diffuses into the nucleus and becomes concentrated there by interacting with a nuclear

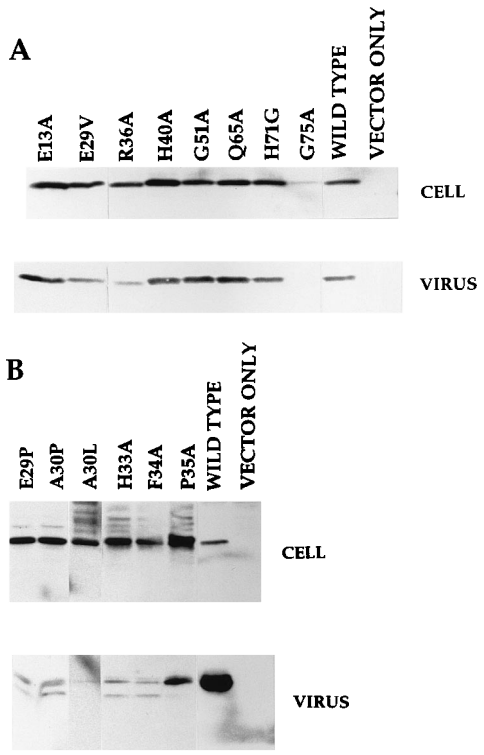

FIG. 5. The amino-terminal alpha helix of Vpr is important for its packaging into HIV-1 virions. 293 cells were transfected with expression vectors encoding HA-tagged Vpr point mutants. 293 cells (2×10^6) were cotransfected with pNL4-3-E^-R^- and with mutant pc-5′-tag-Vpr vectors, as indicated. Lysates were prepared from virions (10 U of reverse transcriptase) secreted into the culture medium and from the transfected cells (100 μg of protein) and were separated by SDS-PAGE and transferred to filters. HA-tagged Vpr was visualized on an immunoblot probed with 12CA5.

component, such as the nuclear matrix. Fractionation studies have shown that Vpr is associated with this structure (20). However, we cannot rule out the possibility that in each of the Vpr fusion proteins tested, the nuclear localization sequence was sterically hindered by the fusion partner, thereby blocking its function.

Virion packaging of Vpr. To define the amino acids of Vpr that are required for its packaging into virions, we tested several HA-tagged mutant Vpr molecules for their ability to be packaged into HIV-1 virions. To do this we used the transient packing system we described previously (29). In this assay, 293 cells were transfected with Vpr expression vector and a *vpr*-mutant HIV-1 provirus, pNL4-3-R^-E^-. Two days later, virions secreted into the culture medium were pelleted by centrifugation and lysates were prepared from the cells. The amount of virion- and cell-associated Vpr was then assessed by immunoblot analysis with anti-HA monoclonal antibody (Fig. 5). The results showed that mutations in the amino-terminal region (E13A and 12-18Δ; not shown) and in the central hydrophobic region (H40A, G51A, Q65A, and H71G) did not affect the ability of Vpr to be packaged into virions. Mutant G75A appeared to be unstable, and therefore its ability to be packaged could not be evaluated. The neighboring amino acid Cys-76 is invariant in Vpr and Vpx and appears to play a structural role in the protein (29). Similarly, Gly-75 may be required for proper folding. Vpr molecules with mutations in the alpha-helical domain (amino acids 17 to 34) showed a somewhat reduced (E29V and R36A; Fig. 5A) or dramatically reduced (E29P, A30P, A30L, H33A, and F34A; Fig. 5B) level of packaging efficiency. Mutant P35A, at the carboxy-terminal boundary of the alpha helix, was packaged but with a reduced level of efficiency. A30L appeared to be completely unable to package into virions. This protein is inactive in both cell cycle arrest and in virion packaging and therefore may be misfolded. Amino acids on both the hydrophilic (E29P) and hydrophobic (A30L and F34A) faces of the helix reduced virion packaging. Taken together, these findings support the importance of the alpha-helical region in Vpr packaging. These results, together with those derived above, are summarized schematically in Fig. 6.

DISCUSSION

We have used a panel of expression vectors encoding mutated Vpr molecules to define the domains of the protein that are required for its ability to arrest the cell cycle, to localize to the nucleus, and to be packaged into virions. Our results show that cell cycle arrest is largely controlled by the carboxy-terminal basic domain, while virion packaging and nuclear localization are controlled by the amino-terminal alpha-helical region (shown schematically in Fig. 6). Our findings on the importance of the alpha-helical domain for virion packaging are consistent with those recently reported by Mahalingam et al. (21, 22). While the carboxy terminus of Vpr is required for cell cycle arrest, it is not sufficient, since Vpr(50-96), which contains the carboxy-terminal half of the protein, was inactive. This could be due either to a failure to dimerize or to localize to the nucleus, since both properties require the amino terminus (36). The amino terminus of Vpr may serve to transport the protein into the nucleus, while the carboxy terminus contains the activation domain that induces cell cycle arrest. We did not find Vpr mutants that arrested the cell cycle but failed to localize to the nucleus, presumably because cell cycle arrest requires the presence of Vpr in the nucleus. Consistent with

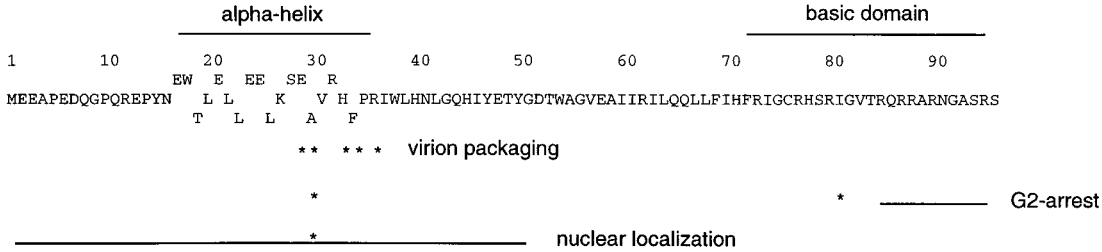

FIG. 6. Regions of Vpr required for cell cycle arrest, nuclear localization, and virion packaging. The amino acid sequence of NL4-3 Vpr is shown, with the alpha-helical and basic domains indicated. Amino acids on the hydrophilic face of the helix are shown raised, while those on the hydrophobic side are lowered. Asterisks indicate critical amino acids, as determined by analysis of mutated Vpr molecules. Horizontal lines indicate affected regions, as determined by analysis of truncation and deletion mutants.

this hypothesis, Vpr-luciferase fusion proteins do not localize to the nucleus and also fail to arrest the cell cycle.

Presumably, Vpr blocks the cell cycle by interacting with a nuclear component involved in cell cycle regulation. On the basis of our previous studies, such a protein may be one of the cellular regulators of the $p34^{cdc2}$-cyclin B complex. The carboxy-terminal activation domain of Vpr would be the likely interaction site for such a factor. Mutants such as R80A, R73A, stop84, stop80, and carboxy-terminally tagged Vpr that localize to the nucleus but fail to arrest the cell cycle are likely candidates for Vpr molecules that fail to interact with a cellular factor involved in the cell cycle regulation.

Interaction of the carboxy terminus of Vpr with a nuclear component does not appear to be required for localization of the protein to the nucleus. Instead, it is likely that a separate interaction of Vpr with the amino terminus of the protein is responsible. However, while we do not detect nuclear localization activity in the carboxy terminus of the protein, it is possible that this region plays a more subtle role. For example, the amino terminus of Vpr might be responsible for transporting it to the nucleus, while the carboxy terminus, by interacting with a nuclear component, could anchor it there.

Our finding that fusion proteins in which Vpr was linked to luciferase or β-galactosidase failed to localize to the nucleus was unexpected. If Vpr is capable of importing a large viral preintegration complex into the nucleus, we expected that it would efficiently import a much smaller fusion partner. Although we cannot rule out the possibility that our fusion proteins folded in such as way as to obscure the nuclear localization sequence of Vpr, it remains a possibility that nuclear import of Vpr is not an active process. As a dimer of 30 kDa, Vpr would still be within the size limitation for diffusion through nuclear pores (27, 28). It is difficult to reconcile these findings with the role of Vpr in nuclear import of the HIV-1 preintegration complex in nondividing cells (14).

While it is clear that Vpr causes cell cycle arrest in transfected and infected cells (13, 32), it is not clear whether this activity is relevant to HIV-1 replication in monocytes/macrophages or to AIDS pathogenesis. Conceivably, cell cycle arrest is an epiphenomenon; the presence of Vpr in the nucleus might nonspecifically interfere with the complicated events of cell division. However, our data suggest that this is not the case, since several mutated Vpr molecules localized to the nucleus but failed to arrest the cell cycle (A30L, R80L, stop84, stop80, and 3′-tag-Vpr). Furthermore, SIV Vpx is similar in sequence to HIV-1 Vpr, localizes to the nucleus, but does not arrest the cell cycle. Thus, cell cycle arrest is not likely to be the result of the accumulation of a foreign protein in the nucleus but probably results from a specific interaction of Vpr with a nuclear component. Defining the amino acids of Vpr that are required for cell cycle arrest, virion packaging, and nuclear localization will be useful for determining which of these properties is important for HIV-1 replication. In addition, it will be useful for identifying the cellular factors that mediate Vpr function.

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