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Conservation and Host Specificity of Vpr-Mediated Cell Cycle Arrest Suggest a Fundamental Role in Primate Lentivirus Evolution and Biology

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The human immunodeficiency virus type 1 (HIV-1) Vpr protein prevents infected cells from passing through mitosis by arresting them in the G_2 phase of the cell cycle. Vpr is conserved among all primate lentiviruses, suggesting an important role in the virus life cycle. Moreover, in this study we show that the ability to cause cell cycle arrest is also conserved in Vpr proteins from a wide variety of both tissue culture-passaged and uncultured human (HIV-1 and HIV-2), sooty mangabey (simian immunodeficiency virus SIV_{SM}), African green monkey (SIV_{AGM}), and Sykes' monkey (SIV_{SYK}) isolates. However, this property is cell type specific and appears to depend on the particular primate species from which the cells are derived. SIV_{AGM} and SIV_{SYK} Vpr proteins are capable of arresting African green monkey cells but are completely inactive in human cells. By contrast, HIV-1, HIV-2, and SIV_{SM} Vpr proteins function in both simian and human cell types, although SIV_{SM} Vpr functions more efficiently in simian cells than it does in human cells. Neither differential protein stability nor subcellular localization explains the species-specific activities of these proteins. These results thus suggest that Vpr exerts its G_2 arrest function by interacting with cellular factors that have evolved differently among the various primate species.

AIDS in humans is caused by two related retroviruses, human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), which are members of a larger group of primate lentiviruses comprised of five lineages that include HIV-1/simian immunodeficiency virus SIV_{CPZ}, HIV-2/SIV_{MAC}/SIV_{SM}, SIV_{AGM}, SIV_{SYK}, and SIV_{MND} (39). Members of each of these five lineages encode an evolutionarily conserved accessory protein called Vpr in the central region of the genome. Vpr amino acid sequences are quite conserved between HIV-1 and HIV-2/SIV_{SM} (roughly 50%) but more diverged between HIV-1 and SIV_{AGM} and SIV_{SYK} (30 and 18%, respectively) (11, 21, 29). In addition, members of the HIV-2/SIV_{SM} group also encode a protein called Vpx which has some amino acid identity with Vpr (38, 43).

Two functions have been described for the Vpr protein of HIV-1 (reviewed in reference 7). First, Vpr is important for targeting the viral preintegration complex to the nucleus of nondividing cells, such as terminally differentiated macrophages (12, 18). A second and less clearly understood role for Vpr is its ability to arrest infected cells in the G_2 phase of the cell cycle (1, 17, 25, 34, 36), precluding chronic infection by HIV-1 (36). Furthermore, in the HIV-2/SIV_{SM} lineage, which has both Vpr and Vpx, only Vpr can arrest cells in G_2, while Vpx, but not Vpr, is needed for efficient infection of macrophages (6, 10, 26). This finding indicates that the ability of Vpr to arrest cells in G_2 is not a consequence of the nuclear import function needed for infection of macrophages.

Previous studies found that although the G_2 arrest function of Vpr was conserved in SIV_{MAC} (6, 26, 33), HIV-2 (6, 26, 33), the amount of G_2 arrest in human cells was attenuated relative to that of HIV-1 Vpr. Moreover, SIV_{AGM} Vpr arrested simian cells in G_2 but not human cells (33). However, one caveat of these studies with HIV-2 and SIV Vpr is that they relied on viruses that had been isolated from chronically infected transformed human T-cell lines (6, 26, 33) which would be expected to select against *vpr* alleles that disrupted cell growth. Indeed, HIV-1 *vpr* accumulates mutations that attenuate or abolish activity of the protein when the virus is passaged in transformed T cells (30, 36).

In this study, we have quantified the ability of Vpr to cause G_2 arrest function from a broad collection of viruses including members derived from four of the five major primate lentiviral lineages. In addition to analyzing *vpr* genes from isolates derived from long-term culture in transformed T-cell lines, we analyzed *vpr* genes from isolates that had been cloned after short-term passage in primary peripheral blood mononuclear cells (PBMC) as well as *vpr* genes amplified directly from tissues of infected humans and monkeys without interim in vitro culture.

We find that primary isolates of HIV-2 can efficiently arrest human cells in G_2. In addition, there is a distinct species specificity in the ability of Vpr from diverse SIV strains to cause G_2 arrest. SIV_{SM}, which is most closely related to HIV-2, is only partially able to arrest human cells in G_2. In contrast, Vpr proteins from the more divergent SIV_{AGM} and SIV_{SYK} strains are completely unable to arrest human cells. Nonetheless, regardless of origin, all of the SIV strains tested were able to efficiently arrest African green monkey cells in G_2. No difference in Vpr cellular localization or stability was observed in cells from different species, suggesting that the ability to function is dependent on the availability of suitable cellular substrates. Taken together, these data suggest that the Vpr-medi-

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TABLE 1. Origin of *vpr* genes characterized in this study

Virus strain	Primate species[a]	Virus isolate[b]	Source of *vpr* gene[c]	Reference
HIV-1_{LAI}	Human	Primary human PBMC	Proviral clone	44
HIV-2_{ROD}	Human	Immortalized T-cell line	Proviral clone	16
HIV-2_{7312A}	Human	Primary human PBMC	Proviral clone	13
HIV-2_{7924A}	Human	Uncultured	PCR of uncultured patient PBMC	13
SIV_{SM}PBj1.9	Sooty mangabey	Primary macaque PBMC	PCR of cultured macaque PBMC	5
SIV_{AGM}ver9063	African green monkey (vervet)	Immortalized T-cell line	Proviral clone	20
SIV_{AGM}ver9648	African green monkey (vervet)	Uncultured	PCR of uncultured monkey PBMC	41
SIV_{AGM}ver9649	African green monkey (vervet)	Uncultured	PCR of uncultured monkey PBMC	41
SIV_{SYK}173	Sykes' monkey	Immortalized T-cell line	Proviral clone	21

[a] Primate species from which virus was originally isolated.
[b] Method of virus propogation before cloning. Uncultured indicates that virus was not propagated in tissue culture.
[c] Source of DNA for cloning the *vpr* gene.

ated G_2 arrest may be an important factor in adaptation of the virus to its host and in cross-species transmission of SIV to humans.

MATERIALS AND METHODS

DNA constructions. PCR was used to amplify the *vpr* genes from the following viral molecular clones (Table 1): HIV-1_{LAI} (44), HIV-2_{ROD} (16), HIV-2_{7312A} (14, 35), SIV_{AGM}9063-2 (20), SIV_{SM}PBj1.9 (5), and SIV_{SYK}173 (21). HIV-2_{7924A} (14) was amplified directly from uncultured patient PBMC genomic DNA by nested PCR.

African green monkeys 9648 and 9649 were caught in Tanzania in 1990. Both were vervet monkeys that had been infected in the wild and likely came from the same troop. Uncultured PBMC DNA from these two animals was isolated in 1994 (41), and viral DNA was amplified by nested PCR using primers designed according to SIV_{AGM} vervet consensus sequences (41): outer primers 5′GGCT TGGTATACTATGTGCAG3′ and 5′CCTGTACTGAGCTGTTTTTCCATAC TGG3′, and inner primers 5′GACGACGTTAACATGGCCTCAGGGAGAGA TCCC3′ and 5′AGGGGATCCTTGTATTGTTCAATCAAGTCC3′.

The influenza virus hemaglutinin (HA) epitope, YPYDVPDYA, was generated by synthesizing and hybridizing the following nucleotides: 5′TCGAGAGG GATACCCATACGATGTTCCAGATTACGCG3′ and 5′GTACCGCGTAAT CTGGAACATCGTATGGGATCCCATC3′. The resulting product was amplified by PCR (PWO, Boehringer Mannheim, Indianapolis, Ind.) and directly cloned into pCRII (Invitrogen, San Diego, Calif.). The linker was then cloned into pSL1180 (Pharmacia, Uppsala, Sweden) to generate plasmid Flu-SL1180. The *vpr* genes from all isolates except HIV-1_{LAI}, HIV-2_{ROD}, SIV_{AGM}9648, and SIV_{AGM}9649 were cloned in frame with the HA tag of Flu-SL1180. The remaining were cloned into plasmids without epitope tags. The *vpr* genes were then inserted into the retrovirus vector LXSH (28). LXSH drives expression of the gene of interest from the murine leukemia virus long terminal repeat (LTR) and codes for hygromycin resistance driven from the simian virus 40 immediate-early promoter. High-level expression vectors were generated by cloning the gene of interest into a vector that drives expression from the HIV-1 LTR (36).

Cells, transfections, and infections. Human embryonic kidney 293T, human epithelial HeLa, macaque mammary tumor CMMT, and African green monkey kidney CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All transfections were done by calcium phosphate coprecipitation. To produce retroviral stocks, 5×10^5 293T cells were transfected with 2 μg of murine leukemia virus Gag/Pol, 2 μg of L-Vpr-SH, 1 μg of L-VSV G, and 0.5 μg of pCMV-Tat as described previously (1). Virus was collected several times per day for 3 days and concentrated 10-fold by ultracentrifugation. Then 2×10^5 HeLa, CMMT, or CV-1 cells plated in 35-mm wells were infected with 300 μl of virus in the presence on 10 μg of DEAE-dextran per ml. Twelve hours after infection, medium containing 0.75 mg of hygromycin per ml was added. After 3 days, no cells remained on the mock-infected wells; nuclei from cells in the wells infected with the retrovirus vectors were isolated and prepared for flow cytometry as previously described (36).

Pulse-chase analysis. CV-1, 293T, or HeLa cells were transfected with the high-level expression vectors described above. At 48 h following transfection, expressed proteins were assayed for stability as previously described (26). Briefly, 5×10^5 cells were labeled with 250 μCi of [^{35}S]methionine-cysteine Trans-label (ICN) for 1 h, washed, and then chased in unlabeled medium containing a 10-fold excess of unlabeled methionine for 1 to 24 h. Cellular lysate was collected, and the proteins of interest were immunoprecipitated with either monoclonal HA antibody (12CA5; Boehringer Mannheim) or rabbit polyclonal antisera directed against HIV-2_{ROD} Vpr (26), SIV_{SM}PBj1.9 Vpr (31), or HIV-1 Vpr (15a). Immunoprecipitated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (National Diagnostics) and quantitated by PhosphorImager analysis (Molecular Dynamics).

Immunofluorescence. HeLa and CV-1 cells were plated 18 to 24 h before transfection at a density of 2×10^5 per well. Cells were transfected by using Lipofectamine reagent (Gibco, BRL) as instructed by the manufacturer with the HA epitope-tagged SIV_{SM}PBj1.9 Vpr and SIV_{AGM}9063 Vpr in the HIV-1 LTR-driven plasmids described above. One day posttransfection, cells were trypsinized and replated onto glass coverslips. Two days posttransfection, coverslips were washed once in phosphate-buffered saline (PBS) and then fixed in PBS–4% paraformaldehyde for 5 min. Cells were permeabilized by treating coverslips for 5 min in PBS–0.2% Triton X-100. Blocking was performed in PBS–1% bovine serum albumin–20% calf serum for 30 min. Mouse monoclonal antibody HA.11 (Berkeley Antibody Co., Richmond, Calif.) and SIV_{SM}PBj1.9 Vpr rabbit polyclonal antiserum were diluted 1:100 in PBS were incubated on cells for 1 h at room temperature in a humidified chamber. Fluorescein isothiocyanate-conjugated sheep anti-mouse and goat anti-rabbit (Jackson Immunoresearch) antibodies were used as secondary antibodies against the HA.11 and the SIV_{SM}PBJ-Vpr antibodies, respectively, incubated in humidified chambers for 30 to 60 min. Images of stained cells were observed and collected with a Delta Vision SA3.1 microscope (Applied Precision Instruments).

Phylogenetic tree construction. Phylogenetic relationships of the viral strains selected for functional analyses were estimated based on Vpr protein sequences. Amino acid sequences were aligned by using CLUSTAL (19), with minor manual adjustments. Sites where there was a gap in any sequence in the alignment were excluded from all comparisons. Phylogenetic trees were constructed by the neighbor-joining method (37), and their reliability was estimated from 1,000 bootstrap replicates (8). These methods were implemented by using CLUSTAL W (42) and programs from the PHYLIP package (9).

Nucleotide sequence accession numbers. Accession numbers for the *vpr* sequences reported here for the first time are U81835, U78790, and U78792 for HIV-2_{7924A}, SIV_{AGM}9648, and SIV_{AGM}9649, respectively.

RESULTS

Selection of viruses for Vpr functional studies. Because of potential artifacts of examining Vpr-mediated cell cycle arrest with *vpr* alleles isolated from viruses that had been selected by long-term passage in tissue culture, we chose here to examine HIV-2 and SIV strains that had been cloned after short-term passage in primary PBMC or cloned directly from infected tissues (Table 1). Therefore, in parallel with analysis of Vpr from HIV-2_{ROD}, an isolate cloned from a chronically infected cell line (16), we also analyzed Vpr function from HIV-2_{7312}, which was cloned after short-term passage in primary PBMC (13, 35), and Vpr from HIV-2_{7924A}, which was PCR amplified directly from uncultured lymphocytes of the infected individual (13).

In addition, we extended previous analyses by examining Vpr function from a more diverse range of isolates of different primate lentivirus lineages. Therefore, the *vpr* gene was cloned from the highly diverse SIV isolates SIV_{AGM} and SIV_{SYK} (Table 1). Three different SIV_{AGM} isolates were used (Table 1). SIV_{AGM}9063 was cloned from a transformed T-cell line and has been shown to be pathogenic in macaques (20); Vpr proteins from two new strains, SIV_{AGM}9648 and SIV_{AGM}9649, were PCR amplified directly from the uncultured lymphocytes of naturally infected African green monkeys (41). The se-

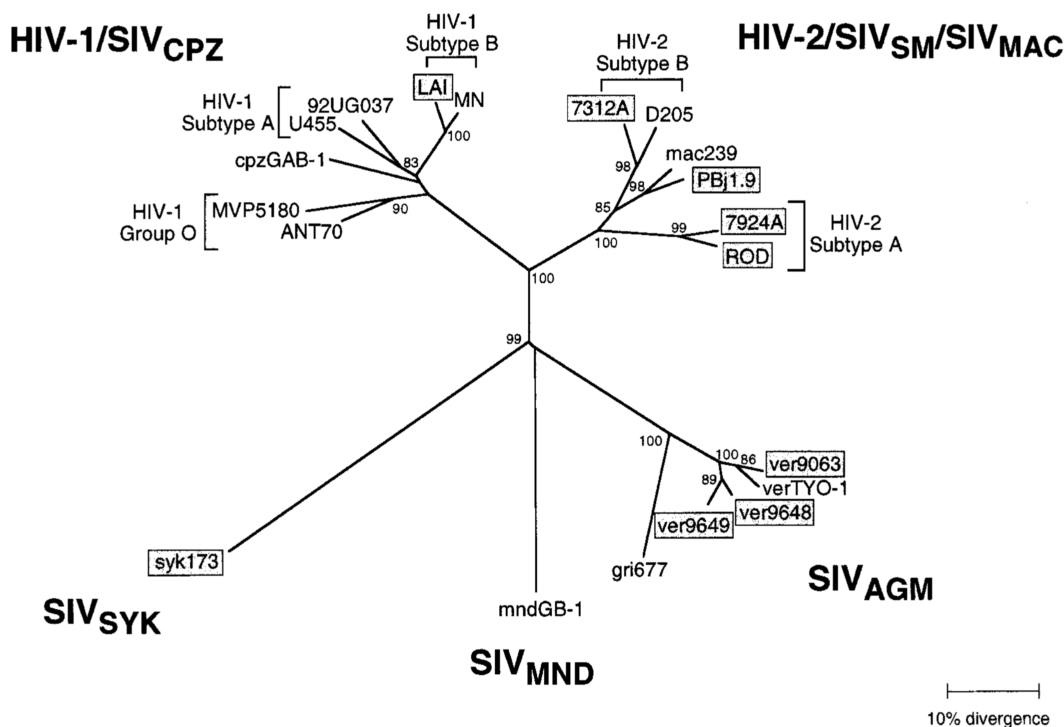

FIG. 1. Phylogenetic relationships of Vpr protein sequences. Vpr amino acid sequences from the nine HIV and SIV strains selected for functional analyses (boxed) were aligned with Vpr sequences from the database (29). Branch lengths are drawn to scale (the bar indicates 0.1 amino acid substitution per site), and the numbers near internal nodes indicate the percentage of bootstraps (out of 1,000) in which the various clades were found (values under 80% are omitted). HIV-1/SIV_{CPZ} group O viruses as well as HIV-1/CPZ and HIV-2/SIV_{SM}/SIV_{MAC} subtype A and B viruses are shown in brackets. The SIV_{AGM} group includes representatives of vervet (ver) and grivet (gri) monkey virus lineages. HIV-2_{7312A} is a mosiac A/B subgroup virus, but the *vpr* region is more related to the B subgroup.

quence relationships of these newly derived Vpr proteins, as well as the subtype/group/lineage classifications of their respective viruses, are shown in Fig. 1.

Both HIV-1 and HIV-2 Vpr proteins can efficiently arrest human cells in G_2. The *vpr* allele from each of the viruses described in Table 1 was cloned by PCR into a retrovirus vector. Infection of target cells by retrovirus vectors was used in place of transient transfection because copy number can be more carefully controlled, and quantitative differences between isolates can more easily be determined. Human HeLa cells were infected with the appropriate expression constructs at a multiplicity of infection of 0.8. At 12 h postinfection, the infected cells were selected in 0.75 mg of hygromycin per ml and were collected at 96 h postinfection. Cells were then stained with propidium iodide and analyzed for DNA content by flow cytometry. Examples of representative cell cycle profiles are shown (Fig. 2A); however, each infection was done at least three times. Results are represented as the ratio of the number of cells in G_2 relative to the number of cells in G_1 of the cell cycle (Fig. 2).

The ratio of cells in G_2 to cells in G_1 when HeLa cells are infected with a vector that does not express Vpr is less than 0.5 (Fig. 2). In contrast, the G2/G1 ratio is 1 to 1.5 when they are infected with a vector that expresses HIV-1 Vpr (Fig. 2). In agreement with previous results (26, 33), HIV-2_{ROD} Vpr, which was cloned from a chronically infected cell line (16), is severely attenuated in its ability to induce a G_2 arrest in human cells relative to HIV-1 (Fig. 2). To determine whether the results obtained for HIV-2_{ROD} were representative of the entire HIV-2 group of viruses, we analyzed two additional strains, HIV-2_{7312A}, and HIV-2_{7924A} (13), which were not propagated in chronically infected T-cell lines (Table 1). In contrast to HIV-2_{ROD}, HIV-2_{7312A} Vpr, and HIV-2_{7924A} were able to arrest human cells to the same level as HIV-1 (Fig. 2). This finding demonstrates that attenuation of Vpr function is not a general property of HIV-2 isolates. Thus, it is possible that the attenuated phenotype of HIV-2_{ROD} Vpr arose from mutations acquired during its passage in tissue culture. Nonetheless, these results indicate the primary strains of both HIV-1 and HIV-2 can induce efficient G_2 arrest in human cells.

Vpr from SIV_{SM}, but not SIV_{AGM} or SIV_{SYK}, can arrest human cells in the cell cycle. SIV_{SM} and SIV_{MAC} are highly related to HIV-2 and belong to the same group of primate lentiviruses (Fig. 1). In contrast to SIV_{MAC}, SIV_{SM}PBj1.9 was not isolated from a chronically infected T-cell line but rather was cloned directly after short-term culture in monkey PBMC (Table 1). Consistent with previous studies (6, 10, 33), we showed that SIV_{SM}PBj1.9 Vpr, like SIV_{MAC}, is able to induce a G_2 arrest in human cells (Fig. 1). However, its activity, like that of SIV_{MAC} (6, 33), is attenuated relative to HIV-1 Vpr.

We also examined the ability of Vpr from SIV_{AGM} or SIV_{SYK} to mediate a G_2 arrest in human cells. SIV_{AGM}gri677, isolated from a grivet monkey, was previously shown to have no Vpr-associated G_2 activity in human cells (33). To determine if this property was general, we examined the Vpr function of SIV_{AGM}9063, isolated from a vervet monkey, which is pathogenic when inoculated into macaques (20). Despite these differences in origin and pathogenicity, Vpr from SIV_{AGM}9063, like that of SIV_{AGM}gri677, was unable to induce a G_2 arrest in human HeLa cells (Fig. 2). Finally, the Vpr from SIV_{SYK}, which is the most divergent relative to HIV-1 Vpr

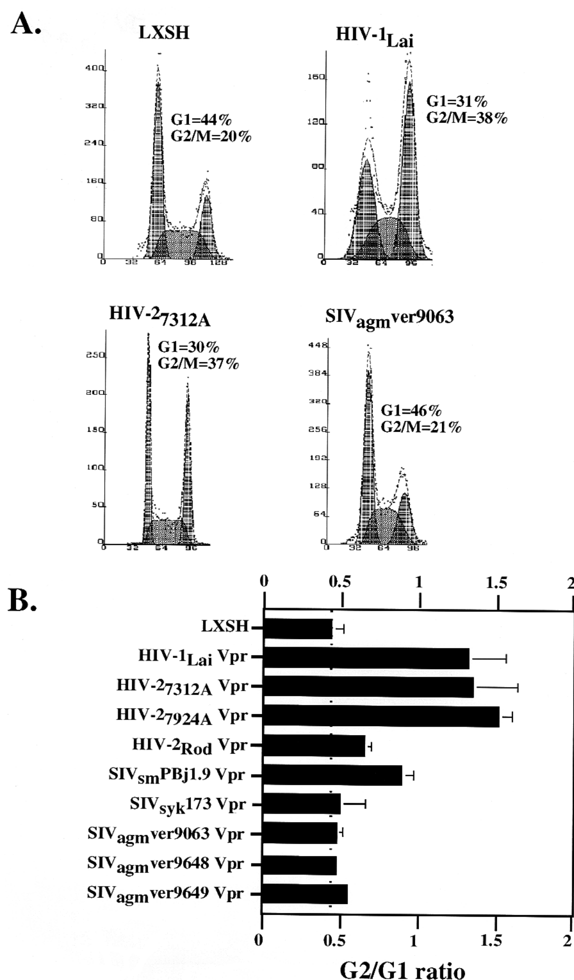

FIG. 2. Only HIV-1 Vpr and HIV-2/SIV_{SM} Vpr are able to induce a G_2 arrest in infected human cells. Human HeLa cells were infected with the control vector LXSH or with LXSH expression vectors encoding the *vpr* genes from several HIV and SIV isolates. Infected cells were selected in 0.75 mg of hygromycin per ml at 12 h postinfection, and cells were collected for analysis at 96 h postinfection. DNA content per cell was determined by propidium iodide staining, followed by flow cytometry of at least 10,000 cells. Shown are representative cell cycle profiles (A) and a graph depicting the G_2/G_1 ratios of the infected cells (B). The graph depicts the average of at least three independent experiments, and standard deviations of the data are marked above the bars.

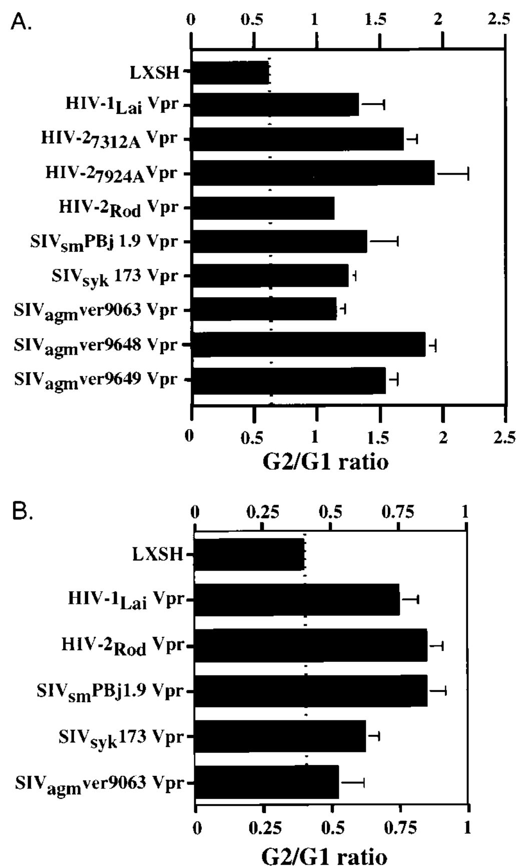

FIG. 3. All primate lentiviruses are able to arrest simian cells in G_2. African green monkey CV-1 cells (A) or macaque mammary tumor CMMT cells (B) were infected with the control vector LXSH or with LXSH expression vectors encoding the *vpr* genes from several HIV and SIV isolates. Infected cells were selected in 0.75 mg of hygromycin per ml at 12 h postinfection, and cells were collected for analysis at 96 h post-infection. DNA content per cell was determined by propidium iodide staining, followed by flow cytometry of at least 10,000 cells. The graph depicts the G_2/G_1 ratios of the infected cells and is a composite of at least three independent experiments.

(Fig. 1), was also unable to induce G_2 arrest in human HeLa cells (Fig. 2).

Both SIV_{AGM}gri677 and SIV_{AGM}9063 were cloned from chronic passage in transformed human cell lines. It was therefore possible that these isolates originally contained a functional Vpr protein that was subsequently lost because of in vitro passage in human cells. Therefore, to determine if the Vpr from the SIV_{AGM} strains tested above faithfully represented the activity of this class of viruses, we used PCR to amplify the *vpr* genes of two additional viruses (41) directly from the PBMC of naturally infected wild-caught vervets (Table 1). These genes were then cloned into retrovirus vectors and tested for the ability to cause G_2 arrest (Fig. 2). Like SIV_{AGM}9063 and SIV_{AGM}gri677, the *vpr* genes of SIV_{AGM}9648 and SIV_{AGM}9649 (Table 1) were unable to cause a G_2 arrest in human cells (Fig. 2). This result indicates that the inability of Vpr from SIV_{AGM} is not selected against by growth in human cells but rather is a general property of these isolates.

Vpr proteins from all primate lentiviruses are capable of arresting simian cells in G_2. Previous work had shown that the Vpr protein of SIV_{AGM}gri677 could arrest African green monkey (CV-1) cells but not human HeLa cells in G_2 (33). Therefore, we speculated that Vpr from other SIV isolates might also function best in this cell type. To test this hypothesis, CV-1 cells were infected with the different constructs as described above. In contrast to the results for human cells, where SIV_{SM} Vpr was attenuated relative to HIV-1 Vpr (Fig. 2), SIV_{SM} Vpr was able to efficiently cause a G_2 arrest in CV-1 cells (Fig. 3A). Similarly, HIV-1 Vpr efficiently caused cell cycle arrest in CV-1 cells. Finally, all of the HIV-2 *vpr* alleles had substantial activity in CV-1 cells (Fig. 3A). These results indicate that Vpr of HIV-2/SIV_{SM} family in general is more active in CV-1 cells than it is in human HeLa cells.

Even more strikingly, although neither SIV_{AGM} nor SIV_{SYK} Vpr caused any detectable G_2 arrest in human cells, all of them were able to cause a significant G_2 arrest in African green monkey CV-1 cells (Fig. 2A). In fact, the level of arrest of CV-1 cells by the Vpr of SIV_{AGM} was nearly as great as that of HIV-1 Vpr (Fig. 2A). Both the passaged and the directly

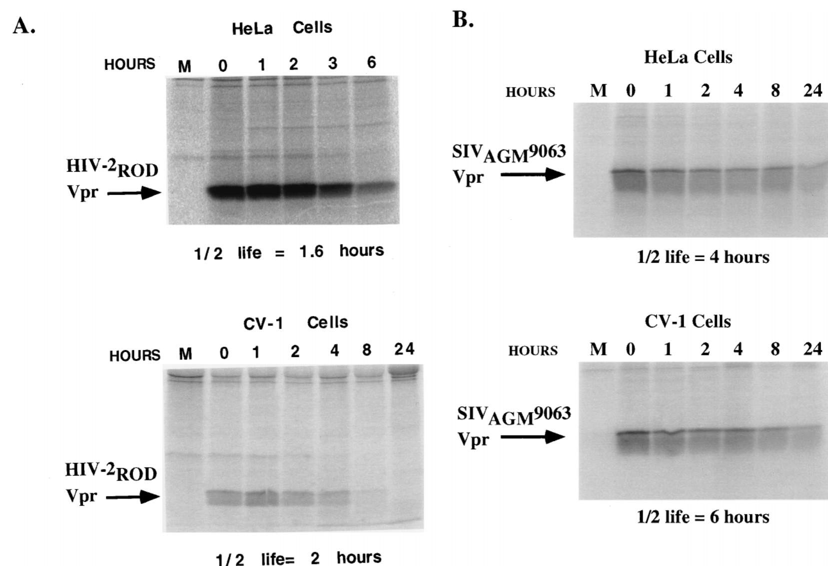

FIG. 4. Subtype and simian specificity of Vpr function is not related to protein stability. A total of 500,000 human HeLa cells or simian CV-1 cells were transfected with the indicated Vpr, expressed from the HIV-1 LTR; 48 h later, the transfected cells were pulse-labeled with [^{35}S]methionine-cycteine for 1 h and chased for indicated intervals. Cell-associated proteins were immunoprecipitated and analyzed by autoradiography after SDS-PAGE. Expression levels were quantitated by PhosphorImager analysis of the entire band width. (A) HIV-2_{Rod} Vpr; (B) SIV_{AGM} Vpr. Lanes M, size markers.

cloned isolates caused G_2 arrest in CV-1 cells (Fig. 2A). These results indicate that SIV_{AGM} and SIV_{SYK} cause cell cycle arrest in a species-specific manner.

We next wished to verify that the Vpr proteins were functional in another simian cell type. We therefore performed the above-described infections in macaque mammary tumor (CMMT) cells. All isolates were able to induce a G_2 arrest in the macaque cells (Fig. 3B), although the effects were less striking in CMMT cells than in the other cell lines, perhaps because CMMT cells grow more slowly than either HeLa or CV-1 cells. Therefore, the species specificity of SIV Vpr is not restricted to CV-1 cells.

Protein stability or cellular localization does not explain differential Vpr activity in cells from different primate species. To determine if the species specificity of HIV-2/SIV Vpr function was due to differences in protein stability, we examined the half-lives of various Vpr proteins in human and simian cells by pulse-chase analysis (Fig. 4 and Table 2). Human HeLa cells or African green monkey CV-1 cells were transfected with the gene of interest, labeled with [^{35}S]Met-Cys, and chased for designated intervals. Consistent with previous results (26), we found that HIV-1 Vpr is highly stable (>20 h) relative to HIV-2_{ROD} Vpr (1.6 h) (Table 2). However, HIV-2_{Rod} Vpr had about the same half-life in HeLa cells as it did in CV-1 cells (Fig. 4A and Table 2). This suggests that the differences in efficiency of Vpr-mediated G_2 arrest in the two cell types are not due to differential protein stability in one cell versus another. Moreover, HIV-2_{7312A} Vpr, which is as efficient as HIV-1 Vpr in inducing G_2 arrest, also has a short half-life of only 2.5 h (Table 2), and SIV_{SM}Pbj Vpr, which is less active than HIV-2_{7312A} Vpr has a longer half-life (4.5 h; Table 2). This suggests that protein levels are not the rate-limiting factor in the ability of Vpr to mediate G_2 arrest.

In order to determine if the reason that SIV_{AGM} Vpr did not cause G_2 arrest in human cells was due to protein instability, we also examined the half-life of SIV_{AGM}ver9063 in HeLa and CV-1 cells. However, we found that SIV_{AGM}ver9063 Vpr protein shows only a modest increase in stability in African green monkey cells relative to HeLa cells (from 6 to 4 h; Table 2 and Fig. 4B). Thus, it appears unlikely that differential protein stability plays a role in the species specificity of the SIV_{AGM} Vpr G_2 arrest function.

HIV-1 Vpr localizes to the nucleus of infected cells (27), and mutant analysis has suggested that nuclear localization is important for the ability of Vpr to cause cell cycle arrest (6). To determine if differential localization of Vpr contributed to the cell type-specific effects of SIV Vpr proteins, their intracellular localization in CV-1 and HeLa cells was visualized by indirect immunofluorescence. These studies showed that SIV_{SM} Vpr localizes to the nucleus and is excluded from nucleoli in both CV-1 and HeLa cells (Fig. 5A and B). Likewise, the localization of SIV_{AGM} Vpr is identical in the simian and human cells (Fig. 5C and D). In both cases, SIV_{AGM} Vpr gave a nuclear punctuate staining pattern that differed from that of SIV_{SM} Vpr. Nevertheless, we could detect no differences in this staining pattern between CV-1 cells and HeLa cells that could explain why the protein was functional in the former cell type but not the latter. Therefore, it is unlikely that attenuation or absence of the ability of Vpr to cause G_2 arrest in human cells is due to incorrect localization of the protein.

TABLE 2. Vpr stability in human and simian cells[a]

Construct	Stability (h)	
	HeLa	CV-1
HIV-1_{LAI} Vpr	>20	>20
HIV-2_{ROD} Vpr	1.6	2
HIV-2_{7312A} Vpr	2.5	ND[b]
SIV_{SM}PBj1.9 Vpr	4.5	ND
SIV_{AGM}ver9063 Vpr	4	6

[a] Vpr stability was determined by pulse-chase analysis. A total of 500,000 human HeLa or African green monkey CV-1 cells were transfected with the indicated construct, expressed from the HIV-1 LTR. The cells were labeled with [^{35}S]Met-Cys and chased for 24 h. Vpr was immunoprecipitated at various time points and analyzed by autoradiography after SDS-PAGE. Expression levels were quantitated by PhosphorImager analysis.

[b] ND, not done.

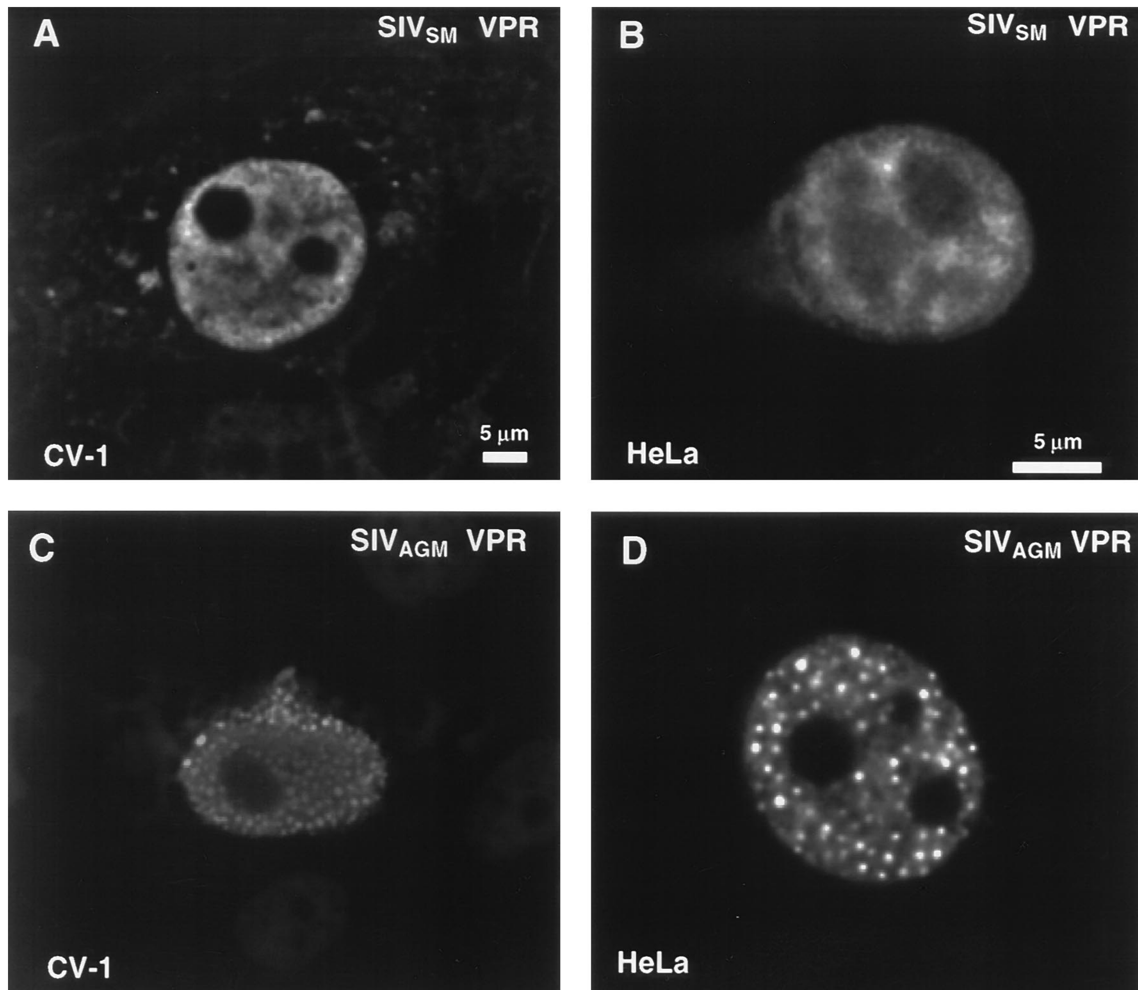

FIG. 5. Localization of SIV_{SM} Vpr and SIV_{AGM} Vpr in simian and human cells. (A and B) HA epitope-tagged SIV_{SM} Vpr from clone PBJ 1.9 probed by using anti-HA and visualized by indirect immunofluorescence; (C and D) HA epitope-tagged SIV_{AGM} Vpr probed with anti-HA. The scale bar is the same for all panels. Optical sections show representative examples of the nuclei of transfected cells. One section from each cell, approximately in the middle of each nucleus, is presented.

DISCUSSION

In this study, we examined the ability of Vpr from a broad range of primate lentiviruses to cause both human and primate cells to accumulate in the G_2 phase of the cell cycle. The isolates examined included members of four of the five major lineages of primate lentiviruses and included both primary and passaged viruses as well as pathogenic (in macaques) and nonpathogenic strains. We found that although Vpr from HIV-1, HIV-2, and SIV_{SM} was able to mediate cell cycle arrest in human cells, SIV_{SM} Vpr was attenuated relative to HIV-1 Vpr. Also, Vpr from primary strains of HIV-2, but not the Vpr from a tissue culture-passaged strain of HIV-2, was as efficient as HIV-1 Vpr in the ability to cause G_2 arrest in human cells. On the other hand, none of the *vpr* genes from several passaged or unpassaged SIV_{AGM} isolates or from SIV_{SYK} could cause cell cycle arrest in human cells. Surprisingly, all *vpr* alleles tested regardless of origin were able to cause efficient cell cycle arrest in African green monkey cells. We found no differences in protein localization and/or stability between human and simian cells for SIV_{AGM} and SIV_{SM} Vpr; therefore, the species specificity of Vpr function is more likely dictated by species-specific cellular substrates.

The best evidence to date for the biological importance of Vpr-mediated G_2 arrest is its conservation among highly divergent primate lentiviral lineages. The *vpr* open reading frame has diverged quite extensively in SIV_{AGM} and SIV_{SYK}. Yet despite this extensive diversity, all SIV Vpr proteins retain the ability to arrest cells in their respective host species. This finding argues strongly for a host-specific selective process in Vpr-mediated G_2 arrest as well as an essential role at some stage of virus replication or transmission.

Although likely important for some step in the viral life cycle, the G_2 arrest function does not appear to affect viral pathogenicity directly. For example, although SIV_{AGM} is most active in cells from African green monkeys, there is no evidence that SIV_{AGM} causes clinical disease or otherwise negatively affects the survival of these animals (for a review see reference 4). Indeed, recent field studies have failed to identify differences in body weight, reproductive success, or mortality between infected and uninfected animals (24, 32). Thus, the fact that SIV_{AGM} Vpr causes a G_2 arrest best in its natural host argues that G_2 arrest of infected target cells by itself is not sufficient for viral pathogenicity. Moreover, deletion of *vpr* in SIV_{MAC239} infection of rhesus monkeys did not abolish patho-

genicity (15). On the other hand, these data suggest that the G_2 arrest function of Vpr is involved in the adaptation of the virus to its host.

The G_2 arrest function of Vpr may also play an important role in viral transmission from one species to another. For example, Fig. 2 shows that only SIV_{SM} Vpr can arrest human cells, albeit with somewhat lower efficiency compared to HIV-1 Vpr and some HIV-2 Vpr proteins. SIV_{AGM} and SIV_{SYK} Vprs, on the other hand, are completely nonfunctional in human cells. To date, no viruses resembling SIV_{AGM} or SIV_{SYK} have been identified in humans, while there is increasing evidence that SIV_{SM} has been transmitted from naturally infected sooty mangabeys to humans on multiple independent occasions (3, 13, 14). This is despite the fact that at least SIV_{AGM}-infected African green monkeys constitute an excellent reservoir: African green monkeys are numerous, geographically widely dispersed, infected at high levels, and frequently hunted for food or kept as pets (see reference 40 for a review). They have also been infected with SIV_{AGM} for long periods of time, possibly even before their speciation and migration into different parts of Africa (22). Finally, African green monkeys have served as the source of viruses detected in feral baboons and patas monkeys (2, 23). The lack of SIV_{AGM}-like viruses in humans thus suggests that transmission does not occur, presumably because of the inability of SIV_{AGM} to establish a productive infection in the human host. Given the data in Fig. 2 and 3, it is intriguing to speculate that the G_2 arrest function of Vpr constitutes one of the determinants that govern cross-species transmission. Studies are under way to further evaluate this hypothesis.

Finally, our results suggest some criteria for testing potential targets of Vpr involved in G_2 arrest. That is, if the mechanism by which all of the Vpr proteins cause G_2 arrest is conserved, then one might expect that the crucial binding partner of HIV-1 Vpr in human cells would also bind to HIV-2 Vpr proteins (but less well to SIV_{SM} Vpr) and would not bind to SIV_{AGM} Vpr. On the other hand, the African green monkey homolog of this protein would be expected to bind to Vpr from all of the primate lentiviruses.

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