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A domain of human immunodeficiency virus type 1 Vpr containing repeated H(S/F)RIG amino acid motifs causes cell growth arrest and structural defects

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ABSTRACT **Vpr is a virion-associated protein of human immunodeficiency type 1 (HIV-1) whose function in acquired immunodeficiency syndrome (AIDS) has been uncertain. Employing the yeast *Saccharomyces cerevisiae* as a model to examine the effects of HIV-1 auxiliary proteins on basic cellular functions, we found that the *vpr* gene caused cell growth arrest and structural defects indicated by osmotic sensitivity and gross cell enlargement. Production of various domains by gene expression showed that this effect arose from within the carboxyl-terminal third of the Vpr protein and implicated the sequence HFRIGCRHSRIG, containing two H(S/F)RIG motifs. Electroporation with a series of peptides containing these motifs caused structural defects in yeast that resulted in osmotic sensitivity. A protein with functions relating to the yeast cytoskeleton, Sac1p [Cleves, A. E., Novick, P. J. & Bankaitis, V. A. (1989) *J. Cell Biol.* 109, 2939–2950], shows sequence similarity to Vpr, and Vpr's effect in yeast may be to disrupt normal Sac1p functions. The Sac1p equivalent has not yet been described in mammalian cells, but in rhabdomyosarcoma and osteosarcoma cell lines Vpr also caused gross cell enlargement and replication arrest [Levy, D. N., Fernandes, L. S., Williams, W. V. & Weiner, D. B. (1993) *Cell* 72, 541–550]. We note that there is a correlation between the region containing the H(S/F)RIG motifs and the pathogenicity of primate lentiviruses and we suggest that the function of Vpr may be to bring about cell growth arrest and/or cytoskeletal changes as an early step in HIV-1 infection.**

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), is a complex retrovirus, like other primate lentiviruses, having genes—*tat, rev, vif, vpr, vpu*, and *nef*—that are not found in simple retroviruses. While the functions of *tat* and *rev* are fairly well understood, the remainder are often referred to as auxiliary genes because they are not essential for *in vitro* infectivity of the virus and have poorly understood roles in pathogenesis.

HIV-1 viral protein R, Vpr (1), is unique among the auxiliary gene products in that it is a virion-associated protein (2, 3). Vpr has also been reported to be a weak transcriptional activator (4, 5) and to bind to the HIV-1 Gag protein (6–8). Although Vpr is not essential for virus replication in established cell lines (5, 9), there is evidence that it may have a critical function for viral replication in primary macrophages (10–12). Because of its virion association it has been suggested that Vpr has an early role in HIV-1 infection, possibly in penetration or uncoating of the virus (2, 3, 13).

Vpr is one of the most highly conserved proteins of HIV-1 and exists as Vpr and/or Vpx in all primate lentiviruses (ref. 14; see Fig. 6*A*). Vpx is similarly virion-associated (2, 3, 13, 15, 16). HIV-2 Vpr is essential for productive infection of human macrophages (17) but, like HIV-1 Vpr, is dispensable for replication in established cell lines (9). Similarly, HIV-2 Vpx is dispensable in established cell lines (15, 18, 19) but is required for infection in fresh macrophages (18, 20) and augments viral infectivity in peripheral blood lymphocytes (21). Perhaps the most convincing evidence for an essential role for Vpr in pathogenesis is the observation that there is a drive *in vivo* for retention of an intact *vpr* reading frame and that mutations in *vpr* lead to a low virus burden in rhesus monkeys (22).

In this study we have utilized the yeast *Saccharomyces cerevisiae* as a model eukaryote to look for basic effects of HIV-1 auxiliary proteins on cell function. We find that of these proteins Vpr is unique in causing profound effects on cell growth and structure. Our findings are discussed in the context of AIDS pathogenesis.

MATERIALS AND METHODS

Molecular Cloning. The HIV-1 genomic clone pNL4-3 (23) was the source of HIV-1 genes that were amplified by polymerase chain reaction (PCR). The cloning of *vpu* and *nef* has been described (24, 25). The cloning of *vpr* is described in Fig. 1, and a similar approach was used to clone PCR-amplified *vif*. Similar cloning of *vpr* into pYEULCGT (26) was designed to direct the copper-inducible production of Vpr fused to glutathione *S*-transferase (GST).

Yeast Growth. Yeast strain DY150 (*MATa ura3-52 leu2-3,112 trp1-1 ade2-1 his3-11 can1-100*) was grown in YEPD medium (1% yeast extract/2% peptone/2% glucose). Yeast were transformed by electroporation (27) and transformants were grown in SD medium [0.67% yeast nitrogen base (Difco)/2% glucose] containing histidine (20 μg/ml), adenine (20 μg/ml), and tryptophan (20 μg/ml) and solidified, when required, with 3% Phytagar (GIBCO). Expression was induced by the addition of $CuSO_4$ as indicated. Cells were examined for osmotic sensitivity by plating onto YEPD and YEPD containing 1.2 M KCl (28). All viable cells, including osmosensitive cells, grew on YEPD, but those that were osmosensitive did not grow on high-osmotic-strength medium.

Sequence Analysis. Genbank (release 82.0) was employed for initial database searches using ALIGN. Subsequent alignments were performed manually.

Flow Cytometry Analysis. Cells were analyzed and sorted with a Coulter EPICS Elite flow cytometer. Illumination was with a 488-nm argon ion laser, and forward angle light scatter (related to cell size) and side scatter were recorded. Cells were sorted on the basis of forward angle light scatter. Live cells

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Abbreviations: GST, glutathione *S*-transferase; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus.

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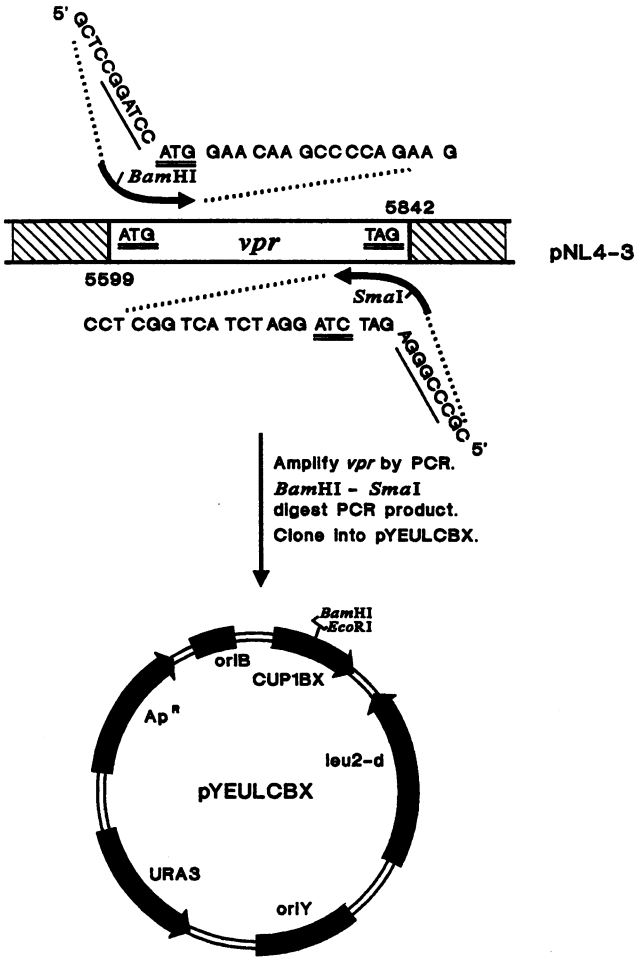

FIG. 1. Cloning of *vpr* for expression in yeast. *vpr* was amplified from the HIV-1 genomic clone pNL4-3 (23) by PCR using the primers shown. The PCR product was cleaved with *Bam*HI plus *Sma*I and cloned into the yeast–*Escherichia coli* shuttle vector pYEULCBX (24) (digested with *Eco*RI, made blunt-ended with phage T4 DNA polymerase, and then digested with *Bam*HI) to produce pYEULCBX.Vpr.

were gated by propidium iodide exclusion, indicated by absence of fluorescence emission at >600 nm following staining with propidium iodide (2 μg/ml).

Peptide synthesis. The peptides (see Fig. 5) were synthesized on an Applied Biosystems 430A peptide synthesizer using the FastMoc solid-phase technique. Protection was as follows: α-amino groups by base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) groups; arginine side chains by 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc); serine and threonine by *tert*-butyl; asparagine, glutamine, histidine, and cysteine by trityl; and glutamic acid by O-*tert*-butyl. Couplings were achieved by using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate activation of amino acids and *N*-methylpyrrolidone as solvent. The peptides were cleaved from the resin with trifluoroacetic acid (plus phenol, ethanedithiol, thioanisole, and water as scavengers).

Electroporation of peptides. Peptides at 2 mg/ml were dialyzed against electroporation buffer (1.5 mM Na_2HPO_4/0.5 mM KH_2PO_4/0.27 M sucrose, pH 7) before electroporation into yeast cells (29). Electroporation conditions in the Baekon 2000 advanced macromolecule transfer system (San Francisco) were as follows: 2^{11} pulses, 8 kV, 0.8-sec burst time, 100-μsec pulse time, 10 cycles, 1-mm gap between solution and upper electrode. The cuvettes contained 3 μl of a late-logarithmic-phase yeast culture, 27 μl of fresh YEPD growth medium, 5 μl of Dulbecco's phosphate-buffered saline, and 5 μl of peptide solution. It was found necessary to achieve a kill of 60–80% in order to achieve uniform penetration of the surviving cells.

RESULTS

Vpr Causes Growth Arrest in Yeast. As part of a general examination of the effects of HIV-1 auxiliary proteins on simple cellular functions, the genes for these proteins were expressed in yeast, a model eukaryote which has been very useful for determining the functions of many proteins. Expression was achieved by cloning *vpr* and the genes of other HIV-1 auxiliary proteins into the expression vector pYEULCBX to produce pYEULCBX.Vpr (Fig. 1), pYEULCBX.Vpu (24), pYEULCBX.Nef27 (25), and pYEULCBX.Vif (this study). Yeast transformed with the vectors were then copper-induced on plates to express the cloned gene and growth was assayed. Profound affects on cell growth were caused by the Vpr protein, whereas the other HIV-1 proteins tested had no effect on vegetative cell growth. Low levels (0.25 mM) of $CuSO_4$ caused total growth arrest to cells expressing *vpr* (Fig. 2), but no adverse effects were caused by the other proteins even with induction levels as high as 1 mM $CuSO_4$ (data not shown). The effect of *vpr* was unrelated to copper toxicity, since with no added $CuSO_4$ [where basal expression from the *CUP1* promoter was 5% of the induced level (reviewed in ref. 30)] *vpr* transformants grew at a slower growth rate than control transformants (data not shown).

The Vpr toxicity was found to be due to growth arrest, not killing, since induced cells (even after 24 hr in the presence of the inducer) formed colonies when plated on medium with no added copper. The DY150(pYEULCBX.Vpr) transformant colonies grown up from the assay were considerably smaller than DY150(pYEULCBX) transformant colonies. These "small" colonies grew like the parental DY150(pYEULCBX.Vpr) transformant upon subsequent culture without added copper (data not shown), indicative of a cell cycle arrest after induction of Vpr synthesis, followed by eventual recovery and return to the normal cell cycle.

Arrested Cells Are Greatly Enlarged. Examination of cells by light microscopy indicated that induced cells producing Vpr had a grossly altered morphology. Vpr-producing cells had a diameter of 16 μm (Fig. 3*B*), more than twice the diameter of the DY150(pYEULCBX) transformants (Fig. 3*A*) grown under the same conditions. It appeared that most of the intracellular space in the large cells was devoid of structure and occupied by a single large organelle or vacuole. The proportion of altered cells was assessed by flow cytometry (Fig. 3*C*), which confirmed that in the cell population Vpr transformants exhibited a greater degree of forward light scattering, indicative of their

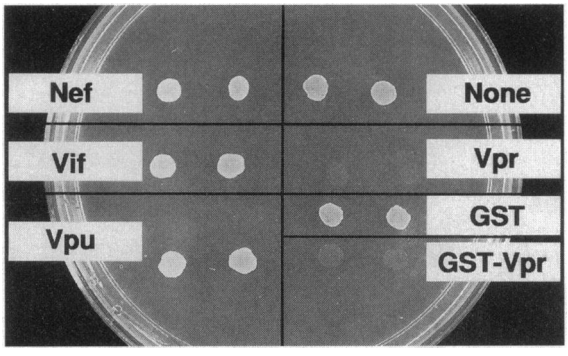

FIG. 2. Expression of HIV-1 auxiliary proteins in yeast. Transformants were suspended in sterile water, aliquoted onto plates, and incubated at 28°C for 3 days. The plates shown are SD medium containing 0.25 mM $CuSO_4$ with histidine, adenine, and tryptophan (each at 20 μg/ml) and solidified with 3% Phytagar (GIBCO). The proteins produced by the transformants are indicated.

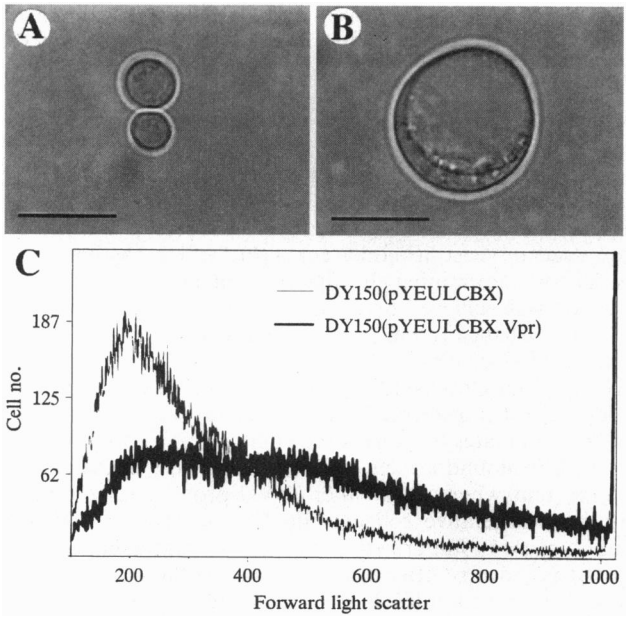

FIG. 3. Morphological changes in yeast cells expressing *vpr*. (*A* and *B*) Copper-induced yeast cells were examined by light microscopy. Shown are DY150(pYEULCBX) control transformant (*A*) and a typical large DY150(pYEULCBX.Vpr) transformant (*B*). (Bars = 10 μm.) (*C*) Induced yeast cells were analyzed by flow cytometry forward angle light scatter (proportional to cell size). Populations containing over 50,000 cells are shown for DY150(pYEULCBX) (thin line) and DY150(pYEULCBX.Vpr) (thick line).

larger size. This suggests that the DY150(pYEULCBX.Vpr) transformants were arrested in growth before cell division.

Location of Sequences Causing Growth Arrest. The sequences responsible for causing the growth arrest were identified by testing various portions of the Vpr protein, particularly as GST fusion proteins, for effects on cell growth since Vpr fused to GST also caused growth arrest (Fig. 2). Deletion of the last 33 aa of Vpr, encoded by an *Eco*RI fragment (constructs VprBE and GST-VprBE; Fig. 4), relieved the growth arrest, whereas the addition of this portion of Vpr to GST (construct GST-VprEE; Fig. 4) caused a growth arrest, suggesting that this domain was responsible for the growth arrest. A partial growth arrest was also seen with the addition of just the last 21 aa of Vpr to GST (construct GST-VprSE; Fig. 4). In each case the growth arrest correlated with cell enlargement, as judged by flow cytometry and light microscopy. This carboxyl-terminal sequence is the region lacking in many laboratory HIV-1 isolates that encode a truncated *vpr* gene product of 73 aa due to a T nucleotide insertion (3, 4, 31). The Vpr in these isolates does not associate with virions (4), presumably because of the truncation. Our findings in this report confirm the importance of the same carboxyl-terminal region, but for another reason.

The region of HIV-1 Vpr that causes cell growth arrest has been compared with known Vpr relatives, the closest relative being the simian immunodeficiency virus (SIV) Vpr followed by HIV-2 Vpr and then Vpx proteins (see Fig. 6*A*). The sequence is composed of 33% arginine, a much higher arginine content than that found in comparable portions of Vpx proteins. A repeated motif, H(S/F)RIG, is conserved in Vpr species. The motif is present at aa 71–75 (encoded in the *Eco*RI–*Sal* I fragment) and at aa 78–82 (encoded in the *Sal* I–*Eco*RI fragment). The greater toxicity caused by the fragment encoding two copies of the H(S/F)RIG motif suggests that the sequence containing the two motifs may be responsible for the growth arrest.

H(S/F)RIG Motifs in Synthetic Peptides Cause Osmosensitivity. We further investigated the function of the region containing H(S/F)RIG motifs by using the synthetic peptides 1–4 described under *Materials and Methods*. These peptides contain the penultimate 14, 21, and 26 aa of Vpr, and aa 71–82 of Vpr. The H(S/F)RIG motif is present at zero, one, two, and two copies, respectively, within these peptides. These peptides were electroporated into yeast cells which were then analyzed for osmosensitivity: growth arrest was not an appropriate assay due to inefficiency of electroporation and the transient presence of the peptide within the cell. Cells electroporated with no peptide or with peptide 1, lacking a H(S/F)RIG motif, were unperturbed. However, peptides 2–4, which contained H(S/F)RIG motifs, caused osmotic sensitivity such that significant proportions of cells were killed on high-osmotic-strength medium (Fig. 5). With peptides 2 and 3, the effects were commensurate with the number of copies of H(S/F)RIG motif present, indicating a direct role for this sequence. Although peptide 4, which comprised the two motifs separated by CR, caused only 20% of cells to be osmotically sensitive, this result clearly reveals that this sequence is important to the osmosensitivity. The lesser effect of the smallest peptide could have been due to rapid turnover or a conformation different from what exists when the remainder of the carboxyl-terminal sequence is also present. Further studies with altered peptides may indicate amino acids that are critical for this effect.

Sac1p Has Sequences Similar to Vpr. In a search for a cellular relative to Vpr we found that a yeast protein, Sac1p (32), has the most significant sequence similarity of cellular proteins listed in the GenBank database (release 82.0). In the alignment of Sac1p and Vpr (Fig. 6*B*) it can be seen that Sac1p has 60% identity in the region of the H(S/F)RIG motifs including the terminal glycines, the part of the motif that is totally conserved in Vpx as well. Over the entire alignment

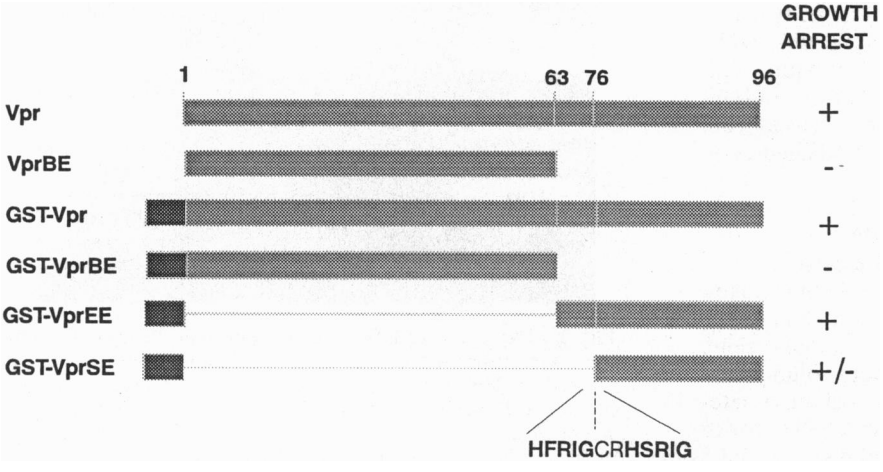

FIG. 4. Delineation of the region in Vpr causing growth arrest. The region of Vpr produced by the construct is represented by light stippling; dark stippling represents GST (not drawn to scale). The approximate positions of *Bam*HI, *Eco*RI, and *Sal*I sites relative to the protein sequence are indicated at amino acid positions 1, 63, and 76, respectively. H(S/F)RIG, shown in bold, is encoded by sequences on either side of the *Sal* I site. Growth of the transformed yeast cells producing these proteins is indicated in the right column.

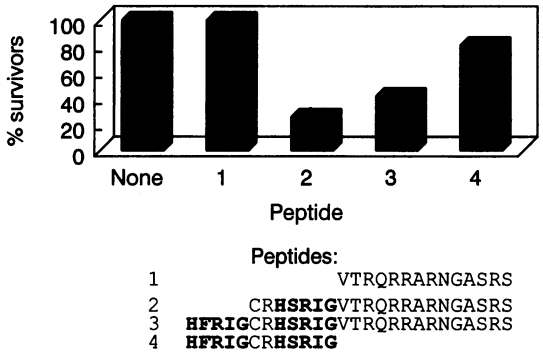

FIG. 5. Osmotic sensitivity of yeast electroporated with Vpr peptides. Colony forming units were determined after induced cells or electroporated cells were plated onto YEPD and YEPD containing high salt. Osmosensitivity was calculated by comparison of colony forming units on the two media. Peptide sequences are shown.

with Vpr there are 32% identical and 45% similar amino acids. The precise function of Sac1p in assembly of the actin cytoskeleton is unresolved; however, *sac1* mutants display profound cytoskeletal defects and growth arrest at low temperature (32, 35, 36).

Motifs in Vpr Correlate to AIDS Pathogenicity. The region of the Vpr protein containing H(S/F)RIG motifs may be correlated to the pathogenicity of HIV and SIV. A brief compilation of sequences of Vpr and Vpx from HIV and SIV is shown in Fig. 7. There is almost total conservation of the 12 aa containing two repeated H(S/F)RIG motifs in HIV-1. In HIV-2 isolates there are between two and five changes from the reference sequence. Most SIV Vpr sequences also show high conservation of the sequence containing the H(S/F)RIG motifs. However, the mandrill virus (SIVmnd) and the Sykes' monkey virus (SIVsykes) show poor sequence conservation, with eight and nine sequence changes, respectively, and are reported to be asymptomatic (37, 38). African green monkey SIV (SIVagm) does not have Vpr and those monkeys remain healthy (39). If the absence of Vpr, and specifically the sequences containing the H(S/F)RIG motif, results in decreased pathogenicity, then Vpr is a very important target for development of anti-HIV drugs.

DISCUSSION

In this study we have shown that the Vpr protein of HIV-1 causes growth arrest and structural defects in yeast. Through a genetic approach and the use of peptides electroporated into yeast we have shown that this effect can be attributed to the sequence HFRIGCRHSRIG within Vpr. Vpr is similar in sequence to yeast Sac1p, and although the precise functions of Sac1p in relation to the yeast cytoskeleton have still to be elucidated, the production of Vpr may disrupt Sac1p functions and lead to cytoskeletal defects, including gross cell size and ultimate growth arrest. Indeed, in the many studies of yeast with cytoskeletal defects, mother cells are abnormally large and daughter cells are abnormally small (see, for example, ref. 40). Time-lapse analysis of newly induced cells producing Vpr shows the same phenomenon (data not shown). Osmosensitivity also indicates that cytoskeletal defects are induced by Vpr. This was demonstrated with synthetic Vpr peptides; however, we also found that in a population of Vpr-producing cells half of the population was incapable of growth on high-osmotic-strength medium, indicative of structural defects in those cells (data not shown).

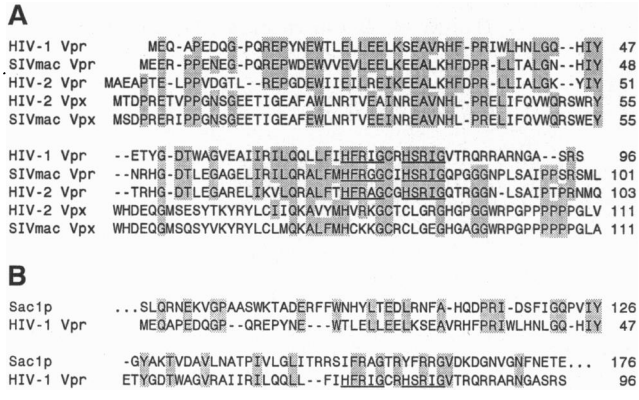

FIG. 6. Alignment of HIV-1 Vpr with Vpr relatives. (*A*) Vpr and Vpx proteins are aligned in their entirety. Sequences are derived from HIV-1 NL4-3 (23), HIV-2ROD (33), and SIVmac239 (34). Three or more identical amino acids are shaded. (*B*) The entire amino acid sequence of Vpr is aligned with aa 77–176 of Sac1p (32). Identical residues are shaded.

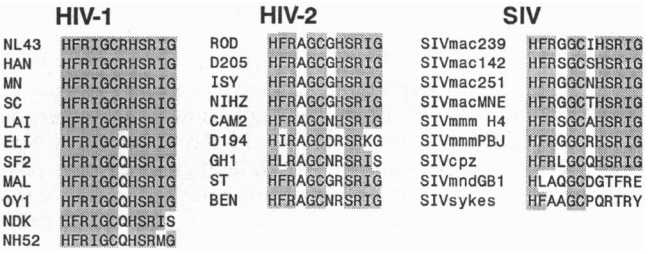

FIG. 7. Relationship of the region containing H(S/F)RIG motifs and pathogenicity. Sequences of the region containing H(S/F)RIG motifs from various HIV-1, HIV-2, and SIV isolates are aligned with the sequence HFRIGCRHSRIG found in pNL4-3. Identical residues are shaded. It should also be noted that the C between motifs is invariant. SIVsykes and SIVmndGB1 sequences show the greatest difference from the reference sequence and are not pathogenic.

A mammalian equivalent to Sac1p has not been reported, but there is evidence that Vpr also produces cytoskeletal defects in mammalian cells. Levy *et al.* (41) showed that *vpr* expressed in osteosarcoma and rhabdomyosarcoma cell lines caused cell replication arrest and gross cell enlargement, indicative of cytoskeletal disruption. Cytoskeletal changes have been observed in HIV-1-infected mammalian cells. In a $CD4^+$ T-lymphoblastoid cell line it was shown that HIV-1 caused ultrastructural changes, including membrane disruption, "ballooning," and vacuolization of the endoplasmic reticulum, during the first hour of infection (42). Also, when infected monocytes are adjacent to epithelial cells they undergo cytoskeletal changes and develop a pseudopod toward the epithelial cells from which HIV-1 buds exclusively (43). It will be of interest to reexamine these phenomena in *vpr* mutants.

What is the role of Vpr in the HIV-1 life cycle, and is induced growth arrest or cytoskeletal changes relevant to this? For some time there has been a dilemma regarding the distinction between HIV-1 and other retroviruses: retroviruses usually require cell proliferation for infection, whereas HIV-1 infects nonproliferating cells such as terminally differentiated macrophages. $CD4^+$ cell lines can be productively infected with HIV-1 when they are arrested in G_2 growth phase (44). Nonproliferation of host cells could therefore be an initial requirement for a productive infection of all or some cell types. The function of Vpr may be to bring about growth arrest so that a process such as genomic integration may occur. If this were so it would account for Vpr (and Vpx counterparts) being virion-associated so that early events can be initiated. Antibodies to Vpr appear in only 17% of AIDS patients but are found in 47% of asymptomatic individuals (1), suggesting that the Vpr is present early in infection and therefore it is probably essential only at that time. It also follows then that specific

inhibitors of Vpr should prevent infection or slow extracellular spread of the virus.

It is also possible that Vpr may affect only certain cell types. We have seen no peptide-induced cytoskeletal changes in $CD4^+$ lymphocyte cell lines (data not shown). However, as stated above there is evidence that in the presence of Vpr several cell types do undergo cytoskeletal changes. It is possible that Vpr may play an important role in enabling virus from infected donor cells to be transmitted to epithelial cells, for example.

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