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Residues within the HFRIGC Sequence of HIV-1 Vpr Involved in Growth Arrest Activities

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HIV-1 Vpr is a virion-associated protein that can cause growth arrest when produced inside the cell but when added externally it can cause cell death. Employing the yeast model system, the C-terminal domain, in particular the sequence HFRIGCRHSRIG ($Vpr_{71–82}$), is essential for both the growth arrest and cytocidal activities. Conservation of this sequence in HIV-2 and SIV suggests that these residues may be functionally important. Using site-directed mutagenesis we show that the most highly conserved aa residues, His71 and Gly75, were important for the cell cycle inhibitory effects. In contrast, we show that the wild-type $Vpr_{71–82}$ peptide and three variants of this peptide with Gly75 changed to Ser, Ala, and Ile all exhibited the same cytocidal activity suggesting that the intracellular and extracellular effects are unrelated.

Viral protein R (Vpr) is a 96-aa protein having the sequence

MEQAPEDQGPQREPYNEWTELLEELKSEAVRHFPRIWLHNLGQHIYE

TYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS.

Vpr is found in virions (1, 2) and in the serum of HIV-1 infected individuals (3). Vpr has been associated with a number of biological functions including nuclear import of the HIV-1 preintegration complex (4), transactivation of cellular genes (1), mitochondrial dysfunction (5, 6), cell structure disturbances (5, 7), induction of cell cycle arrest (8–14), extracellular killing (15) and apoptosis (5, 16) [for review see 17]. The conserved cell growth disturbances of Vpr were believed to contribute to HIV-1 pathogenesis by delaying cells at the point of the cell cycle where viral replication is maximized (18). Similarly the conserved cell killing of externally added Vpr may attribute to the large amount of cell death in AIDS, including cell death of uninfected cells (19).

Both the growth arrest and cytocidal activities are conserved by HIV-1, HIV-2 and most SIV isolates. In yeast both these activities are due to the C-terminal region of Vpr, in particular a repeated H(F/S)RIG aa sequence motif underlined above. Of Vpr and Vpx sequences in HIV-2 and SIV there is high conservation of the first H(F/S)RIG motif (Fig. 1), with most changes occurring in the fourth position of the first motif (8). Among the Vpr and Vpx sequences, the aa that are conserved in the HFRIGCRHSRIG motifs are the first, fifth and sixth residues (underlined), while the third position is always occupied by a basic aa (8). The high conservation of the histidine, glycine and cysteine residues suggests that they may be critical for Vpr's growth arrest and/or cell killing functions.

Using a yeast model system, this study determined the cytostatic and cytocidal activities of HIV-1 Vpr. We systematically investigated a range of Vpr mutants through aa substitutions within the first HFRIGC domain to determine their growth arresting activities. In addition, the cell killing activities of an externally-added $Vpr_{71–82}$ peptide, as well as the $Vpr_{71–82}$ peptide with substitutions at position 75 were examined.

Abbreviations used: aa, amino acid(s); AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus.

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HIV-1 Vpr    HFRIGCRHSRIG
SIVmac Vpr   HFRGGCIHSRIG
HIV-2 Vpr    HFRAGCGHSRIG
HIV-2 Vpx    HVRKGCTCLGRG
SIVmac Vpx   HCKKGCRCLGEG
SIVagm Vpx   HFRCGCRRRQPF
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FIG. 1. Alignment of H(F/S)RIG motifs in Vpr and Vpx of HIV-1, HIV-2, and SIV.

MATERIALS AND METHODS

Mutagenesis strategy and oligonucleotide synthesis. For mutagenesis we targeted highly conserved residues (underlined) of the first HFRIGC motif, substituting aa at positions 71, 72, 73, 75, and 76 with alanine, with the additional changes of F72S and G75S. All seven *vpr* substitutions were made with duplex oligonucleotides that were based on the sequence between unique *Eco*RI and *Sal*I restriction sites of *vpr* (shaded in Fig. 2). Oligonucleotides were synthesized using an Applied Biosystems 394 DNA/RNA synthesizer. Each oligonucleotide was purified by denaturing gel electrophoresis according to the method described by Sambrook *et al.* (20).

Molecular cloning. Using oligonucleotide-duplex replacement, the *Eco*RI + *Sal*I digested YEX-BX.Vpr expression plasmid (21) was ligated to duplexes of purified oligonucleotide and transformed into *E. coli* MC1061 cells {*ara*D139 (*ara*ABC-leu)7679 *galU galK lacX74 rpsL hsdR mcrB*} (20). Transformants were selected by growth on 2 × YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) + Ampicillin plates. Plasmids so generated were subsequently analyzed by DNA sequencing to confirm the mutations.

Yeast transformation. The yeast strain employed, *Saccharomyces cerevisiae* strain DY150 (*MATa ura3*-52 *leu2*-3,112 *trp1*-1 *ade2*-1 *his3*-11 *can1*-100), was transformed by lithium acetate transformation (22). Transformants producing Vpr were initially selected on minimal synthetic medium (0.67% yeast nitrogen base (Difco)/2% glucose/2% agar) containing all supplements required by the host strain except uracil. In subsequent culture leucine was omitted.

Growth arrest. For the examination of cells producing wild-type Vpr and other forms of Vpr, growth was observed on solid media with various levels of inducer. Freshly grown transformed cells were suspended at 10^7 cells/ml in H_2O and 20-μl aliquots were placed onto a series of minimal synthetic medium with varying copper ion concentrations. Growth was scored after 3–4 days 30°C incubation. The growth of each mutant in liquid medium containing 0.25 mM $CuSO_4$ was spectrophotometrically determined by measuring the OD_{600}.

TABLE 1

Growth of Yeast Vpr Transformants

Transforming plasmid	[$CuSO_4$] (mM)				
	0	0.25	0.5	0.75	1
pYEX-BX	++++	++++	+++	+++	+++
pYEX-BX.Vpr	++++	−	−	−	−
H71A	++++	++++	+++	+++	+++
F72A	++++	++	+	−	−
F72S	++++	±	±	−	−
R73A	++++	−	−	−	−
G75A	++++	+++	++	++	++
G75S	++++	++	+	+	−
C76A	++++	−	−	−	−

Cytocidal treatment. A wild-type Vpr peptide, Vpr $_{71–82}$, and three additional peptides with aa substitutions of Gly75 to alanine, serine and isoleucine respectively were synthesized by Auspep (Melbourne, Australia). All four peptides were purified by Auspep and verified by aa composition and mass spectrometry. Peptides were resuspended in water to 2 mg/ml. Serial ten-fold dilutions of each peptide were then added to ~10^3 yeast cells suspensions in a final volume of 100 μl water. After 1 h each mixture was plated out onto solidified YEPD medium and colonies were counted after incubation at 30°C for 2–3 days. Data from multiple plates were averaged and plotted on graphs. The concentration of peptide resulting in 50% survival was determined to be the 50% lethal dose (LD_{50}).

RESULTS

Effects of substitution on the growth arrest activity of Vpr. The pYEX-BX.Vpr is a yeast vector designed for the copper-inducible expression of Vpr. This vector was manipulated to cause changes to residues within the HFRIGC sequence to examine this effect on growth arrest. The unique *Sal*I and *Eco*RI restriction sites on either side of the sequence encoding HFRIGC were cut and then ligated with duplex mutagenic oligonucleotides. The effects of the changes were monitored as Vpr expression was induced. The resulting cell cycle inhibitory effects observed are summarized in Table 1.

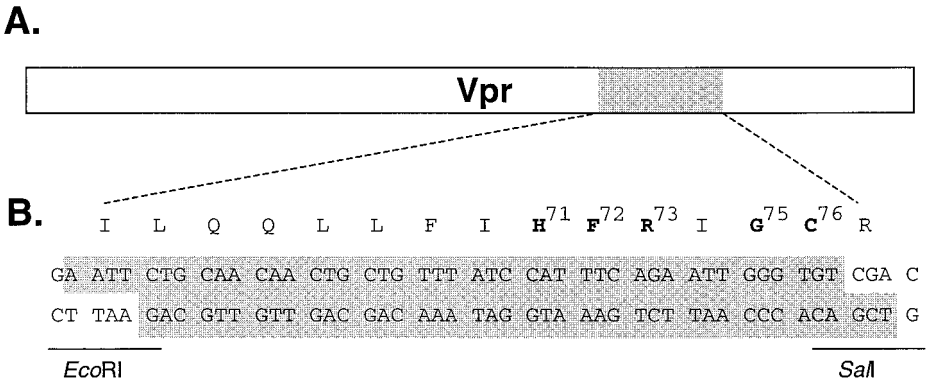

FIG. 2. Mutagenesis of Vpr. (A) Schematic diagram showing Vpr with the region encoded by the *Eco*RI-*Sal*I fragment shaded. (B) Nucleotide sequence of the *Eco*RI–*Sal*I fragment of *vpr* along with the encoded aa sequence. The shaded wild-type *vpr* duplex was replaced with duplex oligonucleotides encoding the desired substitutions. Substituted aa are shown in bold.

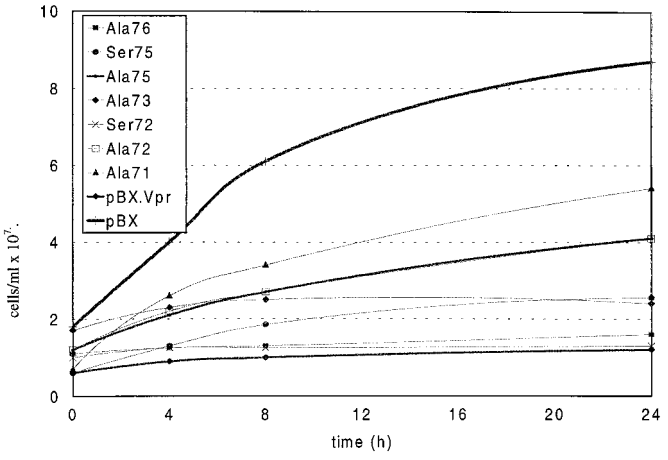

FIG. 3. Growth of yeast transformants producing variants with aa substitutions in the first conserved HFRIGC domain of Vpr.

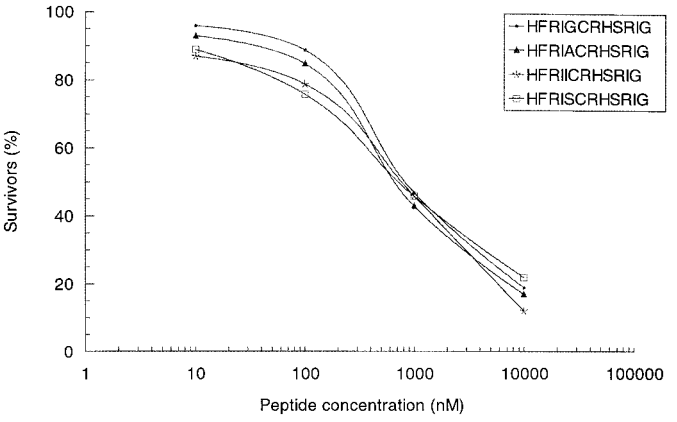

FIG. 4. Cytocidal effects of Vpr_{71-82} peptides and three variants at residue 75.

DY150 [pYEX-BX] transformants, producing no Vpr protein, grew on media with high levels of $CuSO_4$ (1 mM), while DY150 [pYEX-BX.Vpr] transformants producing Vpr grew normally except when induced with copper. Even the lowest level of added copper (0.25 mM $CuSO_4$) led to a total growth arrest as previously observed (8). Cells producing Vpr with specific substitutions in the HFRIGC motif displayed varying degrees of growth arrest over the inducing $CuSO_4$ range. Those constructs producing a mutant Vpr that could grow on media containing in excess of 0.25 mM $CuSO_4$, were H71A, G75A, G75S, and the F72A mutant. In comparison the F72S, R73A, and C76A mutants were growth arrested at 0.25 mM $CuSO_4$.

In liquid cultures growth patterns were similar to those observed on the solid media (Fig. 3). In the presence of 0.25 mM $CuSO_4$, the number of cells containing pYEX-BX alone increased by almost three times over the 24 h while those with pYEX-BX.Vpr showed no increase in growth over the same time period. Similarly, the cells expressing the Vpr substitutions H71A, G75A, G75S, and F72A increased in number over the time period by approximately two fold, whereas cells expressing Vpr with F72S, R73A, and C76A changes did not increase.

Effect of substitution on the cytotoxicity of Vpr peptide. A limited study was carried out to determine if cytocidal Vpr effects were related to the effects seen with Vpr expression. Therefore Vpr_{71-82}, and three peptides with substitutions at Gly75, were compared in dose response studies to determine the effects of substitutions on their toxicity. The dose at which the wild-type Vpr_{71-82} caused 50% killing (Fig. 4) was 800 nM, similar to the 750 nM previously reported for this peptide (15). Similar LD_{50} values of 600, 680, and 750 nM were observed for the glycine peptides substituted to alanine, serine and isoleucine respectively. These values are all within a 15% deviation of the mean value. Therefore they are not considered to be significantly different from each other.

DISCUSSION

Our previous studies in yeast showed that growth arrest and cell killing activities of Vpr were due to the C-terminus and in particular the HFRIGCRHSRIG sequence (8). This study further revealed that several aa residues of this sequence, H71, F72, and G75, are critical for the cell cycle arrest. The fact that the G75A mutation inactivated Vpr agrees with a report using a human cell culture (23). These studies and those of Chen *et al.* (24) support the view that yeast expression systems are relevant for Vpr mutational studies.

The recent NMR derived tertiary structure of the residues of Vpr (25) is used in Fig. 5 to illustrate the residues observed here to be required for growth arrest. As Gly75 is highly conserved in nearly all lentivirus members and with recent structural studies con-

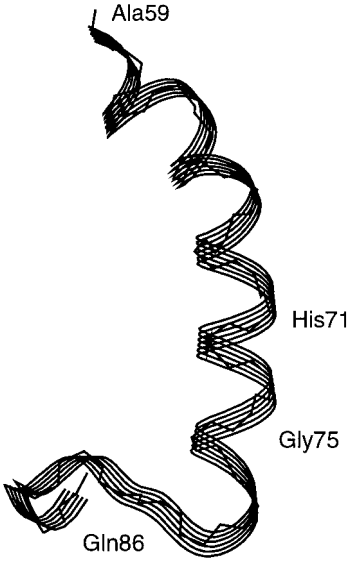

FIG. 5. Tertiary structure of Vpr from aa residue 59 to 86 (23).

firming that the first motif is helical and the second a reverse β turn followed by a less ordered region (25, 26), particularly between residues 74 and 78 (25), the glycine in this region is likely to be functionally important. Therefore the structural alteration here to the Gly75 β turn may explain the loss of Vpr function that is observed.

We changed this Gly75 that we changed to examine the cytocidal effect of externally added Vpr. As all three peptides with changes in this position displayed similar LD_{50} values as the wild-type Vpr peptide this glycine did not seem to be important for cell killing. Although additional cytocidal examination of other altered residues, particularly within the double motif is required, these results suggest that there are different mechanisms for external cytocidal effects and intracellular growth arrest activities of Vpr.

Other highly conserved residues found to be important for growth arrest were the His71 and to a lesser extent the Phe72, as indicated by the change in function when substituted with an alanine. In contrast the R73A mutant displayed an active Vpr cytostatic phenotype suggesting that this arginine may not be as important for growth arrest activity. As this Arg73 is not highly conserved and often is substituted in other lentivirus subfamilies, the R73A substitution may be tolerated. Similarly as the F72S substitution is found in nature, a wild-type phenotype might have been expected.

Residues 71 and 72 are at the C-terminal end of the α-helix, which extends from residues 53–74 (25). Using an eleven residue window, the mean hydrophobicity and α-hydrophobic moment (27) of the H71A mutant at 0.445 and 0.519, respectively, were calculated to be significantly higher than those of the position 71-73 mutants and the wild type (all were, respectively, 0.352 and 0.427). The effect of these increases would be to make the C-terminal end of Vpr in the H71A mutant more amphipathic. It would be worth while to study the effects of other amphipathicity-altering mutants in this segment.

These studies demonstrate that the highly conserved His71, Phe72 and Gly75 may be of future interest to Vpr structural, and more importantly rational anti-Vpr drug development studies. As well as examining the effectiveness of HIV inhibitor drugs in cell culture, these residues may be examined for changes due to the drug's action. These mutants may be useful in raising antibodies against Vpr as a means of immunologically suppressing Vpr's *in vivo* action. Therapies that target the inactivation of these residues of Vpr may alleviate growth arrest and thus possibly reduce HIV viral load.

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