HIV-1 protein Vpr causes gross mitochondrial dysfunction in the yeast *Saccharomyces cerevisiae*

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Abstract The biological effects of the HIV-1 accessory protein, Vpr, have been studied in yeast expression systems. In our previous study [1], employing the *pCUP1-vpr* copper-inducible expression cassette, Vpr was shown to cause growth arrest and structural defects. In this study yeast constitutively expressing *vpr*, through elevated copy number and/or elevated transcription levels, displayed no growth arrest in fermentative growth conditions while Vpr was produced at much lower levels than in the inducible expression system. However, such cells were respiratory deficient and unable to utilise ethanol or glycerol as the sole carbon source. They exhibited gross mitochondrial dysfunction displayed in the loss of respiratory chain complex I, II, III, IV and citrate synthase activities. The effects on mitochondria required a C-terminal domain of Vpr that contains a conserved amino acid sequence motif HFRIGCRHSRIG. These results suggest that the widely observed phenomenon of 'Vpr-induced growth arrest' in human cells could be due to mitochondrial dysfunction.

Key words: AIDS; Apoptosis; Growth arrest; HIV-1; Mitochondria; Respiration; Vpr

1. Introduction

The HIV-1 viral protein R (Vpr) is a virion-associated protein whose mechanism of action has not yet been elucidated. Vpr is thought to influence the nuclear localization of viral nucleic acids in non-dividing host cells [2]; however, two major effects on cell division and survival have not been adequately explained. One effect, cell killing, is seen when Vpr or Vpr-related peptides are added externally [3,4]. The second effect, and the main subject of this report, is a growth arrest when *vpr* is expressed in cells.

When *vpr* is transfected into normally dividing cells on an expression plasmid, *vpr* has the profound effect of blocking cell division. This has been observed in numerous human cells including rhabdomyosarcoma cells [5], osteosarcoma cells [5], lymphocytes [6–10], peripheral blood mononuclear cells [7] and HeLa cells [8,11,12]. The growth arrest would appear to result from an interaction with a basic cellular function because it can be readily observed in simple eukaryotes such as the budding yeast, *Saccharomyces cerevisiae* [1] and the fission yeast *Schizosaccharomyces pombe* [13].

Molecular deletion analysis has shown that the growth arrest in yeast is due to the C-terminal region of Vpr, in particular a repeated H(S/F)RIG amino acid sequence motif [1]. This region of Vpr is also incompatible with growth in HIV-1-infected cells since numerous HIV-1 clones produced in chronically infected, growing cells do not produce the C-terminal, growth inhibitory portion of Vpr [14,6].

This study addresses the mechanism of the growth arrest in a yeast system. Although a number of studies have concluded that Vpr induces an arrest in the G_2 phase of cell growth [7–13], our data provide evidence of an effect on mitochondria that would explain a subsequent G_2 growth arrest. The possible consequences of the above findings for AIDS pathogenesis are discussed.

2. Materials and methods

2.1. Yeast growth and strains

Two strains of the yeast *Saccharomyces cerevisiae* were employed for expression of *vpr*. Strain DY150 (*MATa ura3*-52 *leu2*-3,112 *trp1*-1 *ade2*-1 *his3*-11 *can1*-100) has been described previously and is suited for the copper-inducible expression of *vpr* when preceded by the *CUP1* promoter, *pCUP1*, as occurs in the plasmids utilised in this study [1]. The mutant strain PS145 (*MATa ura3*-1 *leu2*-3,112 *trp1*-1 *ade2*-1 *his3*-11,15 *can1*-100 *HSF1*::*LEU2* [p*hsf1-206*]) allows constitutive expression from *pCUP1* [15]. PS145 has a *HSF1* gene disruption that would be normally be lethal except that in PS145 it is complemented by plasmid p*hsf1-206*, a yeast shuttle plasmid with a *TRP1* marker. Thus, mutant strain PS145 overproduces a mutant HSF1, in the place of a wild-type HSF1. *pCUP1* has a weak HSF1 binding sequence that is bound strongly by the mutant HSF1 leading to increased constitutive transcription from *pCUP1* [15].

Media employed for the growth of yeast were the following: YEPD (1% yeast extract (Difco), 2% peptone, 2% dextrose); YEPE and YEPG were YEPD but with 2% ethanol or 3% glycerol substituted for dextrose; synthetic complete (0.67% yeast nitrogen base without amino acids (Difco), 2% dextrose, 20 μg/ml adenine, 20 μg/ml histidine, 20 μg/ml tryptophan, 20 μg/ml uracil and 20 μg/ml leucine); complete selective medium (synthetic complete lacking uracil and/or leucine) was used for the growth of transformants and was formulated to select for the plasmid in the transformants. Plasmid p*hsf1-206* required no selection since it was essential for viability in strain PS145, which contains a lethal disruption of the chromosomal *HSF1* gene. Media with added copper were solidified by the addition of Phytagar (GIBCO) while other media were solidified by the addition of agar (Difco).

2.2. Cloning of HIV-1 vpr for expression in yeast

The HIV-1 *vpr* gene was derived by DNA amplification using PCR from the molecular clone pNL4-3 and its sequence confirmed by nucleotide sequencing [1]. Cloning of *vpr* into the yeast expression plasmids, pYEULCBX and pYEULCGT, has been described previously [1]. (The vectors pYEULCBX and pYEULCGT are available commercially under the names pYEX-BX and pYEX-4T (AMRAD, Australia, and CLONTECH, USA)). They are designed for the copper-inducible production in yeast of native or glutathione-*S*-transferase (GST) fusion proteins, respectively. Thus, pYEULCBX.Vpr and pYEULCGT.Vpr direct the production of Vpr and GST-Vpr, respec-

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tively. VprBE constructs contain a *Bam*HI–*Eco*RI fragment of *vpr* that directs the synthesis of a truncated protein lacking the C-terminal third of Vpr.

Cloning into the yeast plasmid pYEL2 [16] is described in Fig. 1. The final construct utilised the *CUP1-vpr* expression unit employed in plasmid pYEULCBX.Vpr. pYEL2.Vpr is a vector that replicates to a very high level in absence of leucine. The pYEULCBX and pYEULCGT plasmids described above also have this capacity but pYEL2 plasmids exhibit a 2-fold higher copy number because of their smaller size [17]. Yeast transformants were obtained by lithium acetate transformation [18], with slight modifications. pYEL2 transformants were selected on complete selective medium plates containing all supplements required by the host except leucine. The pYEL2 transformation frequency was low because of selection for the *LEU2-d* marker. Other transformants were initially selected on complete selective medium plates minus uracil, and leucine was omitted in subsequent culturing.

2.3. Mitochondrial enzyme activities

Mitochondria were prepared from cells grown in 500 ml of synthetic complete medium. For induction 0.5 mM copper sulfate was added to cells in exponential growth phase and cultures were maintained for 16 h. Mitochondria were prepared from the cells by the method of Glick and Pons [19], and disrupted by two cycles of freezing and thawing. Samples for assay of respiratory chain complex I and citrate synthase were further disrupted by sonication using a Branson B-30 sonifier with microtip (30 pulses at 30% duty cycle). Respiratory chain complexes I (NADH-coenzyme Q_1 reductase), II (succinate-coenzyme Q_1 reductase), III (decylubiquinol-cytochrome *c* reductase), IV (cytochrome *c* oxidase), and citrate synthase were assayed as described by Rahman et al. [20]. Unlike mammalian cells and as reported previously [21], no rotenone-sensitive complex I activity was detected in *S. cerevisiae*, and the values reported for complex I activity are the total rates of NADH oxidation.

3. Results

3.1. Growth of Vpr transformants

Plasmids described in this report were designed for copper-inducible production of foreign proteins in a variety of laboratory strains of *Saccharomyces cerevisiae*. As shown in Table 1 the copper-induced production of Vpr or GST-Vpr in strain DY150, led to an arrest of cell growth, as observed previously [1].

The growth arrest with copper induction led us to expect that in a constitutive expression system there would be no survivors. As a means of testing the effects of the constitutive production of Vpr we transformed strain PS145, a yeast strain that allows constitutive expression from the *CUP1* promoter. Although *pCUP1* is normally used for copper-inducible expression in yeast, strain PS145 encodes a mutant HSF1 that has high-affinity binding to a *CUP1* HSE (heat shock element)

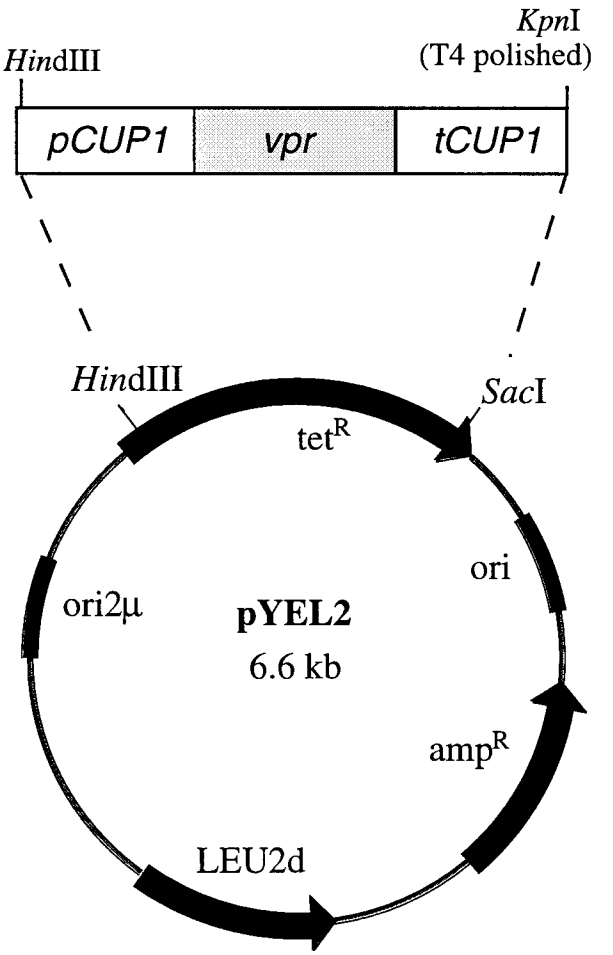

Fig. 1. Cloning of *vpr* expression cassette for higher levels of Vpr production in yeast. The *vpr* expression cassette (top) was obtained from pYEULCBX.Vpr [1] by digesting pYEULCBX.Vpr with *Kpn*I followed by treatment with T4 polymerase to render the DNA blunt-ended. After further digestion with *Hin*dIII, the *Hin*dIII–*Kpn*I (blunt-ended) fragment (*vpr* expression cassette) was isolated. It was ligated into pYEL2 that had been digested with *Sac*I followed by treatment with T4 polymerase to render the DNA blunt-ended, then followed by *Hin*dIII digestion. amp^r, ampicillin resistance gene; CUP1, copper metallothionein-encoding gene; LEU2-d, *LEU2* gene encoding isopropyl-malate dehydrogenase with a truncated promoter; *vpr*, HIV-1 gene encoding Vpr; ori, *E. coli* origin of DNA replication; ori2μ, yeast 2 μm plasmid origin of DNA replication; *p*, promoter; *t*, transcription terminator; tet^r, tetracycline resistance gene.

Table 1
Growth of yeast transformants

Transformant (Strain) [Plasmid]	Growth on (YEPD or complete synthetic)	(complete synthetic+copper)	(YEPG or YEPE)
DY150 [pYEULCBX]	+	+	+
DY150 [pYEULCBX.Vpr]	+	−	+
DY150 [pYEULCBX.VprBE]	+	+	+
PS145 [pYEULCBX]	+	+	+
PS145 [pYEULCBX.Vpr]	+	+	−
PS145 [pYEULCBX.Vpr] following plasmid shedding	+	+	+
DY150 [pYEL2]	+	+	+
DY150 [pYEL2.Vpr]	+	−	−

Freshly isolated yeast transformants were suspended in water and aliquots were dropped onto plates. Growth (+) or lack of growth (−) was scored after 3 days. Identical results were obtained when pYEULCGT (directs the production of a GST fusion protein), was used in place of pYEULCBX (directs the production of a native protein).

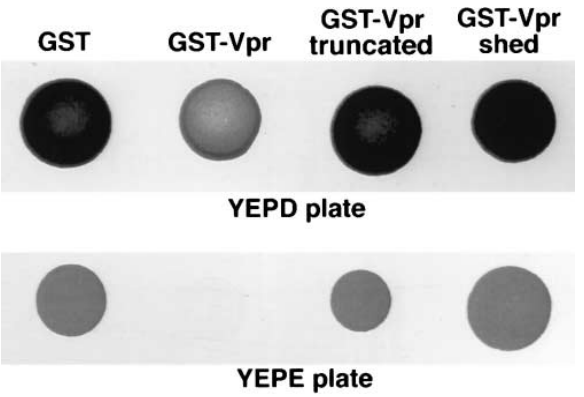

Fig. 2. Respiratory deficiency associated with Vpr. The growth of PS145 yeast transformants on YEPD (fermentable carbon source) and YEPE (non-fermentable carbon source) plates are indicated. The transformants, PS145 [pYEULCGT], PS145 [pYEULCGT.Vpr], PS145 [pYEULCGT.VprBE], and PS145 [pYEULCGT.Vpr] after plasmid shedding, are indicated by the proteins they produce.

and directs transcription from the *CUP1* promoter [15]. pYEULCBX, pYEULCBX.Vpr, pYEULCGT and pYEULCGT.Vpr were transformed into PS145 and URA+ transformants were selected. Transformants were picked onto a master plate of synthetic complete media lacking uracil and following growth they were replica plated to a variety of plates. Growth was normal when glucose was present but when glycerol or ethanol was present as the sole carbon source there was no growth (Table 1 and Fig. 2). This suggests that *vpr* causes a respiratory deficiency. The *vpr*-containing plasmid was shown to cause the respiratory deficiency when it was shed from PS145 [pYEULCGT.Vpr] following growth on non-selective media. Colonies that shed plasmid pYEULCGT.Vpr regained wild-type respiratory function as judged by their growth on non-fermentable carbon sources (Table 1 and Fig. 2).

The respiratory deficiency can be attributed to sequences in the C-terminal third of Vpr since constructs in which this region of Vpr was removed (e.g. pYEULCBX.VprBE and pYEULCGT.VprBE) exhibit normal respiratory growth like pYEULCBX and pYEULCGT transformants (Table 1). This region of Vpr is also involved in the growth arrest and extracellular activities [1,3,4]

In case the respiratory defect could be an anomalous complication resulting from the mutant HSF1 background *vpr* expression was re-examined in a strain wild-type for the HSF. Thus, *vpr* was recloned into pYEL2, a very high copy number plasmid, to direct high constitutive levels of Vpr production in a wild-type HSF1 background (see Fig. 1). Of 48 pYEL2 and 48 pYEL2.Vpr transformants that were sampled at random, all of the pYEL2 transformants were respiratory competent, while none of the pYEL2.Vpr transformants could grow on YEPE or YEPG. This provides a further evidence that Vpr causes a respiratory defect.

3.2. Production of Vpr with a GST fusion

Due to its severe inhibitory effects on cell growth only minuscule amounts of Vpr have previously been produced in eukaryotic cell lines. Our work indicates that cells designed to produce Vpr, although respiratory deficient, can be grown in the presence of glucose and absence of copper. To ascertain that Vpr was being produced, we obtained yeast cell lysates of cells transformed with pYEULCGT.Vpr. First, amounts obtained with copper-induced expression, where the cells become arrested were compared. Fig. 3 shows a comparison of the levels of GST (lanes 2), with GST-Vpr (lane 4) and GST-Vpr truncated (lane 3) lacking the C-terminal portion of Vpr [1]. It is obvious that levels of GST and GST fusions produced during the two hours of copper induction are comparable despite the fact that the DY150 [pYEULCGT.Vpr] cells become growth arrested. Second, the levels of GST-Vpr in PS145 [pYEULCGT.Vpr] were considerably lower (lane 5) with constitutive expression. It appears that such lower levels of GST-Vpr are directly related to Vpr, since levels of GST (lane 6) are more like induced levels of GST and GST fusion proteins seen in lanes 2–4.

In summary it appears that for the eukaryotic production of Vpr, yeast production presents attractive options with either constitutive or inducible systems being demonstrated here as viable alternatives. Considering the growth advantages of respiratory competent strains, however, inducible systems may be preferred to avoid Vpr mutations.

3.3. Transient respiratory deficiency

Although the above-mentioned phenomena are clear, reproducible and well supported by controls, we note that if the master plate is stored for some time and then re-replica plated, colonies that were previously respiratory deficient regrow on the plates with a non-fermentable carbon source. This happens in the absence of any changes to the *vpr* sequence, indicating that the loss of repiratory function is transient and may indicate that Vpr targets a step in mitochondrial function whose loss can be compensated for by another factor. This phenomenon must serve as a warning in the examination and interpretation of data pertaining to the effects of Vpr on cells, including mammalian cells. It is also wise to examine a number of transformants on an individual basis and to examine them without delay as compensatory changes, including deletion of *vpr* or sequences encoding the C-terminal region of Vpr may be employed by the cell to overcome the

Table 2
Mitochondrial enzyme activities in yeast transformants

Transformant (Strain)	[Plasmid]	Inducer	Mitochondrial enzyme activity (Complex I)	(Complex II)	(Complex III)	(Complex IV)	(Citrate synthase)
DY150	[pYEULCBX]	–	655	7.6	7.45	20.9	139
DY150	[pYEULCBX.Vpr]	–	372	5.8	3.2	13.9	62
DY150	[pYEULCBX]	+ Cu^{2+}	325	4.9	2.9	8.1	56
DY150	[pYEULCBX.Vpr]	+ Cu^{2+}	33	2.3	0.9	0.7	7

Yeast transformants were grown in minimal selective medium overnight. Induction (+ Cu^{2+}) was performed overnight with 0.5 mM $CuSO_4$. Units for enzyme activity are nmol/min/mg protein, except for complexes III and IV, which are expressed as apparent first-order rate constants (min^{-1} mg^{-1}).

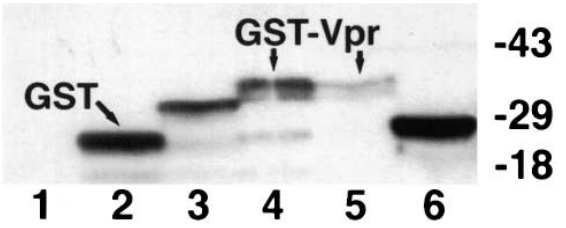

Fig. 3. Expression of GST-Vpr in yeast. DY150 and PS145 transformants were grown in complete selective medium. DY150 transformants were induced for 2 h by the addition of 0.5 mM $CuSO_4$ to the culture media. Cell lysates were prepared by vortexing yeast cells with 0.5 mm glass beads for a total of 2 min. Lysates were then mixed with Laemmli loading buffer and fractionated by SDS-PAGE gels (6–20% gradient gels Novex). The proteins were then transferred to nitrocellulose membranes and blotted with polyclonal antibody to GST. GST binding was detected by enhanced chemiluminescence (Amersham). The position of the pre-stained markers is shown. Lanes contain lysates from the following yeast transformants: 1. DY150 [pYEULCBX]; 2. DY150 [pYEULCGT]; 3. DY150 [pYEULCGT.VprBE]; 4. DY150 [pYEULCGT.Vpr]; 5. PS145 [pYEULCGT.Vpr]; 6. PS145 [pYEULCGT].

toxic effects of Vpr. This has been observed on numerous occasions in the examination of human cells producing Vpr [6,14].

3.4. Multiple mitochondrial defects

To obtain further information on the mitochondrial defect caused by Vpr we especially examined copper-induced cells because of their more reproducible phenotype. Copper addition to the control strain DY150 [pYEULCBX] caused a modest diminution of all mitochondrial enzyme activities measured, with residual activities of respiratory chain complexes I to IV and citrate synthase ranging from 40% to 65% of the 'no copper' sample (Table 2). Basal levels of Vpr expression in uninduced DY150 [pYEULCBX.Vpr] cells also appeared to cause a modest decrease in all mitochondrial enzymes with residual activities ranging from 40% to 75% of the control strain. In a comparison of the induced DY150 [pYEULCBX.Vpr] strain with uninduced DY150 [pYEULCBX] strain, activities of complexes I, III, IV and citrate synthase were decreased by 90–95% and complex II activity was decreased by 70%. This confirms a simultaneous effect on numerous mitochondrial enzyme activities.

4. Discussion

The results presented here show that the production of HIV-1 Vpr in yeast causes a respiratory deficiency due to effects on five or more mitochondrial enzymes. This deficiency causes little interference with yeast growth on fermentable carbon sources such as glucose, but causes a complete block to growth on non-fermentable carbon sources such as glycerol or ethanol where mitochondrial respiratory function is required. Mitochondrial respiratory function has been traditionally studied in yeast systems for almost five decades now so it is appropriate that yeast should provide this new unique insight into Vpr function. The observation of multiple mitochondrial enzymic defects suggests that Vpr may target a major control element such as a mitochondrial import receptor. In this respect it is interesting to note that Vpr contains two potential amphipathic helices. We can speculate that either of these sequences might compete with mitochondrial precursor proteins that utilise amphipathic helical leader sequences for their import into the mitochondrion. Second, it is of interest that there is a precedent for temporary respiratory deficiency of the type observed here. A genetic knockout of the Tom20, one component of the outer mitochondrial membrane import receptor, results in a respiratory deficiency that is only temporary [22]. Further work will be directed toward determining the precise nature of the mitochondrial interaction. Previously, we did not observe the Vpr-related loss of respiratory function because the basal levels were too low to see a significant effect, and with induction we obtained a total growth arrest. The total growth arrest could be due to very high Vpr levels and this could be further complicated by the presence of copper.

It is highly relevant that during HIV-1 infection mitochondrial dysfunction can be observed in lymphocytes and other cells [23–31]. Our results suggest that Vpr could be the cause of such an effect. However, our results also suggest that the effects of Vpr on mitochondrial function could be dependent on a number of variable parameters. The effects could be transient as observed in yeast. In addition the effects would depend on the Vpr levels produced and on whether the Vpr contains the C-terminal domain containing the bioactive HFRIGCRHSRIG amino acid sequence. In our studies with human cells we have determined that the carbocyanine dye, $DiSC_3(5)$ (Molecular Probes), measures a rapid increase in mitochondrial membrane potential ($\Delta\psi m$) preceding total collapse of the potential and apoptosis, following the external addition of Vpr [4]. This result is consistent with a primary effect on mitochondria, as $\Delta\psi m$ disruption and subsequent nuclear apoptosis are strictly correlated [32]. It has been proposed that mitochondrial disruption leads to the swelling, opening of the mitochondrial membrane transition pore and release of a cell suicide protein which causes apoptosis [33].

In summary, while previous studies have reported a Vpr-related cell growth arrest, it is likely that this effect could be a consequence of an initial effect on mitochondrial function. The commonly reported G_2 arrest may be an epiphenomenon, with mitochondrial respiratory function loss leading to apoptosis being the cause of cell cycle arrest, as fragmented DNA would prevent the cells from proceeding to mitosis [34,35]. The finding that Vpr causes mitochondrial dysfunction may aid the development of new strategies to overcome AIDS.

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