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Oxidative stress induced by HIV-1 F34IVpr in *Schizosaccharomyces pombe* is one of its multiple functions

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) exerts multiple effects on viral and host cellular activities during infection, including induction of cell cycle G_2 arrest and cell death in both human and the fission yeast *Schizosaccharomyces pombe* cells. In this study, a mutant derivative of Vpr (F34IVpr), which causes transitent G_2 arrest with little or no effect of cell killing, was used to study the molecular impact of Vpr on cellular oxidative stress responses in *S. pombe*. We demonstrated here that F34IVpr triggers low level of complex and atypical oxidative stress responses in comparison with its parental strain SP223 in early (14-h) and late (35-h) log phase cultures. Specifically, F34IVpr production in *S. pombe* causes significantly elevated levels of reactive oxygen species such as superoxide and peroxides; meanwhile, it also induces decreased levels of glutathione, hydroxyl radical concentrations and specific enzyme activities such as those of antioxidant enzymes including superoxide dismutases, catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and glutathione transferase. These observations may provide functional insights into the significance of Vpr-induced oxidative stress as part of the multifaceted functions of Vpr, and contribute to the development of future new strategies aimed to reduce the adverse Vpr-mediated effects in HIV-infected patients.

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Introduction

Human immunodeficiency virus type-1 (HIV-1) viral protein R (Vpr) is a small, 96 amino acid (14 kDa) protein which is conserved among HIV-1, HIV-2 and simian immunodeficiency virus, suggesting an important role in the viral cell cycle and pathogenesis. Four of the nine genes encoded by HIV-1, *nef*, *vif*, *vpu* and *vpr*, have been named "accessory genes" because deletion of these genes are dispensable for viral replication *in vitro*. Increasing evidence, however, suggest that Vpr plays a pivotal role in viral pathogenesis and disease progression in HIV-infected patients (Goh et al., 1998; Zhao et al., 2002; Somasundaran et al., 2002). Thus it is imperative to understand the molecular actions of Vpr and its effects on cellular functions. The multiple effects of Vpr in human and yeast cells include the nuclear import of the preintegration complex, transactivation of the HIV-1 long terminal repeat, induction of cell cycle arrest in the G_2 phase (also known as the "cdc phenotype" in *S. pombe*) followed by apoptosis of infected cells (Andersen and Planelles, 2005; Andersen et al., 2008; Emerman and Malim, 1998; Vodicka et al., 1998; Zhao and Elder, 2000). Vpr-induced cell cycle G_2 arrest, cell death and apoptosis are also believed contributing to suppression of human immune functions and depletion of $CD4^+$ lymphocytes in HIV-infected patients (for a recent review of this subject, see Li et al., 2009).

To study the effects of Vpr on these highly conserved cellular processes, the genetically well-characterized unicellular eukaryotes such as fission yeast *Schizosaccharomyces pombe* and budding yeast *Saccharomyces cerevisiae* have been widely used. Indeed, Vpr induces cell cycle G_2 arrest, cell death and apoptosis in *S. pombe* (Zhao et al., 1996; Zhao et al., 1998a; Huard et al., 2008). Interestingly, Vpr-induced G_2 arrest and cell death in *S. pombe* can be partly suppressed by H_2O_2-induced adaptive stress, suggesting that Vpr activates an oxidative state of the cells (Antal and Pesti, 2007). Indeed, both wild-type Vpr and F34IVpr trigger the production of reactive oxygen species (ROS), indicating that the cell cycle arrest and apoptotic-like cell death might be mediated by ROS (Huard et al., 2008; Stromajer-Rácz et al., 2007).

Abbreviations: HIV-1 Vpr, human immunodeficiency virus type-1 viral protein R; F34IVpr, mutant Vpr; CAT, catalase; DHR 123, dihydrorhodamine 123; GR, glutathione reductase; SOD, superoxide dismutase; G6PD, glucose-6-phosphate dehydrogenase; GST, glutathione-S-transferase; GPx, glutathione peroxidase; ET, ethidium bromide; nmt1 promoter, no-message thiamine promoter type 1; PBN, N-*tert*-butyl-α-phenylnitrone; ROOH, hydroperoxides; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. pombe*, *Schizosaccharomyces pombe*.

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The mutant F34IVpr, in which phenylalanine at the a.a. residue 34 is replaced with isoleucine, provides a useful tool to study the Vpr effect on oxidiative stress because, unlike the wild-type Vpr, it retains its ability to induce G_2 arrest but partly lost its capability to kill cells in *S. pombe* (Benko et al., 2004, 2007; Elder et al., 2001; Gu et al., 1997; Huard et al., 2008; Iordansky et al., 2004; Zhao et al., 2002). In addition, the F34I*vpr* gene can be expressed on a multicopy plasmids or at the physiologically relevant level as a single copy gene of F34I*vpr*, which was integrated into the *S. pombe* chromosome to form the F34IVpr-carrying strain RE076 (Elder et al., 2001). Interestingly, the activities of Vpr and F34IVpr such as Vpr-induced cell cycle G_2 arrest can be suppressed by elevated production of heat shock proteins (HSPs), e.g., Hsp 16 and Hsp 70 (Benko et al., 2004, 2007; Iordansky et al., 2004). This suppression was further confirmed by suppression of the wild-type Vpr-induced G2 arrest by HSPs in *S. pombe* and human cells (Benko et al., 2004, 2007; Iordansky et al., 2004). Similarily, another small HSP also suppresses F34IVpr-induced cytoskeletal defects in *S. cerevisiae* (Gu et al., 1997). Mechanistically, heat shock-induced elevation or overproduction of the chaperone protein Hsp 16 inhibited HIV-1 Vpr through direct protein–protein interaction (Benko et al., 2004). Since HSP productions can be induced by exposure of cells to thermal and oxidative stresses (Chen et al., 2003; Toone and Jones, 2004), it is possible that Vpr may trigger HSP production through a molecular mechanism involving induction of cellular oxidative stress (Stromajer-Rácz et al., 2007). Therefore, goal of this study was to delineate molecular mechanism of F34IVpr in inducing cellular oxidative stresses.

Induction of oxidative stress has been detected in HIV-infected patients, including (i) depletion of antioxidants, glutathione (GSH) and cysteine in the plasma of HIV-infected patients, or in the peripheral blood T cells and thioredoxin of the lymph of HIV-infected patients; (ii) increased production of ROS in Jurkat, H9 T cells and peripheral blood monocytes; and (iii) changes in mitochondrial transmembrane potential leading to the release of cytochrome *c* and other caspase-activating factors (reviewed by Perl and Banki, 2000). Data published by Aukrust et al. (2005) showed that HIV-infected patients, and particularly those with advanced disease, had increased levels of 8-oxo G (a marker of oxidative DNA damage) in the $CD4^+$ T cells. It is conceivable that the Vpr-induced oxidative stress in the plasma or in various human cell types under different conditions may affect certain physiological processes via signal transduction and/or transcriptional regulators leading to apoptosis and cell death (Chen et al., 2003; Mattson et al., 2005; Toone and Jones, 2004).

A variety of programmed stress processes exist in a single cell organism such as yeast that are involved in the cellular responses to external and/or environmental stimuli. For example, age of the cell cultures could influence the specific activities of antioxidant enzymes. In a late exponential phase culture of *S. pombe*, two or three times higher specific activities of catalase (CAT), glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GR) could be detected than in an early exponential phase culture (Lee et al., 1995), which may in fact contribute to the observed and relative oxidative stress resistance of those resting cells. Low levels of oxidative stress-inducing agents such as redox active metal ions [e.g., Cd(II), Cr(VI) and Cu(II)], or H_2O_2, menadione, lipid peroxides [e.g., *tert*-butil hydroperoxide (*t*-BOOH)] added externally to a cell culture could also trigger an adaptive response, resulting in transient resistance to a higher level of the same stress stimulus. Such an adaptation could also result in increased resistance to other unrelated stresses, i.e., 'cross-protection,' which could be the consequence of overlapping stimulation of stress-regulated signal transduction (Lee et al., 1995; Moradas-Ferreira and Costa, 2000; Toone and Jones, 2004). Moreover, single gene mutations may give rise to various levels of 'internal stress' by influencing the redox state of the cells. For example, in a chromate-tolerant mutant strain *chr1-66T* of *S. pombe*, this single gene mutation led to decreased GSH concentration and specific activity of GR, and simultaneously induced increased peroxide and superoxide ($O_2^{\bullet -}$) concentrations and specific activities of G6PD and manganese superoxide dismutase (Mn-SOD) as consequences of the down-regulation of GR. It turns out that cells carrying this gene mutation are sensitive to H_2O_2, menadione and *t*-BOOH, but it does not influence the generation time or the mating ability of the cells (Czakó-Vér et al., 1999; Gazdag et al., 2003; Koósz et al., 2008).

Our aim in this study was to take advantage of the single gene F34I mutation in Vpr by focusing on the transient effect of F34IVpr on cell cycle G_2 arrest and studying the molecular mechanism underlying the effect of Vpr and F34IVpr on cellular oxidative stress.

Materials and methods

Yeast strains and culture conditions

The F34I*vpr*-carrying *S. pombe* strain RE076 (h^-, *leu1*-32 *ura4*-294::*vpr*(F34I)::*ura4*+ *ade6*-216) has been decribed previously (Elder et al., 2001). Specifically, an F34I*vpr* gene-carrying plasmid, which is under the control of an inducible *nmt1* (*n*o-*m*essage *t*hiamine) promoter (Maundrell, 1990), was integrated as a single copy gene at the *ura4* gene locus in the *S. pombe* chromosome of a wild-type *S. pombe* strain SP223 (h^-, *leu1*-32 *ura4*-294 *ade6*-216) (Lundgren et al., 1991). In this strain, the F34I*vpr* gene expression can be readily achieved by depletion of thiamine from the minimal growth media for about 14 h (Zhao et al., 1996). However, the *nmt1* promoter is leaky, i.e., a low level of F34I*vpr* gene expression can also be observed even high level of thiamine (20 μM) is added to the growth media (Zhao et al., 1998b). Therefore, to avoid potential interference of low level F34IVpr with interpretation of the Vpr effect on oxidative stress, all experiments were carried out by comparison of the effect RE076 under gene-inducing conditions with its parental strain SP223 under the same experimental conditions. To grow these fission yeast cells, minimal medium EMM (Moreno et al., 1991) with 200 mg/l and 75 mg/l of the required amino acids and base, respectively, was used for culturing with or without thiamine (final concentration 20 μM) to achieve nearly full (98%) gene repression or expression, respectively (Maundrell, 1990). Strains were cultured in a 3.3-Hz incubator shaker at 30 °C. Other conditions and the microscopic examination of cells were as described earlier by Antal and Pesti (2007).

Septation index analysis and determination of living cell number

The cells were stained with calcofluor white M2R to enhance the visualization of the septa. After staining, the number of cells passing mitosis was estimated in triplicate by counting the proportion of septated cells in random fields of approximately 500 cells (Zhao et al., 1996). The number of living cells was assessed by using propidium iodide *via* the method described by Lee et al. (1995).

Measurements of reactive oxygen species, specific enzyme activities and other analytical procedures

Cell-free extracts were prepared by using X-pressing (Dybecksgatan 10, S-412 70 Göteborg, Sweden). The protein content of the cell-free extract was measured by a Lowry method with modification (Peterson, 1983). Specifically, to estimate the intracellular H_2O_2 and $O_2^{\bullet -}$ levels, the indicators dihydrorhodamine 123 (DHR 123) and dihydroethidium (10 μM) were used, respectively (Carter et al., 1994; Henderson and Chappell, 1993). The extents of formation of rhodamine and ethidium bromide (ET) were measured and quantified by spectrofluorimetry and flow cytometry (Carter et al., 1994; Henderson and Chappell, 1993).

The *in vitro* formation of hydroxyl radicals ($^{\bullet}OH$) and the reduction of chromate anion [Cr(VI)] to Cr(V) in *S. pombe* were monitored by

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