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Influence of glutathione availability on cell damage induced by human immunodeficiency virus type 1 viral protein R

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ABSTRACT

The human immunodeficiency virus type 1 (HIV-1) encodes for accessory viral protein R (Vpr), which arrests the cell cycle of host cells at G2 and causes mitochondrial dysfunction and alterations in glycolysis. High-level expression of Vpr protein correlates with increased viral production and disease progression. Vpr causes structural and functional injury in many types of eukaryotic cells, whether or not they are permissive for viral replication; among them is the budding yeast *Saccharomyces cerevisiae*. We hypothesized that the dramatic Vpr-induced injuries in yeast could be prevented by strengthening their redox response capacity. We show that exogenous addition of glutathione (GSH) or its prodrug, *N*-acetylcysteine (NAC), protected budding yeasts from Vpr-induced cytopathic effects. Moreover, addition of adenosine triphosphate (ATP) to growing cultures of Vpr-producing yeast returned cellular growth to control levels, whereas the addition dehydroascorbic acid (DHA) had only a minor protective effect. The diminished protein levels of Cox2p and Cox4p in wild typeVpr-producing yeasts together with the acute sensitivity of petite yeasts to Vpr activity may have been caused by low intracellular ATP levels. As a consequence of this energy deficit, eukaryotic cells would be unable to synthetize adequate supplies of GSH or to signal the mitochondrial retrograde response. Our findings strongly suggest that the cytopathogenic effect of Vpr protein in eukaryotic cells can be prevented by increasing intracellular antioxidant stores or, alternatively, supplying external ATP. Furthermore, these results support a potentially promising future for *S. cerevisiae* expression as a modality to search for Vpr-targeted inhibitors.

1. Introduction

Viral protein R (Vpr) is a 14 kDa accessory protein of human immunodeficiency virus type 1 (HIV-1) encoded by *vpr* late gene (Cohen et al., 1990). Vpr protein can be found either inside or outside of host cells, localizing in the nucleus, cytoplasm and mitochondria, packed into virions or free in cerebrospinal fluids and plasma (Lu et al., 1993; Jacotot et al., 2000 Levy et al., 1994). Although dispensable in some *ex vivo* infections, Vpr protein increases viral replication of T cells, is essential for HIV-1 infection of macrophages and determines disease progression (Dedera et al., 1989; Connor et al., 1995; Goh et al., 1998; Mologni et al., 2006).

Newly synthesized Vpr causes pleiotropic effects in eukaryotic cells. Accordingly, Vpr prevents proliferation of infected cells by arresting them in G2 of the cell cycle, increases viral expression, provokes cell death of lymphoid target cells and induces a mitochondria-dependent apoptotic pathway (Goh et al., 1998; Muthumani et al., 2002; Rogel et al., 1995). Extracellular and *de novo* synthesized Vpr protein has been used in a number of cellular models. Among them, the budding yeast *Saccharomyces cerevisiae* was demonstrated to be sensitive to Vpr activity (Zhao and Elder, 2000). The fission yeast *Schizosaccharomyces pombe* reproduces Vpr-induced effects such as cell cycle G2 arrest, changes in cell shape and cell death (Zhao et al., 1998). Similarly, cell growth arrest, size alterations, transient respiratory deficiency and cell death by endogenously expressed or extracellular protein were Vpr-induced effects reported in *S. cerevisiae* (Macreadie et al., 1995, 1996, 1997; Jacotot et al., 2000).

Vpr protein is an apoptogenic protein that induces mitochondrial membrane permeabilization *via* interaction with the adenine

Abbreviations: CFU, colony forming unit; COX, cytochrome c oxidase; DHA, dehydroascorbic acid; ETC, electron transport chain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; GSSG, glutathione disulfide; HIV-1, human immunodeficiency virus type 1; NAC, *N*-acetylcysteine; PGK, phosphoglycerate kinase; V-ATPase, vacuolar ATPase; Vpr, viral protein R; YNB, yeast nitrogen base.

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nucleotide translocator (ANT) in the inner mitochondrial membrane (Jacotot et al., 2001; Vieira et al., 2000). Studies using exogenous Vpr in microglial cells showed a reduction in the cellular levels of GSH and ATP and suggested an oxidative role for the extracellular form of Vpr protein, which is detected in serum and cerebrospinal fluid of HIV-infected patients (Ferrucci et al., 2012). Moreover, the oxidative status of host cells modulates the disease progress and the Vpr-induced death in the *S. pombe* cellular model (Antal and Pesti, 2006; Perl and Banki, 2000). While it is generally accepted that virus production increases in the presence of Vpr protein, the significance for Vpr-induced cytopathicity, in the context of the viral infection, is still debated (Ferrucci et al., 2013; Guenzel et al., 2014). Nevertheless, HIV-1-infected individuals display multiple symptoms of redox imbalance and Vpr is one of the viral proteins that can be involved in these phenomena (Antal and Pesti, 2006; Baruchel and Wainberg, 1992; Porter and Sutliff, 2012; Stromajer-Racz et al., 2010). Thus, HIV-1 infected cells endure elevations in reactive oxygen species (ROS), including superoxide and hydrogen peroxide, and show a decrease in their detoxification mechanisms. Reduction of total antioxidant capacity entails a significant decrease of GSH level in host cells (Nakamura et al., 2002) and recent studies have associated HIV-1 infection with GSH levels (Nguyen et al., 2014; Pang and Panee, 2014; Bhaskar et al., 2015). Notably, drugs that replenish intracellular GSH also counteract oxidative stress and inhibit HIV replication in models of acute and latent infection (Staal et al., 1992).

In this study, we show that exogenous addition of ATP or GSH protected growing yeast from Vpr-induced damage. The positive response of petite mutants to these treatments pointed to an impairment of an energetic pathway by Vpr. Moreover, the suppression of cell growth and resulting cell death induced by Vpr protein correlated with a downregulation of protein expression of the Cox4p subunit of cytochrome c oxidase. Therefore, we propose that Vpr expression leads to an ATP deficit in yeast that impairs the mitochondria retrograde response, a mechanism that would be expected to remedy the mitochondrial ATP deficit and consequent reduction in GSH stores. This *Saccharomyces* heterologous expression system might be a useful tool in searching for inhibitors targeted to Vpr protein.

2. Materials and methods

2.1. Plasmid construction and microbial strains

Escherichia coli DH5α strain (Sambrook and Rusell, 2001) was used for the construction of the *E.coli*-yeast shuttle vector. The *vpr* gene sequence from the NL4-3 clone of HIV-1 (Adachi et al., 1986) was cloned into the *Sma*I and *Bam*HI sites of pEMBLyex, a high copy number yeast expression vector carrying a galactose inducible and glucose repressible promoter (Cesareni and Murray, 1987). Exogenous gene expression is controlled by the *CYCGAL1* promoter, which is tightly repressed by glucose and strongly induced by galactose. Two clones of *S. cerevisiae* W303-1B strain (Thomas and Rothstein, 1989) were used; a wild type clone and a *petite* mutant isolated after growth in the presence of ethidium bromide (Goldring et al., 1970), which was generously provided by M. Remacha, (CBMSO. Universidad Autónoma de Madrid. Spain). The petite mutant preserved characteristics of respiratory deficient mutants, such as slow growth and formation of small (*petite*) colonies in fermentable media (Ephrussi, 1949).

2.2. Yeast media, transformation and induction

Yeast cells were grown at 30 °C, with orbital shaking at 300 rpm, in standard YNB glucose medium supplemented with 20 mg/l L-tryptophan, 40 mg/l adenine and 20 mg/l L-histidine. A standard lithium acetate protocol was used for production of competent yeast and transformation (Burke et al., 2000). Transformants of the W303-1B strain (carrying pEMBLyex or pEMBLyex-*vpr* plasmids) were maintained in selective medium (containing 20 mg/l L-leucine). For *vpr* expression, yeast cells were cultured in YNB medium containing 2% galactose plus required supplements (inducing medium). The pH of media was adjusted with Trizma base. When required, solid medium was prepared by the addition of 1.5% purified agar (Difco) to the broth.

2.3. Assay conditions

All experiments were repeated at least three times using at least three cultures derived from independent colonies, with consistent results. Growth curves represent average data from three experiments. Before initiation of experiments, cells were grown in pH 7-adjusted non-inducing medium to the exponential phase. Following a washing step in MilliQ water, cells were diluted in fresh inducing medium and cell density was adjusted. Cell growth was estimated by measuring optical density at 600 nm (OD_{600}) in a spectrophotometer (Genesys 10 VIS Thermo Scientific). Culturing was carried out at 30 °C, with shaking at 300 rpm. Liquid cultures were initiated with exponentially growing cells diluted in inducing medium to an OD_{600} of 0.01 (corresponding to 2×10^5 cells/ml) or 0.1 (*petite* mutants). In growth kinetic assays, samples were collected after 24 h of incubation ($t = 0$ h) and OD_{600} was determined. Agar plate assays were performed with exponentially growing cells. After a washing step, cultures were diluted with fresh medium to an OD_{600} of 0.2 (glucose) or 0.4 (galactose). Drop tests were carried out by spotting 5 μl of serial dilutions of the cell suspension onto agar plates. Drop tests used the same (non-inducing or inducing) pH- adjusted medium for dilutions and also to prepare agar plates. Plates were incubated for the indicated time at 30 °C and then scanned.

2.4. Protein extraction and western blotting

Exponentially growing cells were diluted in YNB (2% galactose) to an OD_{600} of 0.005. Samples (1 ml) were collected at OD_{600} 1.0 and protein extraction was performed using the following procedure: cells were pelleted and suspended in 1 ml lysis buffer (0.2 M NaOH, 0.1 M β-mercaptoethanol and 0.1 mM PMSF). After 5 min on ice, 2 μl of 100 % TCA was added and cell lysates were incubated at 65 °C for 5 min followed by incubation at 4 °C for 5 min. The non-soluble fraction was collected by centrifugation and washed with cold (−20 °C) acetone (Merck). The pellet was dried in a Speed-vac concentrator (Savant) and the extract was suspended in 100 μl sample buffer (0.16 M Tris–HCl pH 6.8, 13.3% glycerol (Merck), 2% SDS, 1.5% DTT and 0.033% bromophenol blue). Lastly, samples were briefly sonicated and heated to 100 °C for 5 min. Total protein content of each extract was measured using the Protein Assay Kit from Bio-Rad and concentrations were normalized to 1 mg/ml protein prior to loading onto SDS/20% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes by wet blotting. Protein transfer efficiency was monitored by Ponceau S staining. Membranes were subsequently cut into strips with the aid of molecular weight marker bands. Membranes were quickly destained in distilled water and blocked before addition of primary and secondary antibodies (Gonzalez and Carrasco, 2001). Labelled proteins were detected using ECL™ Western Blotting Detection Reagents (Amersham). Membrane striping was carried out in a buffer containing 100 mM 2-Mercaptoetanol, 2% SDS and 62.5 mM Tris–HCl (pH 7) at 50 °C for 30 min. Membranes were subsequently reprobed.