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The malfunction of peroxisome has an impact on the oxidative stress sensitivity in *Candida albicans*

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ABSTRACT

The peroxisome plays an essential role in eukaryotic cellular metabolism, including β-oxidation of fatty acids and detoxification of hydrogen peroxide. However, its functions in the important fungal pathogen, *C. albicans*, remain to be investigated. In this study, we identified a homologue of *Saccharomyces cerevisiae* peroxisomal protein Pex1 in this pathogen, and explored its functions in stress tolerance. Fluorescence observation revealed that *C. albicans* Pex1 was localized in the peroxisomes, and its loss led to the defect in peroxisome formation. Interestingly, the *pex1*Δ/Δ mutant had increased tolerance to oxidative stress, which was neither associated with the Cap1 pathway, nor related to the altered distribution of catalase. However, under oxidative stress, the *pex1*Δ/Δ mutant showed increased expression of autophagy-related genes, with enhanced cytoplasm-to-vacuole transport and degradation of the autophagy markers Atg8 and Lap41. Moreover, the double mutants *pex1*Δ/Δ*atg8*Δ/Δ and *pex1*Δ/Δ*atg1*Δ/Δ, both of which were defective in autophagy and peroxisome formation, showed remarkable attenuated tolerance to oxidative stress. These results indicated that autophagy is involved in resistance to oxidative stress in *pex1*Δ/Δ mutant. Taken together, this study provides evidence that the peroxisomal protein Pex1 regulates oxidative stress tolerance in an autophagy-dependent manner in *C. albicans*.

1. Introduction

Candida albicans is a common commensal fungus of our mucosal surfaces and intestinal tracts, usually without causing disease. But it may cause serious infections in immunocompromised patients (Sudbery, 2011). In the host, *C. albicans* is confronted by phagocytes such as macrophages and polymorphonuclear cells during human infections. A major mechanism of the host defense system against fungal infection is via production of reactive oxygen species (ROS) by phagocytes to kill phagocytosed fungal cells (Mayer et al., 2013). So the tolerance of oxidative stress or the ability of scavenging the ROS is a vital factor determining the virulence of *C. albicans* (Calderone and Fonzi, 2001; Rudkin et al., 2013).

Reactive oxygen species (ROS) are a variety of molecules derived from molecular oxygen. Among ROS, the superoxide anion is a relatively stable cursor of most ROS and a mediator in oxidative chain reactions (Gutteridge and Halliwell, 2010; Miyazaki et al., 2003). During cellular response to oxidative stress, the superoxide anion is enzymatically converted by superoxide dismutases (SODs) to hydrogen peroxide, which, in turn, is converted by catalases or peroxidases to H_2O and O_2 (Foyer and Mullineaux, 1994). Besides antioxidant enzymes, fungi have developed a series of antioxidant mechanisms. For example, Cap1, a key oxidant-related transcription factor, mediates gene expression involved in oxidative stress response (Wang et al., 2006).

Peroxisomes are single-membrane organelles that can import both their matrix and membrane proteins directly from the cytosol. Peroxisomes have a unique variability in enzyme content and have metabolic functions that are adjusted according to cellular needs. Their matrix harbors at least 50 different enzymes that are linked to diverse biochemical pathways, such as catalases and peroxidases. A striking feature of peroxisomes is their

Abbreviations: 5-FOA, 5-fluoroorotic acid; SD, synthetic drop-out; DCFH-DA, 2′,7′-dichlorodihydro-fluorescein diacetate; FM4-64, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide; ROS, reactive oxygen species; SOD, superoxide dismutase; PTS, peroxisomal targeting signal; AAA, ATPase associated with diverse cellular activities; NBT, nitroblue tetrazolium; RF, riboflavin; PI, propidium iodide.

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post-translational import of fully folded, and even oligomeric, matrix proteins (Glover et al., 1994; McNew and Goodman, 1994; Walton et al., 1995). Most peroxisomal matrix proteins exhibit the peroxisomal targeting signal type 1 (PTS1) sequence at the C-terminus, consisting of the tripeptide sequence (S/A/C)-(K/R/H)-L in *Saccharomyces cerevisiae* (Brocard and Hartig, 2006; Gould et al., 1989; Stanley et al., 2006). The tripeptide GKI functions as a peroxisomal targeting signal in *C. albicans* (Aitchison et al., 1991; Strijbis et al., 2009). β-Oxidation of fatty acids and detoxification of hydrogen peroxide are the central functions of peroxisomes. In fungi, the fatty acid degradation pathway is exclusively localized in the peroxisomal compartment, and peroxisome proliferation and decomposition in yeast can be induced by manipulation of the carbon source (Baker et al., 2006; Wanders and Waterham, 2006). In *C. albicans*, the unique catalase Cat1, which has a PTS1 sequence, localizes at peroxisomes (Herrero et al., 2008; Kamigaki et al., 2003). The peroxisomal peroxidases reduce inorganic and organic peroxides into the corresponding alcohols using cysteine thiols at the active sites (Herrero et al., 2008).

Autophagy, a conserved degradation and recycling process in eukaryotic cells, occurs constitutively and is induced by stress. It is now well established that autophagy is a very sensitive process induced by almost every stressful condition, such as nutrient deprivation, viral infection and genotoxic stress (Hale et al., 2013; Kroemer et al., 2010). A growing amount of evidence in recent years argues that oxidative stress acts as the converging point of these stimuli, with ROS being among the main intracellular signal transducers sustaining autophagy (Filomeni et al., 2015).

In *S. cerevisiae*, Pex1, a member of the large AAA-protein family, is involved in a wide range of different cellular processes. Deletion of *ScPEX1* results in altered distribution of peroxisomal matrix proteins from peroxisome to cytosol (Miyata and Fujiki, 2005; Platta et al., 2005). In addition, deficiency of the exportomer component Pex1 causes enhanced pexophagy (Nuttall et al., 2014). However, in *C. albicans*, the homolog of *Sc*Pex1 remains to be investigated.

In this study, we identified a homologue of *S. cerevisiae* Pex1 in *C. albicans*, also termed as Pex1, and investigated its role in oxidative stress response. This protein is also a peroxisomal protein participating in matrix protein import, suggesting the functional similarity between *Ca*Pex1 and *Sc*Pex1. We further demonstrated that the decreased sensitivity of the *pex1*Δ/Δ mutant to oxidative stress was intimately associated with the enhanced autophagy.

2. Materials and methods

2.1. Strains and growth conditions

All strains used in this study are listed in Table 1. SN76 was the parental strain and used as the wild-type strain in subsequent experiments. The *C. albicans* strains were grown in YPD medium (2% glucose, 2% peptone, 1% yeast extract) supplemented with 80 μg/mL uridine, or in synthetic drop-out (SD) medium (2% glucose, 0.67% yeast nitrogen base, 0.2% amino acid mixture) for the selection of transformants. To obtain the homozygous strain without *URA3* gene, the SD medium supplemented with 0.1% 5-fluoroorotic acid (5-FOA) was used for counter-selecting. H_2O_2 was added to achieve oxidative stress conditions. The STY medium (0.67% yeast nitrogen base, 0.05% yeast extract, 1% tergitol, 0.2% amino acid mixture) containing 0.1% oleate was used to induce peroxisome proliferation.

2.2. Construction of strains and plasmids

Plasmids and primers used in our study are listed in Tables 2 and 3, respectively.

For generating the reconstituted plasmid pDB78-PEX1-HIS1, the *PEX1* complementary fragment which was composed of 783 bp promoter region, 3276 bp ORF and 524 bp terminator region was amplified by the primers PEX1-5com and PEX1-3com, digested with *Mlu* I and *Kpn* I and recombined into the plasmid pDDB78. The reconstituted plasmids, pDDB78-PTS-CAT1, pDDB78-d3-PTS-CAT1 whose C-terminal PTS was shortened by three amino acids, and pDDB78-d7-PTS-CAT1 whose C-terminal PTS was shortened by seven amino acids, were constructed in the same way.

The Pex1-localization plasmid pAU34M-GFP-Pex1 was constructed as follows. Firstly, the *PEX1* fragment containing its terminating code was amplified from wild-type genome, and then N-terminally fused with GFP fragment obtained from the plasmid pGFP-URA3 using the PCR method. The fusion fragment was digested by *Xho*I/*Bam*H I, and then inserted into pAU34M, obtaining the localization plasmid pAU34M-GFP-PEX1. The Cat1-localization plasmids pAU34M-PTS-CAT1-GFP, pAU34M-d3-PTS-CAT1-GFP and pAU34M-d7-PTS-CAT1-GFP were constructed in the same way.

To generate the *CAT1* reporting plasmid P_{CAT1}-GFP, the promoter of *CAT1* (1545 bp) was amplified from the wild type genome, and then inserted into the plasmid pGFP (Barelle et al., 2004), obtaining the corresponding plasmid P_{CAT1}-GFP.

To obtain the *PEX1* disrupted mutant *pex1*Δ/Δ, the *pex1*::*ARG4* cassette was amplified from pRS-ARG4Δ*Spe*I with the deletion primers PEX1-5DR and PEX1-3DR, and then transformed into SN76. The obtained heterozygous mutant was transformed with the *pex1*::*URA3-dpl200* cassette, obtaining the homozygous mutant. The recombinant strains were confirmed with the detection primers PEX1-5det and PEX1-3det. To obtain the *pex1*Δ/Δ strain without *URA3* selectable marker, the homozygous mutant was streaked on the SD medium containing 5-FOA. To construct the *PEX1* complemented strain *pex1Δ/Δ+PEX1*, the *pex1Δ/Δ* strain without *URA3* was transformed with the *Nru*I-digested pDDB78-PEX1. The double mutant *pex1Δ/Δatg8Δ/Δ*, in which both *PEX1* and *ATG8* were disrupted, was constructed in the *atg8*Δ/Δ background (Yu et al., 2015). Firstly, the *URA3*-depleted *atg8Δ/Δ* mutant was transformed with the *pex1*::*URA3-dpl200* cassette amplified from the plasmid pDDB57, generating the *PEX1* heterozygous mutant, followed by *URA3* depletion, obtaining heterozygous mutant without *URA3*. This strain was then transformed again with the *pex1*::*URA3-dpl200* cassette, generating the *PEX1* homozygous mutant. Finally, the *PEX1* homozygous mutant was plated on the 5-FOA-contained SC agar, obtaining the *URA3*-depleted *pex1*Δ/Δ*atg8*Δ/Δ double mutant. The double mutants *pex1*Δ/Δ*atg1*Δ/Δ, *pex1*Δ/Δ*cat1*Δ/Δ and *pex1*Δ/Δ*cap1*Δ/Δ were obtained in the same way.

2.3. Analysis of growth under stress conditions

To study the effect of *PEX1* deletion on the growth rate, overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1, grown to mid-exponential phase at 30 °C and 37 °C. Cultures were adjusted to an OD_{600} of 0.6 and then treated with or without 5 mM H_2O_2, OD_{600} was measured every 0.5 h for 3–4 h. For the oxidative stress tolerance analysis, overnight cultures were resuspended in YPD medium supplemented with 80 μg/mL uridine and grown to mid-exponential phase at 30 °C. Cultures were adjusted to an OD_{600} of 0.2. Series of 10-fold dilutions were prepared in sterile distilled water. 3 μL of each dilution were spotted onto solid YPD plates supplemented with H_2O_2 at the indicated concentrations. The plates were incubated at 30 °C for 2–3 days before being photographed.

2.4. Measurement of ROS levels

DCFH-DA (2′,7′-dichlorodihydro-fluorescein diacetate) was used to measure the intracellular ROS levels (Kobayashi et al.,