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Isocitrate Dehydrogenase Is Important for Nitrosative Stress Resistance in *Cryptococcus neoformans*, but Oxidative Stress Resistance Is Not Dependent on Glucose-6-Phosphate Dehydrogenase▽

Sarah M. Brown,[1,2] Rajendra Upadhya,[3] James D. Shoemaker,[2] and Jennifer K. Lodge[3]*

Division of Laboratory and Genomic Medicine, Department of Pathology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, Missouri 63110[1]; Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1100 S. Grand Boulevard, St. Louis, Missouri 63104[2]; and Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, Missouri 63110[3]

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The opportunistic intracellular fungal pathogen *Cryptococcus neoformans* depends on many antioxidant and denitrosylating proteins and pathways for virulence in the immunocompromised host. These include the glutathione and thioredoxin pathways, thiol peroxidase, cytochrome *c* peroxidase, and flavohemoglobin denitrosylase. All of these ultimately depend on NADPH for either catalytic activity or maintenance of a reduced, functional form. The need for NADPH during oxidative stress is well established in many systems, but a role in resistance to nitrosative stress has not been as well characterized. In this study we investigated the roles of two sources of NADPH, glucose-6-phosphate dehydrogenase (Zwf1) and $NADP^+$-dependent isocitrate dehydrogenase (Idp1), in production of NADPH and resistance to oxidative and nitrosative stress. Deletion of *ZWF1* in *C. neoformans* did not result in an oxidative stress sensitivity phenotype or changes in the amount of NADPH produced during oxidative stress compared to those for the wild type. Deletion of *IDP1* resulted in greater sensitivity to nitrosative stress than to oxidative stress. The amount of NADPH increased 2-fold over that in the wild type during nitrosative stress, and yet the *idp1*Δ strain accumulated more mitochondrial damage than the wild type during nitrosative stress. This is the first report of the importance of Idp1 and NADPH for nitrosative stress resistance.

The alveolar macrophage can produce microbicidal amounts of toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS) following phagocytosis (27, 53). Despite this, the opportunistic fungal pathogen *Cryptococcus neoformans* is able to inhabit and replicate within phagocytes of the mammalian host and to exit these cells unharmed (1, 2, 40). The intracellular pathogenicity of *C. neoformans* is most likely facilitated by stress resistance mechanisms, including a number of antioxidant proteins and pathways involved in the detoxification of ROS and RNS. Specifically, these include the synthesis of mannitol, a free radical scavenger (9, 20); the small protein flavohemoglobin denitrosylase (Fhb1), which is essential for resistance of *C. neoformans* to nitrosative stress (10, 14, 32); and the glutathione and thioredoxin antioxidant systems, which are both important for stress resistance and virulence (42, 43, 45).

Even with different mechanisms of catalysis and/or cellular localization, one thing that these stress resistance proteins and pathways have in common is the requirement for NADPH as a cofactor. NADPH is used as an electron donor either in recycling of oxidized, inactive enzymes to reduced, active forms or directly in catalytic activity. For example, Fhb1 binds NADPH during its catalytic activity and uses it directly as an electron donor for the reduction of $NO\cdot$ to NO_3 (21). Catalases, which are highly conserved antioxidants that dismute H_2O_2 to molecular oxygen and water, consist of four units each with a molecule of NADPH bound in the core (18, 36, 59). The tripeptide glutathione (GSH) is oxidized to glutathione disulfide (GSSG), a homodimer held together by a disulfide bridge, during its oxidative state. GSSG can be reduced back to GSH by glutathione reductase, an enzyme that requires NADPH for electrons used in reduction. Similarly, glutathione peroxidase and thiol peroxidase ultimately depend on NADPH for recycling from an oxidized, inactive form back to a reduced, active form (57).

NADPH is classically recognized as being produced by the highly conserved, cytosolic pentose phosphate pathway. This pathway has been shown to be important for reductive biochemistry during oxidative stress in many organisms. The pentose phosphate pathway is an essential factor in maintaining health of erythrocytes, cells that, due to their biological function, have considerable risk for oxidative damage. Humans deficient in the pathway have hemolytic anemia, as their erythrocytes are unable to maintain sufficient pools of reduced glutathione (68). Also, the pressure of oxidative stress can stimulate the pentose phosphate pathway. This has been shown in human lymphocytes (56); in the rat adrenal gland, liver, and pancreas (15, 16); and in bacteria (63).

In fungi, the pentose phosphate pathway has been impli-

* Corresponding author. Mailing address: Department of Molecular Microbiology, Box 8230, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63104. Phone: (314) 286-2125. Fax: (314) 367-6666. E-mail: lodgejk@wustl.edu.

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cated in both oxidative stress resistance and adaptation to oxidative stress. In the model yeast *Saccharomyces cerevisiae*, NADPH-generating systems, including the pentose phosphate pathway, are critical for the ability of this organism to resist and adapt to high levels of oxidative stress (35, 47). It has also been shown that the cytosolic copper/zinc superoxide dismutase and the pentose phosphate pathway have overlapping roles in protecting *S. cerevisiae* from oxidative stress and that both systems are critical for maintaining the intracellular redox state (62). Furthermore, fungi may rely on the pentose phosphate pathway for more than reducing oxidative stress. *Aspergillus nidulans* requires a functional pentose phosphate pathway for nitrogen metabolism. Four *A. nidulans* mutants with independent defects in the pentose phosphate pathway were unable to grow on nitrite, nitrate, or various carbon sources, including 1% glucose, D-xylose, or D-glucoronate (28).

The pathway has two phases, the oxidative phase and the nonoxidative phase. The oxidative phase consists of two successive oxidations and results in the production of NADPH. The first enzyme in the oxidative phase of the pentose phosphate pathway is glucose-6-phosphate dehydrogenase (Zwf). Zwf catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate and is highly specific for $NADP^+$ as a cofactor (49, 67). There is abundant evidence supporting the role of Zwf in oxidative stress resistance. In addition to Zwf deficiency causing hemolytic anemia, Zwf has been also been implicated in maintenance of DNA repair systems during oxidative stress, as some cancers and aging disorders have also been linked to Zwf deficiency (30). For instance, Chinese hamster ovarian cells that are Zwf null have enhanced radiation sensitivity and a reduced ability to repair double-strand breaks due to the inactivation of Ku, a heterodimer DNA repair protein. In this case, the inactivation of Ku is the result of overoxidation of key cysteine residues on the protein due to the lack of sufficient reduced GSH (3). In the model yeast *Saccharomyces cerevisiae*, deletion of *ZWF1* results in sensitivity to oxidative stress. *ZWF1* is also important for the adaptive response to oxidative stress in *S. cerevisiae*. *ZWF1*-null mutants and wild-type cells were pretreated with 0.2 mM H_2O_2 and then challenged with 2 mM H_2O_2. While a large increase in tolerance to the high level of H_2O_2 was observed in the wild-type cells pretreated with 0.2 mM H_2O_2, the *zwf1*Δ strain was unable to tolerate the higher concentration (33). In *Candida albicans*, another pathogenic fungus, *ZWF1* is upregulated during oxidative stress (38).

Another source of NADPH is $NADP^+$-dependent isocitrate dehydrogenase (Idp) (55), a ubiquitous enzyme that in systems ranging from humans to yeasts to plants has been found in the cytosol, peroxisomes, or mitochondria (12, 19, 70). Although this enzyme can be targeted to mitochondria, it is distinct from the NAD^+-dependent isocitrate dehydrogenase (Idh) that functions in the mitochondria as part of the Krebs cycle. However, similarly to Idh, Idp catalyzes the decarboxylation of isocitrate to α-ketoglutarate (29). This reaction can be performed in the mitochondria, in the cytosol, or in peroxisomes using isocitrate formed from citrate exported across the mitochondrial membrane. This allows for the production of NADPH in cellular compartments without reliance of active transport of NADPH across membranes (11). It is important to have reductive power produced directly within organelles for protection from exogenous as well as endogenous stressor. For example, NADPH is consumed in peroxisomes by enzymes such as catalase and uric acid oxidase, that counteract the ROS produced during breakdown of lipids (4, 5, 31). Mitochondria particularly require reductive capability, as these organelles are susceptible to endogenous ROS produced during cellular respiration and also to exogenous RNS (52). The proteins that make up the electron transport chain are prone to damage by nitric oxide, peroxynitrite, and *S*-nitrosothiols (6). Nitric oxide and peroxynitrite have been shown to cause irreversible damage to cytochrome *c* reductase, NADH dehydrogenase, and the succinate-ubiquinone complex; the common mechanism of damage is sequestration of iron/sulfur centers of the proteins (54, 69). Thus, without a means of detoxification, the mitochondrial membrane loses potential and the ability to continue respiration, leading to death of the stressed cell. In *C. neoformans*, some antioxidant enzymes that are located at the mitochondria and dependent on NADPH for function include catalases, superoxide dismutases, cytochrome *c* peroxidase, and flavohemoglobin denitrosylase (7, 24, 25, 26). These enzymes are important for stress resistance or virulence of *C. neoformans* due to their role in high-temperature growth (24, 25) or nitrosative stress resistance (10, 14, 26).

In humans, there is one *IDP* gene that results in mitochondrial and peroxisomal products (22). In *S. cerevisiae*, there are three *IDP* genes, which encode mitochondrial (*IDP1*), cytosolic (*IDP2*), and peroxisomal (*IDP3*) forms of the protein. Deletion of both *ZWF1* and any one of the *IDP* genes in *S. cerevisiae* results in sensitivity to oxidative stress, likely due to a substantial decrease in NADPH produced in these double deletion mutants (41). In *C. neoformans* there is one predicted *IDP* gene (*IDP1*). Microarray data have indicated that this gene is upregulated 2.5-fold during nitrosative stress and thus may have a role in resistance to this stressor (44).

Since so many factors essential for stress resistance in *C. neoformans* utilize NADPH, we hypothesize that the sources of this cofactor are likewise critical for stress resistance. Although Zwf1 is important for adaptation to oxidative stress in the fungi *S. cerevisiae* and *C. albicans*, we had previously found that *C. neoformans* is unable to adapt to oxidative stress (S. M. Brown and J. K. Lodge, unpublished data), and thus we had reason to suspect that the role of Zwf1 in *C. neoformans* may be different than that in other organisms. The role of Idp1 in stress resistance, especially in resistance to nitrosative stress, is relatively unknown. In this study we used biochemical and genetic approaches to compare the roles of Zwf1 and Idp1 in resistance to oxidative and nitrosative stress in *C. neoformans*. We found that the Zwf1 is dispensable for viability, for resistance to oxidative and nitrosative stress, and for NADPH production. In contrast, we found that Idp1 is important for resistance to nitrosative stress, specifically for maintaining healthy mitochondria during exposure to nitrosative stress.

MATERIALS AND METHODS

Fungal strains, media, and reagents. KN99α (50), *C. neoformans* serotype A, was used for all experiments. All mutant strains were made in the KN99α background. Reagents, including 6-phosphogluconate potassium salt and rhodamine 123, were from Sigma Chemical Company (St. Louis, MO). MitoTracker Green FM was from Invitrogen (Invitrogen Corporation, Carlsbad, CA).

Gene deletions. Gene-specific deletion cassettes containing specific drug resistance markers were created using overlap PCR technology (13). The primer pair idp1-1/idp1-4 was used to amplify a 690-bp genomic region corresponding to

TABLE 1. Primers used for generating deletion constructs for *IDP* and *ZWF* genes

Primer	Sequence
idp1-1	AGGGGGTATAAGCTGGACAGAAGCCTGC
idp1-2	CTCGTGGAGCAGAAACAGTCTTGGTGGT
idp1-3	TCCCACAGCTCGCTCGAAGCA
idp1-4	TGCTTCGAGCGAGCTGTGGGA
idp1-5	TTCAACAGGGTCACAGGATGC
idp1-6	GCATCCTGTGACCCTGTTGAA
zwf1-1	CCAGGCAGCTGAAGGCCCTTATTAG
zwf1-2	CAGACCGCCCCTCTCGTACTGTATCT
zwf1-3	TATCCGAGAATGGCGGCGCCTCAGG
zwf1-4	CATGGTCATAGCTGTTTCCTGAGGCG
zwf1-5	CACTGGCCGTCGTTTTACAACGATGC
zwf1-6	TGGCTCCGGCTTAGGCGCATCGTTG

the 5′ region of *IDP*1. Primer pair idp1-2/idp1-5 was used for the 3′ region of the gene (Table 1). An entire *IDP1* gene open reading frame (1.65-kb genomic fragment) was deleted. Primer pair zwf1-1/zwf1-6 (Table 1) was used to amplify a 3.48-kb region of the genome encompassing the *ZFW* locus; the entire *ZWF1* gene open reading frame was deleted. Drug marker cassettes used were Geneticin (*zwf1*Δ) and hygromycin (*idp1*Δ). KN99α was transformed using biolistic techniques (64). Cells were grown in rich medium (yeast extract-peptone-dextrose [YPD]) to late log phase, concentrated, and plated onto YPD agar for transformation. The cells were bombarded with 0.6-μm gold beads (Bio-Rad, Richmond, CA) which were coated with DNA of the target construct according to the manufacturer's recommendations. Following the transformation, the cells were incubated at 25°C for 4 h on nonselective medium to allow for recovery and then transferred with 0.8 ml sterile phosphate-buffered saline (PBS) to the appropriate selective medium. Transformants were observed in 3 to 5 days.

Analysis of transformants. To isolate stable transformants, all transformants were passaged three times on nonselective YPD agar and then tested for resistance to the appropriate selective marker. Only those transformants that grew equally well on the selective medium as on nonselective medium were considered stable transformants. A three-primer PCR screen was used to prove homologous integration on both the 5′ and 3′ ends of the deletion construct (46). In this manner, homologous recombinants can be distinguished from the wild type. Genomic DNA for the screen was prepared by a modification of the glass bead DNA extraction protocol described by Fujimura and Sakuma (17). A PCR screen using the primers outside the deletion construct will amplify the entire gene region, demonstrating that a single copy of the transforming DNA had been inserted at the desired locus. Southern blots were performed to screen for single integration in the genome. A single band of characteristic size (after *in silico* analysis) was observed for all the deletion colonies used for the experiments. The colonies in which Southern blot analysis revealed multiple insertion of the drug cassette were excluded from further analysis.

Complementation of the *C. neoformans idp1* mutation. The *IDP1* gene for complementation was amplified from strain K99 using primers idp1-3/idp1-6 (Table 1). A 1.65-kb *IDP1* gene flanked by 5′ and 3′ regions (700 bp and 600 bp, respectively) was cotransformed into *idp1*Δ cells with supercoiled G418-containing plasmid. After biolistic transformation, cells initially were incubated at 30°C for 4 h and later were incubated in the presence of G418 at 37°C. The colonies growing at 37°C were passaged immediately on YPD in the absence of drugs and were used for colony PCR. The colonies in which the *IDP1* gene was reinserted at its genomic locus replacing the hygromycin cassette were confirmed by diagnostic PCR and also tested sensitive for hygromycin. The two complemented strains designated *idp1*Δ::*IDP1*-A8 and *idp1*Δ::*IDP1*-C4 were used for further analysis.

***In vitro* stress resistance assays.** Cells were plated in 10-fold serial dilutions on solid minimal medium (yeast nitrogen base [YNB], pH 4.0) with or without a stressor (1 mM H_2O_2 for oxidative stress and 0.75 mM $NaNO_2$ for nitrosative stress). Plates were incubated and observed at 2, 3, 5, and 7 days.

NADPH assays. For the measurement of $NADP^+$ and NADPH, cells were grown overnight at 25°C in YNB, pH 4.0. A stressor (1 mM H_2O_2 or 1 mM $NaNO_2$) was added to the cell cultures at an optical density at 650 nm (OD_{650}) of 1.0 and incubated for 1 h. Cells were pelleted by centrifugation and frozen at −80°C prior to measurements. The measurements were made using the EnzyChrom $NADP^+$/NADPH assay kit from BioAssay Systems (ECNP-100; BioAssay Systems, Hayward, CA). Briefly, cells were homogenized by bead beating in either acidic extraction buffer for extraction of $NADP^+$ or alkaline extraction buffer for extraction of NADPH. Homogenates were heated at 60°C for 5 min and then neutralized by addition of the opposite extraction buffer. For the measurement, a working reagent mix including glucose-6-phosphate dehydrogenase was added, and an initial reading at 565 nm was taken immediately. Another reading was taken after 30 min of incubation at room temperature. A standard curve of $NADP^+$ was used for the calculation of total pyridine nucleotide.

For the measurement of NAD^+ and NADH, cells were prepared as for the $NADP^+$ measurements, using the EnzyChrom NAD^+/NADH assay kit from BioAssay Systems (ECND-100; BioAssay Systems). The working reagent for this assay contained alcohol dehydrogenase. The first reading at 565 nm was immediately following addition of the working reagent, and the final reading was taken after 15 min of incubation at room temperature. A standard curve of NAD^+ was used for the calculation of total pyridine nucleotide.

GC/MS. 6-Phosphogluconate (6-PG) was measured by gas chromatography-mass spectrometry (GC/MS) (61). The standard used for comparison was 6-phosphogluconate potassium salt. This standard was run at multiple concentrations to confirm the retention time of silylated 6-PG and the *m/z* of the corresponding fragments. The derivatized standard eluted at 30.984 min and gave distinct fragments of *m/z* 299, 315, 333, 357, and 387. The ion of *m/z* 387 was followed for quantification. Spiked samples were also run to determine matrix effects. For measurement of 6-PG produced in wild-type and *zwf1*Δ strains under normal conditions, oxidative stress, and nitrosative stress, 50-ml cultures of each strain were incubated in YNB (pH 4.0) in a 30°C shaking incubator overnight. One millimolar H_2O_2 (oxidative stress), 1 mM $NaNO_2$ (nitrosative stress), or 1 ml PBS as a control for no stress was added to the cultures, and the cultures were incubated for an additional 2 h at 30°C. Following treatment, cells were pelleted by centrifugation at 3,220 × *g* for 8 min. The cells were washed three times in sterile PBS. The washed cells were disrupted by vortexing with 0.5 mM glass beads (BioSpec Products, Inc.) for 15 min in sterile, deionized water. The disrupted cells and beads were spun at 16,000 × *g* for 10 min and the supernatants collected and passed through a 45-μm filter.

A specified volume of filtered lysate was transferred to a Reactivial containing the following 11 internal standards: 500 nmol d_3-creatine; 10 nmol d_3-methylmalonic acid; and 100 nmol each of [$^{13}C_3$]lactate, [$^{13}C_3$]pyruvate, d_3-serine, d_5-phenylalanine, [$^{15}N_2$]orotate, d_4-sebacic acid, [$^{13}C_6$]glucose, d_6-inositol, and d_5-tryptophan. Forty microliters of triethylammonium trifluoroacetate was added to each vial, and acetonitrile was added to the tops of the vials. The samples were then placed under a nitrogen stream and held at 70°C in a heating block. Acetonitrile was added as the volume was reduced until a precipitate appeared. Methylene chloride was added, allowed to blow off, and then added again until the sample was anhydrous and reduced to constant volume. Up to 500 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was added, depending upon the appearance of the original sample, and the suspension was capped and heated at 70°C for 1 to 2 h. A splitless glass insert was used with the valve program, which turns off the purge for 1 min. The injection temperature was 200°C. A 25-m DB5 capillary column (film thickness, 0.5 μm; inner diameter, 0.32 mm) was used with a temperature program of 80 to 130°C at 4°C/min, 130 to 200°C at 6 C/min, and 200 to 285°C at 12°C/min, with holds of 1 min at 80°C and 10 min at 285°C for a total run time of 42.5 min. The mass spectrometer, in EI+ mode, scanned from 50 to 650 amu every 2.46 s after a 3.5-min delay.

Fluorescence microscopy. For staining with fluorescent dyes, 1 mM H_2O_2 or 1 mM $NaNO_2$ was added to 50-ml cultures of KN99α and the *zwf1*Δ, *psi11*Δ, and *idp1*Δ mutants that had been incubated in YNB (pH 4.0) at 25°C overnight. The cultures were incubated for an additional 2 h with the stressors, and then the cells were collected by centrifugation at 1,800 rpm for 8 min. The cells were washed in fresh YNB and then resuspended in 30 ml of YNB prewarmed to 37°C. To 1 ml of each culture, 10 μg rhodamine 123 (1-mg/ml stock solution, diluted to 10 μg/ml), 100 μg MitoTracker Green FM (lyophilized MitoTracker resuspended in fresh dimethyl sulfoxide [DMSO] to 1 mM, diluted to 100 μg/ml), or both were added and incubated with shaking at 37°C for 30 min. The cells were collected by centrifugation and washed in fresh YNB three times or until the supernatant was clear. The stained cells were resuspended in 1 ml YNB, and 10 μl of the suspension was placed on a microscope slide. The cells were visualized by confocal microscopy at a magnification of ×100.

RESULTS

Identification and deletion of *ZWF1* and *IDP1* in *C. neoformans*. The Zwf1 enzyme in *C. neoformans* (CNAG_03245.1) has been previously purified and characterized as a glucose-6-phosphate dehydrogenase by Niehaus and Mallett (49). We

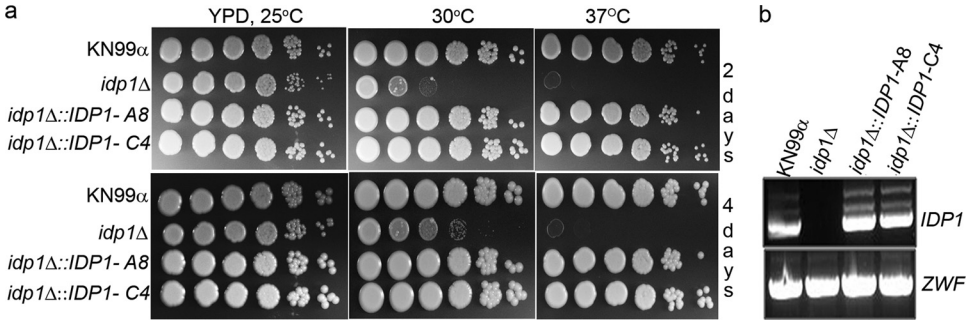

FIG. 1. *C. neoformans IDP1* is essential for growth at higher temperature. (a) The temperature-sensitive phenotype of an *idp1*Δ mutant is completely restored in complemented strains. Tenfold serial dilutions of cell suspensions of wild-type *C. neoformans* (KN99), the *idp1*Δ mutant, and two independent *idp1* mutants reconstituted with the wild-type *IDP1* gene (A8 and C4) were inoculated on YPD and incubated at the indicated temperatures. Pictures were taken at 2 and 4 days. (b) Real-time PCR confirmation of the presence of the *IDP* gene-specific transcript in the complemented strains.

compared the sequence of this gene to the current *C. neoformans* genome (Broad Assembly 1, May 2006; www.broad.mit.edu/annotation/genome/cryptococcus_neoformans/) and also to the current *S. cerevisiae* genome database (SGD). There are two genes in *S. cerevisiae* (51), and one in *C. neoformans*. This gene was disrupted by overlap PCR, and deletion of the gene was confirmed by PCR and Southern blotting.

While there are three *IDP* genes in *S. cerevisiae*, BLAST homology searches resulted in only one homologue in the *C. neoformans* genomic sequence. This gene, *IDP1* (CNAG_03920.1), was homologous to all three of the *S. cerevisiae IDP* genes, with highest homology to the mitochondrial *IDP1*. Mitoprot, a mitochondrial targeting sequence prediction server (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html), was used to identify a putative mitochondrial signal sequence in *C. neoformans IDP1*. A full-length cDNA of *IDP1* was isolated, and the sequencing results confirmed the presence of putative mitochondrial signal sequence. *IDP1* is only 17% similar to the *C. neoformans* NAD^+-dependent isocitrate dehydrogenase gene *IDH*.

Loss of *IDP1* influences growth at 37°C, a major virulence factor. Deletion of the *IDP1* and *ZWF1* genes and confirmation of the deletions were carried out as described in Materials and Methods. A total of 12 *idp1* deletion mutants, generated by four independent biolistic transformations, were selected for phenotypic analysis. Individual colonies grown in YPD were plated in 10-fold serial dilutions on solid YPD plates. Plates were incubated at 25, 30, and 37°C; a representative result is shown in Fig. 1a. Nine of the individual deletion mutants exhibited a consistent temperature-sensitive growth phenotype. The *idp1*Δ cells exhibited growth similar to that of the wild type after 2 and 4 days of incubation at 25°C. However, they showed a significant impairment of growth at 30°C even after 4 days of growth. Loss of Idp1 completely abolished the growth at 37°C. The temperature-sensitive growth phenotype of *idp1*Δ was completely restored in the two independent *idp1*Δ::*IDP1*-A8 and *idp1*Δ::*IDP1*-C4 complemented strains. Figure 1b confirms the presence of the *IDP* gene-specific transcript in the complemented strains. Temperature-sensitive phenotypes were not observed in the *zwf1*Δ strain (data not shown).

NADPH levels in wild-type, *zwf1*Δ, and *idp1*Δ cells under normal growth conditions, oxidative stress, and nitrosative stress. We measured absolute values of $NADP^+$ and NADPH produced in *zwf1*Δ, *idp1*Δ, and wild-type cells during normal growth (YNB, pH 4.0, 25°C) and growth during oxidative and nitrosative stress (YNB, pH 4.0, with 1 mM H_2O_2 or 1 mM $NaNO_2$, 25°C). $NADP^+$ and NADPH were extracted from whole-cell lysate and measured by absorbance at 565 nm. Amounts were quantified by comparison to a standard curve of $NADP^+$, and the data were processed as a ratio of NADPH to $NADP^+$. The ratio of NADPH to $NADP^+$ is indicative of the demand for NADPH by the cell. If there was no demand for NADPH, then the ratio of the reduced pyrimidine to the oxidized form would be 1. The requirement for NADPH, whether for stress resistance or for biosynthesis, decreases the ratio, as NADPH is used in cellular processes (15, 48, 58). Each measurement was taken in triplicate from six biological replicates.

In wild-type cells, the same NADPH/$NADP^+$ ratio was maintained regardless of growth condition, whether without a stressor or with an oxidative or nitrosative stressor (Fig. 2A, B, and C). We expected the ratio to decrease in this strain during oxidative stress, due to decreased production of NADPH by the oxidative phase of the pentose phosphate pathway in the absence of the rate-limiting enzyme. However, the same ratio found in the wild type was maintained in the *zwf1*Δ strain under all growth conditions (Fig. 2A, B, and C). In contrast, the ratio was significantly increased in the *idp1*Δ strain during oxidative and nitrosative stress compared to in both the wild-type and *zwf1*Δ strains (Fig. 2B and C). The increase was most dramatic during nitrosative stress (Fig. 2C).

The *idp*Δ strain, but not the *zwf1*Δ strain, is sensitive to nitrosative stress. Despite the increase in the NADPH/$NADP^+$ ratio in *idp1*Δ cells during nitrosative stress, the *idp1*Δ strain was sensitive to nitrosative stress in an *in vitro* stress assay. Serial dilutions were plated onto solid YNB (pH 4.0) with 1 mM $NaNO_2$ or 1 mM H_2O_2 or without a stressor. Plates were incubated at 25°C and observed at 3, 5, and 7 days. Compared to the wild type, the *idp1*Δ strain exhibited a stress phenotype on nitrosative stress plates. The *idp1*Δ strain was not sensitive to oxidative stress *in vitro* (Fig. 3).

To test whether Zwf1 is important for resistance to oxidative

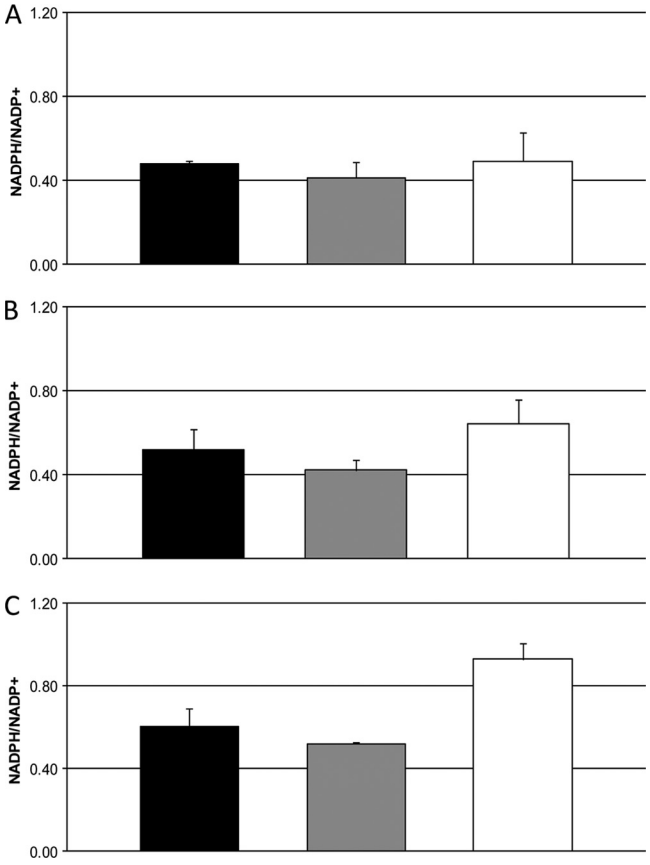

FIG. 2. NADPH in *idp1*Δ cells increases during nitrosative stress but stays consistent in wild-type and *zwf1*Δ cells during stress. Absolute amounts of NADPH and $NADP^+$ were plotted as a ratio. Measurements were taken from 1×10^7 wild-type (black bars), *zwf1*Δ (gray bars), and *idp1*Δ (white bars) cells. (A) NADPH/$NADP^+$ ratio during normal growth. (B) NADPH/$NADP^+$ ratio during oxidative stress (1 mM H_2O_2). (C) NADPH/$NADP^+$ ratio during nitrosative stress (1 mM $NaNO_2$). Values are means ± standard deviations ($n = 12$). *, $P < 0.05$ compared with control (two-tailed Student *t* test).

or nitrosative stress, serial dilutions of *zwf1*Δ and wild-type cells were plated on solid YNB (pH 4.0) with 1 mM H_2O_2 or 0.75 mM $NaNO_2$ or without a stressor. Plates were incubated at 30°C and observed at 3, 5, and 7 days. Unlike *S. cerevisiae*, the *C. neoformans zwf1*Δ strain did not exhibit sensitivity to

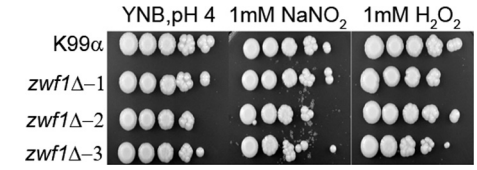

FIG. 4. The *zwf1*Δ strain is not sensitive to oxidative or nitrosative stress. Three independent null isolates (*zwf1*Δ-1, *zwf1*Δ-2, and *zwf1*Δ-3) and the wild type (KN99α) were plated on minimal medium with or without a oxidative (H_2O_2) or nitrosative ($NaNO_2$) stressor and incubated at 30°C. Compared to the wild type, the null strains did not have a sensitive phenotype with either stressor. The picture was taken after 7 days of incubation.

either nitrosative or oxidative stress (Fig. 4). Previous reports on *S. cerevisiae* have indicated that the *zwf1*Δ strain is a methionine auxotroph (62), but the *C. neoformans zwf1*Δ strain was able to grow in the absence of methionine (data not shown). The *zwf1*Δ and *idp1*Δ strains were both tested with lower (0.5 mM) and higher (2.0 mM) concentrations of H_2O_2, and there was no difference in the phenotypes seen (data not shown).

Production of 6-phosphogluconate in the wild-type and *zwf1*Δ strains. Deletion of *ZWF1* did not cause a stress-sensitive phenotype, as seen in other systems such as *S. cerevisiae*, or a change in NADPH during stress compared to the wild type. Thus, we investigated whether the second reaction of the oxidative phase of the pentose phosphate pathway could be a source of NADPH in the absence of Zwf1.

The two reactions of the oxidative phase of the pentose phosphate pathway (see Fig. 8) produce NADPH. The first reaction is catalyzed by Zwf1 and converts glucose-6-phosphate to 6-phosphogluconate. 6-PG is then converted to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase (Gnd), with the concurrent reduction of $NADP^+$ to NADPH. Gnd is highly specific for 6-PG; so far there is no reported evidence that Gnd utilizes any other phosphosugar *in vivo* (8, 39). Based on current evidence, this reaction should proceed, thereby producing NADPH, only if 6-PG is provided. In eukaryotes, with the exception of *Schizosaccharomyces pombe* (65, 66), the source of 6-PG for the second reaction of the pentose phosphate pathway is the Zwf-catalyzed conversion of glucose-6-phosphate to 6-PG. Therefore, we reasoned that deletion of Zwf1 would

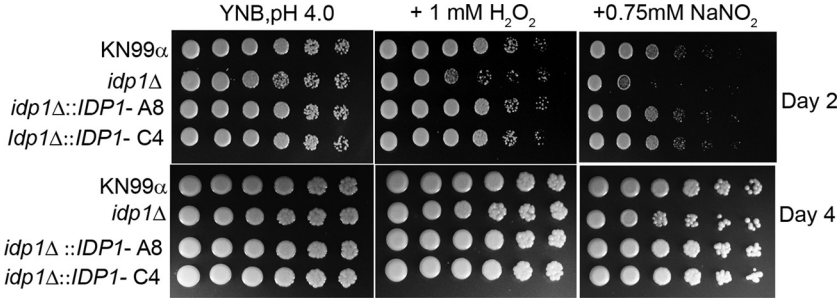

FIG. 3. The *idp1*Δ mutant displays greater sensitivity to nitrosative stress than to H_2O_2 stress. The stress phenotype is completely rescued in the two independent complemented strains. Serial 10-fold dilutions of the wild type (KN99), the *idp1* mutant, and two reconstituted strains were spotted on YNB (pH 4.0) and YNB (pH 4.0) supplemented with H_2O_2 (1 mM) or $NaNO_2$ (0.75 mM). Plates were incubated at 25°C, and pictures were taken at days 2 and 4. The *idp1*Δ::*IDP1*-A8 and *idp1*Δ::*IDP*-C4 strains contain the wild-type *IDP1* gene reinserted at its endogenous locus.

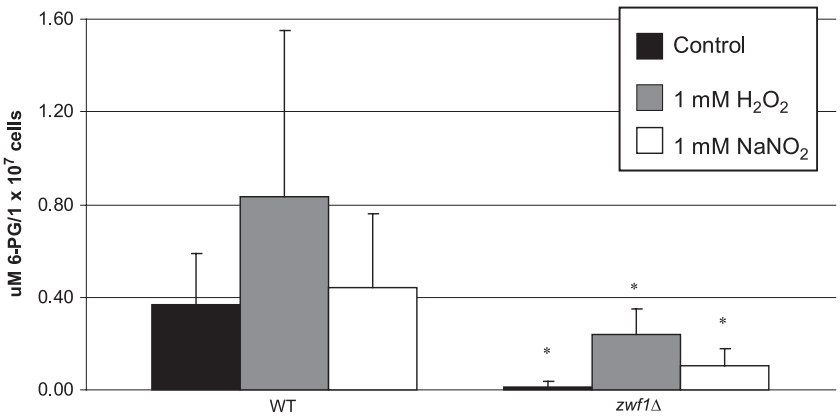

FIG. 5. Production of 6-PG is attenuated in the *zwf1*Δ strain compared to the wild type. Trimethylsilyl derivatives of 6-PG were measured by gas chromatography-mass spectrometry from lysates of 1×10^8 wild-type (KN99α) and *zwf1*Δ cells. Triplicate measurements were made from six biological replicates of each strain grown in minimal medium without a stressor or after 2 h of exposure to oxidative stress (1 mM H_2O_2) or nitrosative stress (1 mM $NaNO_2$). Values are means ± standard deviations. *, $P < 0.05$ compared to control, using Student's *t* test.

eliminate production of 6-PG and thus NADPH by the 6-phosphogluconate reaction.

To test this hypothesis, trimethylsilyl derivatives of 6-PG were measured by gas chromatography-mass spectrometry of wild-type and *zwf1*Δ strains grown with or without oxidative or nitrosative stressors. The results are shown in Fig. 5. The amounts of 6-PG in wild-type cells under normal growth conditions (no stressor) and during nitrosative stress were the same. There was a statistically significant increase in 6-PG produced by wild-type cells during oxidative stress, indicating that the pressure of oxidative stress does increase flux through the first reaction of the oxidative phase of the pentose phosphate pathway in wild-type cells. In contrast to the case for wild-type cells, there was no 6-PG found in the *zwf1*Δ cells under normal growth conditions. However, some 6-PG was found in *zwf1*Δ cells during oxidative and nitrosative stress. This amount was 4-fold less than that in the wild type under the same conditions. Thus, in *C. neoformans*, deletion of *ZWF1* attenuates production of 6-PG but does not completely abolish it.

Deletion of *IDP1* does not affect NADH production. *IDP1* is distinct from *IDH*, the NAD^+-dependent isoform of isocitrate dehydrogenase that functions in the tricarboxylic acid (TCA) cycle. We predict that Idp1 does not participate in this energy-producing cycle; however, we needed to confirm that the slow-growth phenotype of the *idp1*Δ strain was not due to disruption of the TCA cycle. NADH is a product of the cycle, and so to confirm that deletion of *IDP1* does not interfere with the cycle, we measured NADH in wild-type and *idp1*Δ cells during normal growth and nitrosative stress. We found no difference in the amounts of NAD^+ and NADH in the *idp1*Δ strain compared to the wild type, indicating that deletion of *IDP1* does not interfere with normal production of NADH (data not shown).

Integrity of the mitochondrial membrane is compromised during nitrosative stress. If Idp1 indeed is localized to mitochondria, it may have a critical role in supplying mitochondrial antioxidants with NADPH during nitrosative stress. The sensitive phenotype despite the increase in NADPH in the *idp1*Δ strain during nitrosative stress could be caused by a lack of sufficient reducing equivalents within the mitochondria due to the absence of a direct source and insufficient membrane transport. Thus, NADPH would pool within the cytosol and yet not be accessible to the enzymes essential for nitrosative stress resistance. We decided to use the mitochondrial stains rhodamine 123 (Sigma) and MitoTracker Green FM (Invitrogen) to test loss of mitochondrial membrane integrity in *idp1*Δ cells during nitrosative stress. Rhodamine 123 passively diffuses across mitochondrial membranes and will be sequestered within the organelles if the membrane is potentiated, as it is in the healthy state. However, if the membrane has lost potential, this dye will efflux out of the organelle (34). Conversely, MitoTracker mitochondrion-selective probes passively diffuse across mitochondrial membranes and will remain within mitochondria regardless of membrane potential (Invitrogen). As a result, MitoTracker can be used to identify mitochondria by fluorescence microscopy, and rhodamine 123 can be used to determine whether mitochondria have intact, potentiated membranes.

For this experiment, we used the wild-type, *idp1*Δ, and *zwf1*Δ strains. For a control, we used the *psi11*Δ strain, which is sensitive to nitrosative stress but in which this sensitivity is likely because of poor cell wall integrity and/or cell signaling (K. J. Gerik and J. K. Lodge, unpublished data) and not primarily mitochondrial damage. We looked at the retention of both MitoTracker and rhodamine 123 in strains exposed to oxidative (1 mM H_2O_2), and nitrosative (0.75 mM $NaNO_2$) stressors for 1 h compared to that in cells not exposed to stress (Fig. 6). To rule out the nonspecific effect of incubating cells at 37°C (employed in this experiment for labeling), we investigated the effect of brief exposure of cells (90 min) to 37°C on their viability. By counting the CFU after the exposure of either the wild-type or mutant cells to a temperature of 37°C for up to 90 min, we observed no significant difference in cell viability (data not shown). We found that 100% of wild-type cells not exposed to a stressor that stained with MitoTracker also retained rhodamine 123, indicating healthy mitochondria. Conversely, after exposure to nitrosative stress, only 30% of wild-type cells that stained with MitoTracker also retained rhodamine 123, indicating that nitrosative stress does induce

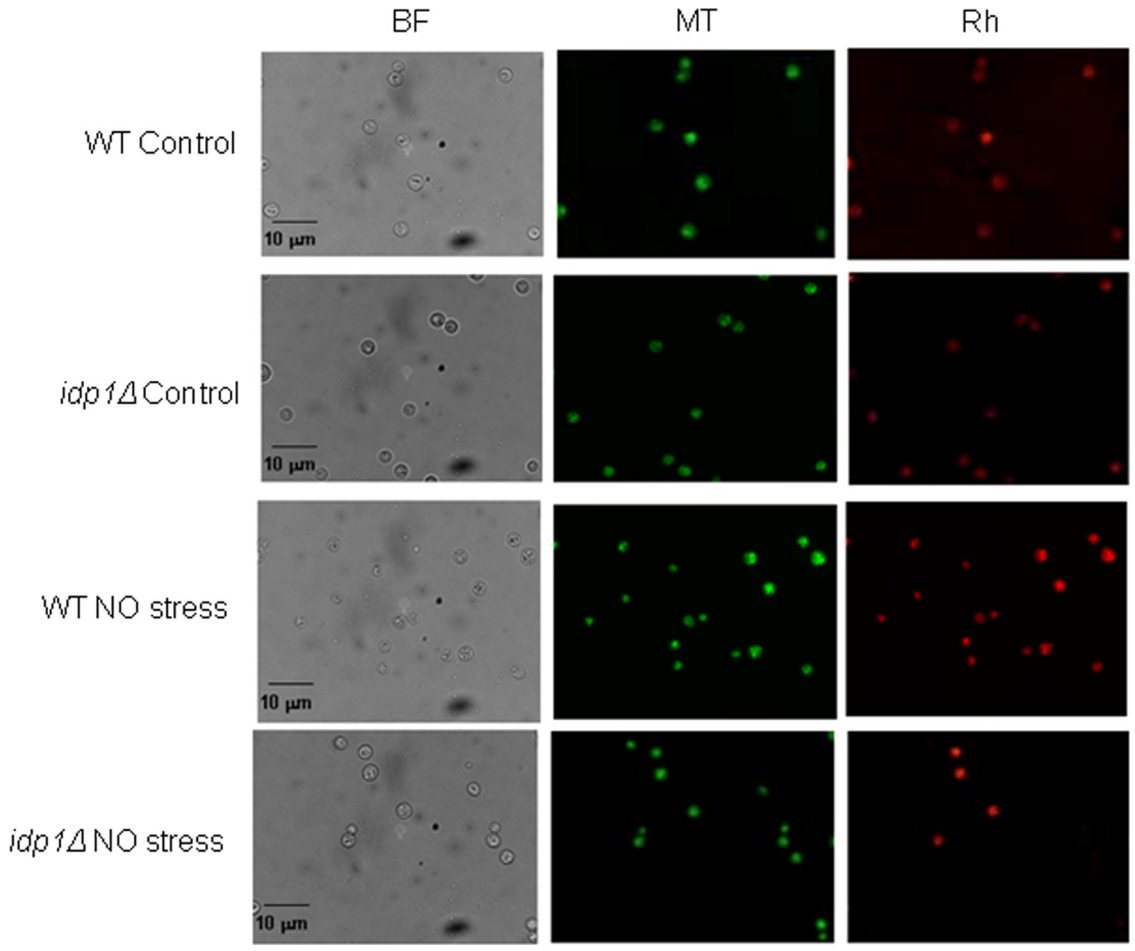

FIG. 6. Nitrosative stress induces mitochondrial damage that is pronounced in the *idp1*Δ strain compared to the wild type. Images were taken by confocal microscopy at a magnification of ×100. During exposure to nitrosative stress (0.75 mM $NaNO_2$), the *idp1*Δ cells retain MitoTracker (MT) (green images) but poorly retain rhodamine 123 (Rh) (red images) compared to the wild type (KN99α) under the same conditions. During normal conditions (control), both wild-type and *idp1*Δ cells retain MT and Rh. BF, bright field.

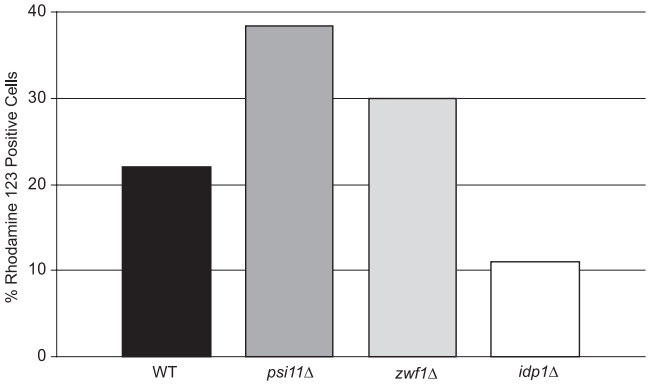

FIG. 7. Percentage of cells that were rhodamine 123 positive after exposure to nitrosative stress. The percentage of cells that retained MitoTracker and also retained rhodamine 123 after 1 h of exposure to nitrosative stress (0.75 mM $NaNO_2$) is shown. Cells that retained both dyes are considered to have mitochondria with intact, potentiated membranes, while cells that did not retain rhodamine 123 do not have mitochondria with fully potentiated membranes. Three hundred cells of each strain that retained MitoTracker were counted, and the percentage of these 300 that also retained rhodamine 123 is represented graphically.

mitochondrial damage (Fig. 7). This is in contrast to oxidative stress, which did not result in a decrease in the number of cells that retained both dyes. Similar results were seen with the *zwf1*Δ and *psi11*Δ strains. However, while 100% of the *idp1*Δ cells that took up MitoTracker also retained rhodamine 123 after exposure to no stress or oxidative stress, only 9.0% of cells that retained MitoTracker retained rhodamine 123 as well (Fig. 7). This indicates that the *idp1*Δ strain is more susceptible to mitochondrial damage during nitrosative stress than any of the other strains tested, supporting the hypothesis that Idp1 is localized to the mitochondria and that its presence there is essential for production of the NADPH used by the antioxidant proteins located in the mitochondria.

DISCUSSION

The ability of the fungal pathogen *C. neoformans* to survive within macrophages of the mammalian host is presumably due to the ability of the fungal cell to protect itself from toxic reactive oxygen and nitrogen species that can be produced by macrophages following phagocytosis. It is well established that several antioxidant proteins are essential for stress resistance

and virulence. Regardless of mechanism or localization, a common requirement of many essential protective enzymes is the reductive cofactor NADPH. Since NADPH crosses membrane bilayers only by facilitated transport, it is important that NADPH be produced within the various cellular compartments so that there is a pool of reducing equivalents readily available for stress resistance. Mitochondria particularly are susceptible to damage from $NO^{\cdot}$, as several of the components of the electron transport chain contain metal centers that can be sequestered by $NO^{\cdot}$, leading to disruption of the membrane, impaired function, and ultimately cell death.

Two potential sources of NADPH for stress resistance are the cytosolic pentose phosphate pathway and $NADP^+$-dependent isocitrate dehydrogenase. In many other systems, disruption of the pentose phosphate pathway by deletion of the first and rate-limiting enzyme, Zwf1, leads to poor growth and insufficient resistance to oxidative stress, presumably due to a decrease in NADPH. Likewise, in *S. cerevisiae* the three Idp proteins are important for protection from ROS under various conditions, including peroxisomal fatty acid oxidation (Idp3). Given the need for antioxidant function and the number of NADPH-dependent enzymes essential for nitrosative stress resistance and virulence in *C. neoformans*, we investigated the roles of Zwf1 and Idp1 as sources of NADPH for resistance to nitrosative stress as well as oxidative stress.

We first investigated the role of Zwf1 in production of NADPH and resistance to stress by creating a *zwf1*Δ strain, measuring NADPH in this strain, and testing *in vitro* stress sensitivity. Our results showed that the role of Zwf1 in production of NADPH and oxidative stress resistance in *C. neoformans* is unlike that in other fungi. In *S. cerevisiae*, deletion of Zwf1 results in a slow-growth phenotype and methionine auxotrophy (35, 47, 62). In contrast, our *C. neoformans zwf1*Δ strain had no growth phenotype on rich, minimal, or methionine-deficient media, nor was it sensitive to changes in temperature. In *S. cerevisiae*, *ZWF1* has been shown to be upregulated during oxidative stress at both the transcriptional and translational levels (33, 60), and in both *S. cerevisiae* and *C. albicans*, it is essential for adaptation to oxidative stress (37, 38). In contrast, the *C. neoformans zwf1*Δ strain was not sensitive to oxidative stress, and further, we found no changes in mRNA expression during oxidative stress (data not shown). We also measured the amount of NADPH produced by the *zwf1*Δ strain under normal conditions (no stress), oxidative stress, and nitrosative stress. Finding no difference compared to results for the wild type under any condition, we considered the possibility that NADPH is still made by the pentose phosphate pathway even in the absence of Zwf1.

While the first two decarboxylation reactions of the pentose phosphate pathway can produce NADPH, the second reaction, driven by Gnd1, is dependent on 6-PG, the substrate produced by the first reaction, and therefore would proceed only if provided this substrate. Thus, without an alternative source of 6-PG, Gnd1 would not be able to reduce $NADP^+$ in the *zwf1*Δ strain. We found that deletion of *ZWF1* in *C. neoformans* did not completely abolish production of 6-PG, and therefore we propose that there is an alternative source of this phosphosugar in *C. neoformans*. To date, the only reported source of 6-PG other than the decarboxylation of glucose-6-phosphate by Zwf1 is gluconate kinase (Fig. 8, arrow 3) in the "gluconate route," a pathway that, while described mainly for prokaryotes, has been reported for *Schizosaccharomyces pombe*. While the bacterial gluconate kinase is NAD^+ dependent, the *S. pombe* ortholog (CAA93562.1) is $NADP^+$ dependent. Despite the authors' suggestion that the active gluconate route in *S. pombe* is a distinguishing factor between fission and budding yeasts (65, 66), bioinformatic searching of the *C. neoformans* genome (www.broad.mit.edu/annotation/genome/cryptococcus_neoformans/) resulted in identification of a possible homologue (CNAG_03048; E value = 9e−27) to the *S. pombe* gluconate kinase, annotated only as "cytoplasm protein." We suggest that this enzyme may an alternative source of 6-PG in the absence of Zwf1 as well as another source of cytosolic NADPH (Fig. 8) and that while the amount of 6-PG produced in the absence of Zwf1 is still 4-fold less than that in the wild type, this is enough to support antioxidant proteins and biosynthetic pathways. This hypothesis is supported by the lack of an oxidative stress phenotype in the *zwf1*Δ strain.

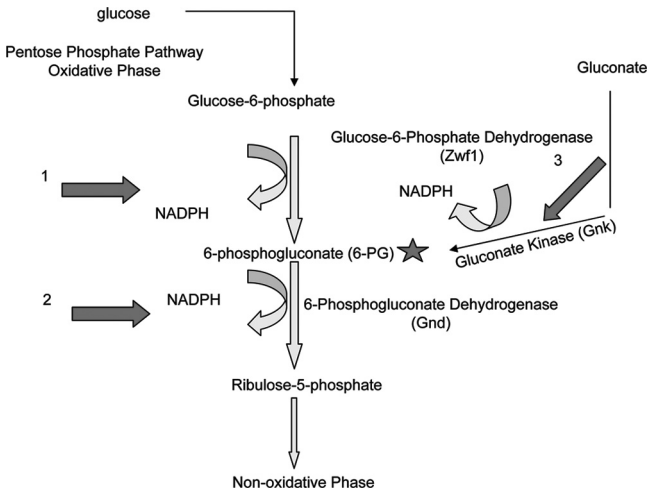

FIG. 8. Possible cytosolic sources of NADPH in *C. neoformans*. NADPH is produced during the oxidative phase of the pentose phosphate pathway after glucose is converted to glucose-6-phosphate, which is then converted to 6-phosphogluconate (6-PG) by glucose-6-phosphate dehydrogenase (Zwf1). This reaction produces NADPH (arrow 1). 6-PG (star) is then converted to ribulose-5-phosphate by 6-phosphogluconae dehydrogenase (Gnd) in a reaction that also produces NADPH (arrow 2). The Gnd reaction requires 6-PG (star) to proceed. In *C. neoformans*, an alternative source of 6-PG may be gluconate kinase (Gnk), an enzyme that converts gluconate to 6-PG in a reaction that produces NADPH (arrow 3).

Second, we looked at the role of Idp1 in stress resistance. Our *idp1*Δ strain was not susceptible to oxidative stress but was sensitive to both high temperature and nitrosative stress. Despite the phenotype, the *idp1*Δ cells accumulated NADPH during nitrosative stress, as indicated by the increase in $NADPH/NADP^+$. In an effort to explain the phenotype, we predicted that the sensitivity to nitrosative stress is due to an inability to reduce mitochondrion-damaging RNS in the absence of Idp1. Containing a mitochondrial signal sequence, *C. neoformans*' Idp1 is likely to be localized to the mitochondria, and as such it could produce intracompartmental NADPH for use by mitochondrial antioxidant and denitrosylating proteins. We found that the *idp1*Δ strain accumulated significantly more inviable mitochondria during exposure to nitrosative stress

than either the wild-type or *zwf1*Δ strain. Thus, we propose the following working model. Wild-type cells, with both intracompartmental and cytosolic sources of NADPH, have normal growth and stress resistance. In the absence of Zwf1, *C. neoformans* maintains normal stress resistance through cytosolic NADPH produced by an alternative protein or pathway and maintaining intracompartmental levels with Idp1. However, in the absence of Idp1, the cells sustain considerable mitochondrial damage during nitrosative stress, leading to cell death, while NADPH produced by cytosolic sources pools in the cytosol. The presence of antioxidant enzymes in the cytosol prevents the *idp1*Δ strain from being completely inviable when exposed to nitrosative stress. Of note, we were not able to obtain a *zwf1*Δ *idp1*Δ double deletion strain, suggesting that these enzymes have some redundant, essential role.

Our study shows that despite overwhelming evidence for a role of Zwf1 in oxidative stress resistance in many, various systems, its role in *C. neoformans* is exceptional. One indication from the results is that recycling of oxidized antioxidants, which is purported to be a significant use of NADPH, is not the main source of active enzymes in *C. neoformans* and that instead, increased protein turnover may be the prominent source of reduced enzymes in this species. This hypothesis is supported by proteomic data showing that there are very few changes in steady-state levels of proteins in *C. neoformans* during oxidative stress (S. M. Brown and J. K. Lodge, unpublished data). We also provide evidence of a functional gluconate kinase in *C. neoformans*. Finally, our results suggest that compartmentalization of NADPH production is important for stress resistance in *C. neoformans* and reveal a key role for Idp1 in nitrosative stress.

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