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The lysine biosynthetic enzyme Lys4 influences iron metabolism, mitochondrial function and virulence in *Cryptococcus neoformans*

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Abstract

The lysine biosynthesis pathway via α -amino adipate in fungi is considered an attractive target for antifungal drugs due to its absence in mammalian hosts. The iron-sulfur cluster-containing enzyme homoaconitase converts homocitrate to homoisocitrate in the lysine biosynthetic pathway, and is encoded by *LYS4* in the model yeast *Saccharomyces cerevisiae*. In this study, we identified the ortholog of *LYS4* in the human fungal pathogen, *Cryptococcus neoformans*, and found that *LYS4* expression is regulated by iron levels and by the iron-related transcription factors Hap3 and HapX. Deletion of the *LYS4* gene resulted in lysine auxotrophy suggesting that Lys4 is essential for lysine biosynthesis. Our study also revealed that lysine uptake was mediated by two amino acid permeases, Aap2 and Aap3, and influenced by nitrogen catabolite repression (NCR). Furthermore, the *lys4* mutant showed increased sensitivity to oxidative stress, agents that challenge cell wall/membrane integrity, and azole antifungal drugs. We showed that these phenotypes were due in part to impaired mitochondrial function as a result of *LYS4* deletion, which we propose disrupts iron homeostasis in the organelle. The combination of defects are consistent with our observation that the *lys4* mutant was attenuated virulence in a mouse inhalation model of cryptococcosis.

Keywords

C. neoformans; Iron; Lysine; *LYS4*; Mitochondria; Virulence

1. Introduction

Lysine is an essential amino acid for animals and must be obtained from diet. Bacteria, fungi and plants, however, can synthesize lysine using two distinct pathways [1,2]. In bacteria and plants, lysine is synthesized via the diaminopimelate (DAP) pathway, whereas the majority

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of fungi use the α -aminoadipate (AA) pathway [3]. The DAP pathway has been well studied in bacteria, and is considered a novel antibacterial target, especially in light of the absence of lysine biosynthesis in mammalian hosts [4]. Similarly, the AA pathway in fungi has been proposed as a valuable target for antifungal therapy. Indeed, several researchers have developed antifungal agents that specifically inhibit the AA pathway in pathogenic fungi [5,6,7].

In this study, we identified the gene encoding the putative homoaconitase in the human fungal pathogen *Cryptococcus neoformans*, which causes pneumonia and life-threatening meningoencephalitis, mainly in immunocompromised patients [8]. Homoaconitase converts homocitrate to homoisocitrate in the lysine biosynthesis pathway [9]. The orthologous gene encoding homoaconitase, *LYS4*, in the model yeast *Saccharomyces cerevisiae*, is essential for lysine biosynthesis [10]. The protein encoded by *S. cerevisiae* *LYS4* contains amino acid residues associated with an iron-sulfur (Fe-S) cluster and shows evolutionary conservation with the aconitase family of proteins [3,11]. The *S. cerevisiae* ortholog of *LYS4* in *C. neoformans* was initially found in our transcriptome analysis to identify differentially expressed genes in a mutant lacking *CFO1*, the gene encoding a ferroxidase for high-affinity iron transport at the plasma membrane [12]. The expression of *LYS4* was significantly down-regulated in the *cfo1* mutant, suggesting that Lys4 function may be associated with iron metabolism in *C. neoformans*. The fact that iron metabolism and homeostasis have a large influence on the virulence of *C. neoformans* [13], and that Lys4 is a possible antifungal drug target, led us to characterize its functions and roles in the physiology and virulence of the pathogen.

2. Materials and Methods

2.1 Strains, growth conditions and expression assays

The *C. neoformans* var. *grubii* strains (serotype A; MAT α) used in this study are listed in Table S1. The strains were maintained in yeast extract-bacto peptone (YPD) medium with 2.0% glucose or yeast nitrogen base (YNB; Sigma, Saint Louis, MO, USA) with 2.0% glucose. To test the phenotype of the lysine auxotroph, 0.2 mg/mL of lysine (Sigma, Saint Louis, MO, USA) was added to YNB medium. The YNB low iron medium was prepared as described [14]. Construction of the *lys4* mutant, the reconstituted strain, and the strain harboring the Lys4-Gfp fusion protein are described in the Supplementary materials and methods. For Northern and western blot analyses, strains were cultured in the media listed above, and total RNA and proteins were extracted, as previously described [15].

2.2 Microscopy

The mitochondria and Lys4-Gfp fusion protein were visualized using an Axioplan 2 imaging system (Zeiss, Germany) with 100 $\times$ magnification [15]. Metamorph imaging software (version 6.1r6, Universal Imaging Corp.) was used to acquire the differential interference contrast (DIC) and fluorescent images.

2.3 Mitochondria isolation and aconitase assay

The strain expressing the Lys4-Gfp fusion protein was grown in YPD at 30°C overnight, and the mitochondrial fraction was isolated using differential centrifugation as described previously [16]. The wild-type strain and the *lys4* mutant were grown in YPD medium at 30°C overnight and resuspended in triton/citrate lysis buffer. The activity of aconitase was estimated as previously described [17].

2.4 Determination of iron and ergosterol contents

The iron content in isolated mitochondria was measured as previously described [18]. Lipids were extracted as previously described [19]. Ergosterol content was determined using GC-MS (Agilent Technologies, CA, USA).

2.5 Virulence assay

The virulence assay (protocol A13-0093), approved by the University of British Columbia Committee on Animal Care, was performed using female 4–6-week-old BALB/c mice, procured from Charles River Laboratories (Ontario, Canada), as previously described [20].

3. Results

3.1 *LYS4* is required for lysine biosynthesis in *C. neoformans*

Our previous transcriptome data showed that the gene CNAG_02565 was significantly downregulated in the *cfo1* mutant, which suggested a possible connection between the gene function and the iron homeostasis and metabolism in *C. neoformans* [12]. The protein sequence of CNAG_02565 is highly homologous to Lys4, an enzyme in the lysine biosynthesis pathway in *S. cerevisiae*, with 64% similarity and 52% identity [21]. We therefore designated CNAG_02565 as *LYS4* and constructed a mutant strain lacking the gene for further characterization in *C. neoformans*. The *lys4* mutants were generated using biolistic transformation with a gene-specific knock-out cassette, and confirmed by southern blot analysis (Fig. S1). The wild-type *LYS4* gene was introduced at the original *LYS4* locus in the *lys4* mutant to construct a *LYS4* reconstituted strain for inclusion throughout the study.

The *C. neoformans lys4* mutant was first challenged for lysine auxotrophy to confirm the requirement of *LYS4* in lysine biosynthesis. Indeed, the *lys4* mutant displayed growth defects when grown on YNB medium without lysine, and addition of lysine restored the growth of the mutant (Fig. 1A). The same experiment also showed that lysine uptake was more efficient in medium containing asparagine versus ammonium sulfate indicating that the nitrogen source influenced restoration of lysine uptake in the *lys4* mutants. These results suggested the possibility of nitrogen catabolite repression (NCR) of lysine uptake and we noted that NCR of leucine uptake in *C. neoformans* was observed in our previous study [15]. To study the influence of NCR on lysine uptake in further detail, we investigated the transcript levels of eight putative amino acid permeases, *AAP1* ~ *8*, that were identified in the recent study by Fernandes *et al.* [22]. Wild-type cells were grown in YNB medium with ammonium sulfate or lysine as a sole nitrogen source and total RNA was extracted for evaluation of transcript levels of each *AAP* gene by quantitative real-time PCR. As shown in

Fig. 1B, *AAP2* and *AAP3* were significantly up-regulated by approximately 13- and 293-fold, respectively, in YNB medium with lysine as sole nitrogen source, compared with cells grown in the presence of ammonium sulfate. Fernandes *et al.* [22] showed increased transcript levels for *AAP2* upon addition of several amino acids including tryptophan, methionine, and histidine suggesting that the gene encodes an amino acid permease with broad substrate specificity. Our observations when combined with the results of Fernandes *et al.* [22] implied that among the identified amino acid permeases in *C. neoformans*, Aap3 is specific for lysine uptake and lysine is one of the substrates of Aap2. To address the effect of NCR on amino acid permeases responsible for lysine uptake, the transcript levels of *AAP2* and *AAP3* were compared in cells grown in YNB medium containing either lysine and ammonium sulfate, or lysine and asparagine as nitrogen sources. The transcript levels of *AAP2* and *AAP3* were dramatically reduced by approximately 6- and 12.8-fold, respectively, in cells grown in medium containing ammonium sulfate, compared with cells grown in the medium containing asparagine (Fig. 1C). Collectively, these results suggested that *LYS4* is required for lysine biosynthesis, and lysine uptake is regulated by NCR in *C. neoformans*.

3.2 Expression of *LYS4* is regulated by iron levels and iron regulatory transcription factors

Our previous studies indicated an influence of iron availability on *LYS4* expression [12]. To confirm iron-dependent regulation, *LYS4* transcript levels were assessed by Northern blot analysis using RNA extracted from wild-type cells grown in low- and high-iron medium. The transcript levels of *LYS4* in the mutant strains lacking *CIR1*, *HAPX*, or *HAP3* (transcription factors that control the genes involved in iron transport and iron metabolism) were also analyzed. No *LYS4* transcripts were observed in cells grown in low-iron medium, whereas abundant transcripts were observed in the cells grown in the high-iron medium. These results confirmed the regulation of *LYS4* by iron availability and indicated that the *LYS4* gene is mainly transcribed when sufficient iron is present in the environment. Furthermore, the mutant strains lacking *HAP3* or *HAPX* exhibited significantly increased transcript levels of *LYS4*, indicating that these transcription factors negatively regulate expression of the gene, but only in iron-depleted conditions. That is, these transcription factors did not influence *LYS4* transcript levels upon iron repletion (Fig. 2A). In contrast, deletion of the *CIR1* gene encoding an additional iron regulator did not influence *LYS4* transcript levels in either condition. Regulation of the protein levels of Lys4 by iron was also investigated. For this analysis, *LYS4* was fused with the gene encoding the green fluorescent protein (Lys4-Gfp) and introduced into the *lys4* mutant. The strain expressing the Lys4-Gfp fusion protein restored the wild-type growth level in minimal medium without lysine suggesting that the fusion protein is functional (data not shown), and was used for the western blot analysis. As with transcript levels, the abundance of the Lys4 protein was significantly increased in cells grown in high-iron medium (Fig. 2B). Therefore, we conclude that iron availability influences both transcript and protein levels for Lys4.

3.3 The *lys4* mutant showed impaired mitochondrial functions

The Lys4 protein in *S. cerevisiae* is reported to be localized in mitochondria [23]. Using fluorescence microscopy, we also found that the Lys4-Gfp protein is indeed localized in mitochondria in *C. neoformans* (Fig. 2C). Additionally, mitochondrial localization of Lys4

was confirmed using western blot analysis to detect the protein in an isolated mitochondrial fraction (Fig. 2D). Mitochondria play important roles in the biogenesis of Fe-S proteins such as Lys4 and in response to various stress conditions such as oxidative stress and antifungal drug treatment [24,25]. We therefore examined the impact of *LYS4* deletion on phenotypes related to mitochondrial function. Initially, we investigated the sensitivity of the *lys4* mutant to several stress conditions and found that the mutant cells displayed increased sensitivity to oxidative stress caused by hydrogen peroxide (Fig. 3A). We expected this phenotype to be caused increased accumulation of reactive oxygen species (ROS) in the mitochondria of mutant cells, and we confirmed this with MitoSOX, a fluoroprobe that can selectively detect ROS in the mitochondria of live cells. Specifically, a higher signal intensity for MitoSOX was found in mutant versus wild-type cells (Fig. 3B) [26]. The *lys4* mutant also showed increased sensitivity to the cell wall/membrane disrupting agents congo red and sodium dodecyl sulfate (SDS), as well as the azole antifungal drugs fluconazole and miconazole (Fig. 3C). Azole drugs inhibit Erg11, which is a cytochrome P450 enzyme having heme as a cofactor [27,28]. Heme is primarily synthesized in the mitochondria and previous studies revealed that mitochondrial mutants of *Candida* species have reduced ergosterol levels [29,30]. Consistently with these observations, we also found that the *lys4* mutants have significantly reduced ergosterol contents compared with the wild-type cells (Fig. 3D).

In a further analysis of connections between mitochondrial function and Lys4, we also examined the growth of *lys4* mutants in media containing inhibitors for the mitochondrial electron transport chain. We found that the growth of the mutant was significantly reduced compared with the wild-type strain in medium containing salicylhydroxamic acid (SHAM; an inhibitor of alternative oxidase), diphenyleneiodonium (DPI; an inhibitor of complex I of the mitochondrial respiratory chain) and potassium cyanide (KCN; an inhibitor of complex IV of the mitochondrial respiratory chain) (Fig. 3E). Taken together, these results indicated that *LYS4* deletion impairs mitochondrial functions in *C. neoformans*.

The activity of aconitase has been used to estimate Fe-S cluster synthesis in mitochondria [31]. We hypothesized that *LYS4* influences iron metabolism in mitochondria and that loss of Lys4 resulted in organelle dysfunction due to a potential impact on Fe-S levels. To examine this hypothesis, the activity of the Fe-S enzyme aconitase was measured in the *lys4* mutant and the wild-type strain. As shown in Fig. 4A, the aconitase activity was reduced to $80.03 \pm 12.45\%$ ($p = 0.0499$) in the *lys4* mutant compared to the wild-type strain, which suggests that Fe-S biosynthesis may be perturbed in the *lys4* mutant. Furthermore, we observed significantly reduced iron content in the isolated mitochondrial fraction from the mutant cells compared with the wild-type cells (Fig. 4B). Thus, we conclude that Lys4 plays an important role in mitochondrial iron metabolism, and is required for proper mitochondrial function in *C. neoformans*.

3.4 *Lys4* is required for full virulence in *C. neoformans*

Capsule formation, melanin synthesis and growth at 37°C are well known virulence factors that help *C. neoformans* survive within the host environment [32,33]. Although, we found no difference in capsule formation and melanin synthesis compared to the wild-type strain (data not shown), the *lys4* mutant showed a significant growth defect in YPD medium at 37°C

(Fig. 4C). This result suggested that the *lys4* mutant would be avirulent. We therefore inoculated ten BALB/c mice per strain each with the wild-type strain, the *lys4* mutant and the reconstituted strain, and monitored the survival of the mice daily. We observed 100% mortality in mice infected with the wild-type and reconstituted strains after 21 days and 20 days post-infection, respectively. However, the mice infected with the *lys4* mutant showed significantly attenuated disease with prolonged survival by ~11 days compared to mice infected with the wild-type strain (Fig. 4D). These results indicated that *LYS4* is required for full virulence in *C. neoformans* and that the temperature sensitivity observed in culture did not completely prevent the mutant from causing disease.

4. Discussion

In this study, we identified and characterized *LYS4* in *C. neoformans*, and demonstrated that the gene is expressed only when sufficient iron is available. The major iron-regulatory transcription factors HapX and Hap3 negatively regulated the expression of *LYS4* in the low iron condition. We hypothesize that *C. neoformans* might down-regulate *LYS4* expression to adapt to iron deficiency in the host, perhaps as part of a broader remodeling of metabolism. Similar remodeling and an impact on amino acid biosynthesis in response to iron deprivation has been characterized in *S. cerevisiae* [34]. Our previous study also showed similar patterns of iron-dependent regulation of *LEU1*, a homolog of isopropylmalate dehydrogenase that is required for leucine biosynthesis in *C. neoformans* [15]. Therefore, we believe that iron deprivation might signal the down-regulation of amino acid biosynthesis in *C. neoformans*, particularly lysine and leucine biosynthesis, to regulate fungal fitness in nutrient-restricted conditions within the host. *LYSF*, a homolog of *LYS4* in *Aspergillus nidulans*, showed a similar pattern of iron-dependent regulation indicating that down-regulation of *LYS4* expression might be a common response in fungi [35].

The *lys4* mutant was more sensitive to oxidative stress and more susceptible to cell wall and membrane-disrupting agents. We hypothesized that *LYS4* deletion influenced mitochondrial iron metabolism, Fe-S cluster biosynthesis, and homeostasis in particular, and caused deficiency in mitochondrial functions. Previous studies have suggested that mitochondria play a role in oxidative stress responses, and cell wall and membrane integrity in *C. glabrata*, *C. albicans*, and *S. cerevisiae*, which agree with the phenotypes we observed in the *C. neoformans lys4* mutant [24]. Moreover, we obtained strong evidence throughout our study that the *lys4* mutant impaired mitochondrial functions and disrupted mitochondrial iron metabolism. In particular, the *lys4* mutant had reduced mitochondrial iron levels and decreased aconitase activity compared to the wild-type strain. The *lys4* mutants also showed increased sensitivity to inhibitors of the respiratory complexes in the mitochondrial electron transport chains that requires iron cofactors. Dysfunctional mitochondrial iron metabolism could also increase the sensitivity of the *lys4* mutant to various oxidative stressors, similar to other mutants with impaired mitochondrial functions [12,15]. Furthermore, deletion of *LYS4* reduced ergosterol content in the mutant cells compared to the wild-type strain and increased sensitivity to azole antifungal drugs. This suggested that *LYS4* directly influences production of cell membrane constituents, as well as its structure and integrity.

Previously, Kingsbury *et al.* [36] studied the virulence of a lysine biosynthesis deficient mutant of *C. neoformans*, using the murine inhalation infection model. The mutant lacked *LYS9*, which encodes saccharopine dehydrogenase in the lysine biosynthesis pathway, and exhibited attenuated virulence compared to the wild-type strain. In the current study, we also observed similar attenuation of the virulence in the *lys4* mutant using the murine inhalation infection model. Kingsbury *et al.* [36] argued that attenuated virulence of the *lys9* mutant was mainly caused by reduced growth of the cells due to the host body temperature of 37°C, and not by a deficiency in lysine synthesis. However, in our study, deletion of *LYS4* caused more detrimental effects beyond reduced growth at 37°C and including disrupted mitochondrial Fe-S cluster biosynthesis and iron homeostasis, resulting in increased sensitivity of the mutant cells to oxidative stress. Therefore, we believe that in addition to sensitivity to the host body temperature, mitochondrial dysfunction also contributes to the attenuation of the virulence in the *lys4* mutant. *Lys4* has also been characterized in *C. albicans* but does not appear to contribute to virulence [37]. Overall, we conclude that *LYS4* is not only required for lysine biosynthesis but is also necessary for the proper mitochondrial function and for the full virulence. Therefore, *Lys4* can be considered as a promising target of antifungal drugs to treat cryptococcal infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *LYS4* encoding homoaconitase in *Cryptococcus neoformans* was identified.
- *LYS4* expression is regulated by iron.
- *LYS4* plays critical roles in mitochondrial function, and antifungal sensitivity.
- *LYS4* is required for the full virulence in a mouse inhalation model of cryptococcosis.

Fig. 1.

Lys4 is required for lysine biosynthesis. (A) The growth of the *lys4* mutant in YNB media containing different nitrogen sources with or without lysine was monitored. (B) Transcript levels of the putative amino acid permease genes in the wild-type strain, grown in YNB medium with ammonium sulfate (AS) or lysine (Lys) as sole nitrogen source, were measured by quantitative real-time PCR with primers listed in Table S2. (C) Transcript levels of the amino acid permease genes, *AAP2* and *AAP3*, in the wild-type strain grown in YNB medium containing asparagine (Asn) and Lys, or AS and Lys. Data were normalized with *TEF2* as an internal control and represent the averages from three independent experiments. Error bars indicate standard deviations.

Fig. 2.

Expression of *LYS4* is regulated by iron and iron regulatory transcription factors. (A) Transcript levels of *LYS4* in the wild-type strain and the mutants lacking *CIR1*, *HAP3*, and *HAPX*, grown in low (- FeCl₃) or high iron (+ 100 μM FeCl₃) medium, were analyzed by northern blot. Ethidium bromide (EtBr) staining of rRNA was used to assess equal loading in each lane. (B) Western blot analysis using the strain expressing Lys4-Gfp. Staining with CPTA (copper phthalocyanine-3,4,4',4''-tetrasulfonic acid tetrasodium) was used to demonstrate equal loading of each sample. (C) The cells expressing Lys4-Gfp were grown 30°C for 6 hours and the fluorescence was monitored. Mitotracker was used to stain mitochondria a final concentration of 100 nM. (D) Western blot analysis using total lysates and isolated mitochondrial fraction of the cells expressing Lys4-Gfp with anti-Gfp antibody. PSTAIR was used for the cytosolic protein control.

Fig. 3.

Deletion of *LYS4* causes mitochondrial dysfunction. (A) The growth of the *lys4* mutant in YPD medium containing hydrogen peroxide (H_2O_2) was monitored. (B) Mitochondrial ROS level were measured by FACS analysis after MitoSOX treatment. The *lys4* mutant showed increased ROS levels, $186.48 \pm 6.75\%$ ($p < 0.0001$) compared to the wild-type strain. (C) The growth of the *lys4* mutant in YPD containing congo-red and sodium dodecyl sulfate (SDS) was monitored. (D) Ergosterol levels were measured using GC-MS analysis. The *lys4* mutant showed reduced ergosterol content, $21.66 \pm 4.27\%$ ($p = 0.0012$) compared to the wild-type. (E) The growth of the *lys4* mutants in YPD containing salicylhydroxamic acid (SHAM), diphenyleneiodonium (DPI) and potassium cyanide (KCN) was monitored.

Fig. 4.

LYS4 is required for mitochondrial iron metabolism and virulence. (A) Aconitase activity was determined using zymography. (B) The mitochondrial iron level in the *lys4* mutant was reduced to $55.97 \pm 23.15\%$ ($p=0.0301$) compared to the wild-type strain. (C) Growth of the *lys4* mutant at 30°C and 37°C. (D) Ten female BALB/c mice were infected intranasally with each of the strains indicated, and the survival of the mice was monitored twice per day.