Fungal Genetics and Biology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

The *Neurospora crassa* CPS-1 polysaccharide synthase functions in cell wall biosynthesis

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ARTICLE INFO

Article history:
Received 31 March 2014
Accepted 21 May 2014
Available online xxxx

Keywords:
Fungal cell wall
Cell wall polysaccharide
Neurospora crassa
Cell wall biogenesis
Polysaccharide synthase
Cell wall glucan

ABSTRACT

The *Neurospora crassa cps-1* gene encodes a polysaccharide synthase with homology to the *Cryptococcus neoformans* hyaluronic acid synthase Cps1p. Homologs of the *cps-1* gene are found in the genomes of many fungi. Loss of CPS-1 results in a cell wall defect that affects all stages of the *N. crassa* life cycle, including vegetative growth, protoperithecia (female mating structure) development, and conidia (asexual spore) development. The cell wall of *cps-1* deletion mutants is sensitive to cell wall perturbation reagents. Our results demonstrate that CPS-1 is required for the incorporation of cell wall proteins into the cell wall and plays a critical role in cell wall biogenesis. We found that the *N. crassa* cell wall is devoid of hyaluronic acid, and conclude that the polysaccharide produced by the CPS-1 is not hyaluronic acid.

1. Introduction

The cell wall is an important organelle for bacteria, archaea, fungi, and plant cells. Cell walls protect cells from environment stress and play a critical role in the interactions of cells with the environment and with other cells. Fungal cell walls consist of a cross-linked matrix of glucans, chitin, and cell wall proteins (Free, 2013; Gastebois et al., 2009; Klis et al., 2006, 2001; Latge, 2007). Research has shown that cell wall polysaccharides are needed for the formation and functionality of the fungal cell wall. Mutants affected in chitin synthases, glucan synthases, and in the post-translational modification of cell wall proteins have shown the importance of these elements for cell wall biogenesis. Fungi contain multiple chitin synthase genes, and the mutational loss of chitin synthase genes can affect the ability of the fungus to generate a normal cell wall (Free, 2013; Klis et al., 2006; Latge, 2007). Fungal cell walls have been shown to contain a variety of glucans. The most abundant glucans are β-1,3-glucan, β-1,6-glucan, and α-1,3-glucan. Most fungi have a single β-1,3-glucan synthase, and the β-1,3-glucan synthase gene is needed for cell wall synthesis. Cell wall β-1,6-glucans are found in yeast cell walls, but are absent from filamentous fungi. In the yeasts, β-1,6-glucans are used to cross-link cell wall elements together in generating a cell wall matrix (Kapteyn et al., 1996; Kollar et al., 1997; Lu et al., 1995). Cell wall α-1,3-glucans are found in many cell types and their role varies from being a critical component in *Schizzosacchromyces pombe* and *Cryptococcus neoformans* to being a cell-type specific cell wall component in *Neurospora crassa* (Free, 2013; Grun et al., 2005; Hochstenbach et al., 1998; Reese et al., 2007). The glucans and chitins are cross-linked together into a cell wall matrix by cell wall proteins having glucanase and chitinase activity. Presumably, these enzymes cleave the polymers (forward reaction) and then attach the cleaved polymers to other cell wall polymers (reverse reaction) to generate a cross-linked matrix (Cabib et al., 2007; Goldman et al., 1995; Mouyna et al., 2000). The cell wall proteins have associated O-linked and N-linked mannans or galactomannans. These polysaccharides are post-translationally added to the proteins as the proteins pass through the secretory pathway. The post-translationally added N-linked galactomannan has been shown to be needed for the incorporation of cell wall proteins into the *N. crassa* cell wall (Maddi and Free, 2010). The *dcw-1* and *dfg-5* mannanases have been shown to function in cross-linking the cell wall protein galactomannan into the glucan/chitin matrix (Maddi et al., 2012).

C. neoformans, the causative agent of fungal meningitis, has an outer capsid that extends beyond the canonical fungal cell wall, and functions as an important virulence factor. The major constituents of the outer capsid are glucuronoxylomannan, galactomylomannan, and mannoproteins. Some of the enzymes needed for the synthesis of the capsid have been identified (Doering, 2009). The

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http://dx.doi.org/10.1016/j.fgb.2014.05.009
1087-1845/

CPS1 gene encodes a hyaluronic acid synthase. Mutational and biochemical analyses of the *C. neoformans CPS1* mutant showed that Cps1p was needed for the formation of the outer capsid and that purified Cps1p was capable of synthesizing hyaluronic acid (a repeating polymer of β-1,4-glucuronic acid-β-1,3-N-acetylglucosamine) using UDP-glucuronic acid and UDP-N-acetylglucosamine as substrates (Jong et al., 2007). CPS-1 is also related to the *Streptococcus pneumoniae* type 3 polysaccharide synthase, which directs the synthesis of the type 3 polysaccharide (Chang et al., 2006). The type 3 polysaccharide is a β-1,3-glucuronic acid-β-1,4-glucose repeating polymer (Arrecubieta et al., 1996; Cartee et al., 2005).

In screening the *N. crassa* single gene deletion library for mutants that were defective in the protoperithecia (female mating structure) production, we identified the Δ*cps-1* mutants as being unable to produce protoperithecia. Our analysis of the mutants showed that the *cps-1* gene (NCU00911) plays an important role in cell wall biogenesis. In particular, we found that the incorporation of cell wall glycoprotein into the cell wall was defective in the *Δcps-1*. The mutant was affected in vegetative growth, and was unable to grow in the presence of cell wall perturbation reagents. In addition to being affected in vegetative growth and sexual development, the mutant was also affected in asexual development (production of conidia). We were unable to detect any hyaluronic acid in the *N. crassa* cell wall, and an analysis of the cell wall carbohydrates failed to detect any uronic acids. The composition of the polysaccharide produced by the CPS-1 polysaccharide synthase remains to be determined.

2. Materials and methods

2.1. Strains and growth conditions

The Δ*cps-1* mutant isolates (FGSC#16861 and #16862) were taken from the single gene deletion library, which was obtained from Fungal Genetics Stock Center (Kansas City, MO). The wild type parental *N. crassa* strains ORS-SL6 a (FGSC#4200) and 74-OR-23-IV A (FGSC# 2489), the *his-3 A* mutant (FGSC#6103), and the pBM60 plasmid used in the cloning experiments were also supplied by the Fungal Genetics Stock Center. Cultures were routinely grown on either Vogel's medium with 2% sucrose or on synthetic crossing medium with 0.5% sucrose (Davis and DeSerres, 1970). When testing for growth in the presence of cell wall perturbation reagents, the cells were inoculated onto Vogel's agar medium that was supplemented with caspofungin (10 μg/ml), calcofluor white (10 mg/ml), SDS (0.01%), glycerol (2 M) or NaCl (10%) as previously described (Maddi et al., 2012). The media were inoculated with conidia from *Δcps-1* or wild type cultures and incubated at 30 °C for 4 days. Transformation of *N. crassa* was carried out as described by Margolin et al. (1997).

N. crassa mating experiments were done as described by Davis and DeSerres (1970). To generate a *Δcps-1, his-3* strain for the *N. crassa* transformation experiments, the *Δcps-1 a* mutant (FGSC#16862) was mated with a *his-3 A* isolate (FGSC#6103) and individual ascospore progeny were isolated and tested for histidine auxotrophy, the *Δcps-1* mutant phenotype, and for hygromycin resistance. The *cps-1* deletion was created by replacing the *cps-1* coding region with a hygromycin-resistance cassette (Colot et al., 2006). A *Δcps-1* (hygromycin resistant and mutant phenotype), *his-3* (histidine auxotroph) progeny isolate was used for the transformation experiments.

To measure vegetative hyphal growth rates, a drop of conidia was placed near the edge of a Petri dish containing Vogel's sucrose medium and the Petri dish was incubated at 30 °C. The distance traveled by the growing edge of the colony was then measured as a function of time.

2.2. Cloning, RIP mutagenesis, complementation, and Western blot experiments

To clone the *cps-1* gene, the genome sequences from 1538 bp upstream to 678 bp downstream of the *cps-1* coding region were PCR amplified using the *cps-1* 5′ end forward and 3′ end reverse primers (Table 1). The PCR product was digested with *SpeI* and *NotI* and cloned into a *SpeI* and *NotI* digested pBM60 vector to create pCPS-1. The pCPS-1 plasmid contains sequences from the *his-3* gene and the intergenic region 3′ of the *his-3* gene. Upon transformation, homologous recombination between the vector and genome generated a *his3+* allele and inserted the *cps-1* gene into the intergenic region 3′ of *his-3*. Individual transformants were picked from unsupplemented sorbose agar plates as *his3+* isolates and further characterized.

The *N. crassa* RIP phenomenon can be used to create multiple mutations in a targeted gene (Selker, 1999). In the *N. crassa* RIP process, genes that are present in two or more copies in the haploid genome receive multiple C-to-T mutations during the sexual cycle. To demonstrate that mutations in *cps-1* give rise to the mutant phenotype, the 5′ end of the gene was cloned and used in a RIP experiment. For this experiment, *cps-1* RIP forward and reverse primers (Table 1) were used to PCR amplify the 5′UTR, the first exon and a portion of the first intron of *cps-1*. The PCR product was digested with *XmaI* and *SpeI* and ligated into an *XmaI* and *SpeI* digested pBM61 vector to create pCPS1-5′. pCPS1-5′ was used to transform the *his-3A* mutant (FGSC#6103) to generate an isolate having two copies of the *cps-1* sequences (the endogenous copy and the transforming copy at the *his-3+* locus). Transformants were mated with a *his-3* isolate to activate the RIP process. The *his-3* progeny from this mating will contain a single copy of *cps-1* at the endogenous locus. Progeny having the *his-3, cps-1* mutant phenotype were isolated and the *cps-1* gene sequences were amplified from the genomic DNA and sequenced.

To detect CPS-1 inside the cell, an HA-tagged CPS-1 protein was generated by inserting the HA-tag sequence YPYDVPDYA right before the stop codon. This was done in a two-step process. First, two PCR products were generated using primer pair *cps-1* 5′ end forward primer and *HA-cps-1* reverse primer and primer pair *HA-cps-1* forward primer and *cps-1* 3′ end reverse primer (Table 1). Second, these two PCR products were combined and used as template for a second PCR reaction using primer set *cps-1* 5′ end forward primer and *cps-1* 3′ end reverse primer. The final PCR product was digested with *SpeI* and *NotI* and cloned into *SpeI* and *NotI* digested pBM60 to create pHA-CPS1. The HA-tagged construct was then transformed into a Δ*cps-1, his-3* isolate. To detect HA-tagged CPS-1 protein expression, Western blot analyses were performed. The transformed cells were inoculated on cellophane filters placed on top of Vogel's sucrose agar medium plates. After 24 h of growth at room temperature, the cellophane filters were peeled off of the agar and ground in liquid nitrogen. Protein extraction buffer (100 mM Tris/HCl pH 7.4, 1% SDS, supplemented with 1× protease inhibitor cocktail (P-8340 Sigma Aldrich, St. Louis, MO)) was added to the samples and the supernatant was collected after centrifugation. Protein concentrations were determined by using the *DC* protein assay kit (BioRad, Hercules, CA). Samples containing 60 μg of protein were subjected to SDS–PAGE and transferred to nitrocellulose membranes. The nitrocellulose membranes were subjected to Ponceau S red (Sigma Aldrich, MO) staining to verify equal loading of the different protein samples. Mouse monoclonal anti-HA (Covance, Princeton, NJ) and Rabbit anti-mouse IgG-HRP (Sigma Aldrich) were used to assess the levels of HA-CPS-1 protein expression. The ChemiDoc XRS + chemiluminescent detection system was used for the Western blots and the images were analyzed with Bio Rad Image Lab software.

Please cite this article in press as: Fu, C., et al. The *Neurospora crassa* CPS-1 polysaccharide synthase functions in cell wall biosynthesis. Fungal Genet. Biol. (2014), http://dx.doi.org/10.1016/j.fgb.2014.05.009