Fungal Genetics and Biology 47 (2010) 855–868

Contents lists available at ScienceDirect

Fungal Genetics and Biology

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

Genes encoding a striatin-like protein (*ham-3*) and a forkhead associated protein (*ham-4*) are required for hyphal fusion in *Neurospora crassa*

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ARTICLE INFO

Article history:
Received 31 March 2010
Accepted 16 June 2010
Available online 1 July 2010

Keywords:
Cell fusion
Neurospora
Striatin
Far complex
Forkhead associated (FHA) protein
STRIPAK

ABSTRACT

Cell–cell fusion during fertilization and between somatic cells is an integral process in eukaryotic development. In *Neurospora crassa*, the hyphal anastomosis mutant, *ham-2*, fails to undergo somatic fusion. In both humans and *Saccharomyces cerevisiae*, homologs of *ham-2* are found in protein complexes that include homologs to a striatin-like protein and a forkhead-associated (FHA) protein. We identified a striatin (*ham-3*) gene and a FHA domain (*ham-4*) gene in *N. crassa*; strains containing mutations in *ham-3* and *ham-4* show severe somatic fusion defects. However, *ham-3* and *ham-4* mutants undergo mating-cell fusion, indicating functional differences in somatic versus sexual fusion events. The *ham-2* and *ham-3* mutants are female sterile, while *ham-4* mutants are fertile. Homozygous crosses of *ham-2*, *ham-3 and ham-4* mutants show aberrant meiosis and abnormally shaped ascospores. These data indicate that, similar to humans, the HAM proteins may form different signaling complexes that are important during both vegetative and sexual development in *N. crassa.*

1. Introduction

Cell signaling resulting in cell–cell fusion is integral to a multitude of eukaryotic processes. Examples include fertilization of an egg by sperm and osteoclast development in mammals (Primakoff and Myles, 2007; Vignery, 2008; Zeng and Chen, 2009), pollen tube and ovary fusion in plants (Higashiyama et al., 2003), and the development of filamentous colonies of fungi (Buller, 1933; Fleissner et al., 2008; Read et al., 2010). Unlike unicellular yeasts, such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, where cell fusion is only associated with mating, in filamentous fungi, cell fusion also occurs between vegetative cells. Somatic cell fusion is important in colony establishment and development of the interconnected hyphal network characteristic of these organisms. Connectivity in fungal networks is presumed to be necessary for maintaining proper intercolony communication, resource exploitation, virulence in pathogens, and maintaining homeostasis (Reviewed in Read et al. (2010), Fleissner et al. (2008), Glass et al. (2004), Rayner (1991) and Rayner (1996)).

A number of mutants deficient in hyphal fusion and/or signaling have been characterized in the filamentous ascomycete fungus, *Neurospora crassa* (Read et al., 2010). Many of these mutants have a decreased growth rate and are female sterile. One gene required for vegetative and germling fusion in *N. crassa* is *ham-2*, which encodes a protein with multiple transmembrane domains and a C-terminal domain of unknown function that is conserved in fungi and animals, but not in plants (Xiang et al., 2002). The *ham-2* mutant does not form conidial anastomosis tubes (CATs) and neither attracts nor responds to the presence of a wild-type germling (Roca et al., 2005).

Homologs of *ham-2* have been identified in protein complexes in both yeast and humans. Far11, a protein encoded by the *S. cerevisiae* homolog of *ham-2*, was shown to be part of a complex composed of Far3, Far7, Far8, Far9 and Far10 (Kemp and Sprague, 2003; Uetz et al., 2000). Far proteins are required for maintenance of G1 cell cycle arrest after pheromone stimulation (Kemp and Sprague, 2003). In humans, homologs of *ham-2* have been identified in a complex that acts as a regulatory subunit to protein phosphatase 2A (PP2A), and which also includes a protein similar to Far8 (striatin), and a protein similar to Far9/10 (similar to sarcolemmal membrane-associated protein, SLMAP) which contains a forkhead-associated domain (Goudreault et al., 2008). In addition, this complex contains a MOB3 homolog. Intriguingly, *mob-3* mutants were shown to be hyphal fusion mutants in *N. crassa* (Maerz et al., 2009).

In addition to *ham-2*, a genome wide search revealed that only two additional homologs to the *S. cerevisiae FAR* genes were present in the *N. crassa* genome (Glass et al., 2004), a homolog of *FAR8* (similar to striatin) and a homolog of *FAR9/10* (SLMAP). No homologs of *FAR3* or *FAR7* were identified. In this study, we

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1087-1845/$ - see front matter © 2010 Published by Elsevier Inc.
doi:10.1016/j.fgb.2010.06.010

evaluate whether mutations in the *FAR8* and *FAR9/10* homologs in *N. crassa*, termed *ham-3* and *ham-4* respectively cause a similar cell fusion phenotype to that observed in *ham-2* mutants. We show that *ham-2*, *3* and *4* mutants share a similar vegetative fusion defect, but undergo sexual cell fusion. However, the *ham* mutants show an abnormal meiosis phenotype as well as aberrant ascospore development. The phenotypic similarities between *ham-2*, *3* and *4* mutants suggest they are in the same pathway and regulate diverse cellular processes during both vegetative growth and sexual reproduction in *N. crassa*.

2. Materials and methods

2.1. Strains, growth media and conditions

The strains used in this study are listed in Table 1. Strains were grown on Vogel's minimal medium (Vogel, 1956) with required supplements. BDES medium was used to induce colonial growth (Brockman and de Serres, 1963). Crosses were performed on Westergaards's medium (Westergaard and Mitchell, 1947). The *ham-4* (NCU00528; *Δham-4*) deletion strains, the *ham-3* (NCU08741; *Δham-3*) deletion strains, and the *ham-2* (NCU03727; *Δham-2*) deletion strain (Table 1) were obtained from The Fungal Genetics Stock Center (FGSC) (Colot et al., 2006; McCluskey, 2003). The strain FGSC 4564 was used as a helper for crosses when the female could not produce sexual structures (Perkins, 1984). Growth rates were assessed using the race tube method (Ryan et al., 1943). Electroporation was performed according to (Margolin et al., 1997) with 1.5 kV setting.

2.2. Nucleic acid techniques

Genomic DNA was isolated as described (Lee et al., 1988). Southern hybridization was performed as described (Sambrook and Russell, 2001). Sequencing was performed by the Berkeley DNA sequencing facility (http://mcb.berkeley.edu/barker/dnaseq). Oligonucleotides were obtained from MWG Biotech (www.mwg-biotech.com) and IDT (www.idt.com). The following primers were used: YDR200CFOR371 AGCAACAGCAGCCATCATCG, YDR200CREV2193 TTGTATCAACGCACGCTCCTG, vps64-16 FOR TCTAGACATGTTCATCTCGCAAAATCTCTTCAAC, vps64-16 REV TTAATTAAATTCTTCGCCTGCGGCTGCCACC. Taq polymerase (Promega) was used for routine PCR and PfuTurbo (Stratagene) or Phusion (Finnzymes) was used for high fidelity PCR for cloning.

Repeat induced point (RIP) mutation (Selker, 2002), a naturally mutagenic process in *N. crassa* was used to create *ham-4* point mutation mutants. A fragment amplified by oligonucleotides YDR200CFOR371 and YDR200CREV2193 was cloned into pCB1004, and transformed into strain I-1-83 (Table 1). Hygromycin-resistant transformants were crossed to FGSC 988 to obtain RIP mutants. Progeny were screened for sensitivity to hygromycin (to insure loss of the ectopic transformed *ham-4* fragment and retention of the native mutated *ham-4* locus) and morphological defects. Resulting mutant progeny identified by short aerial hyphae (~24%) were screened for restriction length fragment polymorphisms (RFLPs) at the *ham-4* locus (NCU00528) using a PCR product amplified using oligonucleotides *vps64*-16 FOR and *vps64*-16 REV and digested with *Sau3A* (New England Biolabs).

Cassettes were kindly provided by Hildur Colot for knocking out *vps39* (NCU01539). Deletion strains were constructed as previously described (Colot et al., 2006) and Southern blotting was used to confirm correct integration of the *hph* cassette (data not shown).

The striatin domain, WD repeats, and N221 like protein domain were identified according to Pfam (http://pfam.sanger.ac.uk/). Transmembrane sequences were identified using TopPred (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py) and verified using TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html). Coils (http://www.ch.embnet.org/software/COILS_form.html) and Paircoil2 (http://groups.csail.mit.edu/cb/paircoil2/paircoil2.html) were used to predict coiled-coil domains in the protein sequences. The calmodulin-binding motif was identified using The Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/pub_pages/search/search.htm). A caveolin-binding motif was found in the striatin domain of *ham-3*, which is consistent with the sequence of the striatin domain in *F. verticillioides* FSR-1 and *S. macrospora* PRO11 (Shim et al., 2006).

2.3. Quantitative heterokaryon and conidia formation test

A heterokaryon test between mutant strains and a wild-type tester strain was performed to assess hyphal fusion frequency as previously described (Xiang et al., 2002). A conidial suspension of ~10^7 conidia of the heterokaryon tester strain FGSC 4564 was mixed with ~10^3 conidia of the strains CR16-7 (*ham-4*^{RIP1}), CR16-16 (*ham-4*^{RIP3}), AS2-4 (*his-3 ham-3*::*hph*), AS1-40 (*his-3 ham-4*::*hph*), the negative control strain CR1-10 (*ham-2*^{RIP1}) and the wild-type positive control strain I-1-83 or FGSC 6103. The mixed conidial suspensions were plated on BDES minimal media and grown for 6 days. The colonies resulting from fusion events to form a heterokaryon were counted. The amount of viable conidia in each suspension was determined by germinating the conidia on BDES plates containing supplements (adenine and histidine) and counting the number of resulting colonies. This experiment was repeated three times with similar results.

The amount of conidia each strain produced was determined by inoculating conidia into Vogel's MM tubes and allowing strains to grow for 5 days at 25 °C. After 5 days, 1 ml of water was added to the tube and vigorously vortexed (30 s) to release conidia. The conidia were then counted using a hemacytometer. This experiment was repeated three times with similar results.

2.4. Microscopy

Conidial fusion was assessed using 3–5 day old cultures grown in Vogel's MM (Vogel, 1956) tubes at room temperature (~22 °C). One ml of water was added to the tubes and vigorously vortexed. Conidia were filtered through cheesecloth to remove mycelia,

Table 1
Strains used in this study.

Strain	Genotype	Origin
FGSC 988	ORS 8-1 *a*	FGSC
FGSC 2489	74 OR23 *A*	FGSC
FGSC 6103	*his-3 A*	FGSC
FGSC 4564	*ad-3B cyh-1 a*^{ml}	FGSC
FGSC 11299	*ham-3*::*hph A*	FGSC
FGSC 11300	*ham-3*::*hph a*	FGSC
FGSC 12081	*ham-4*::*hph A*	FGSC
FGSC 12080	*ham-4*::*hph a*	FGSC
FGSC 12091	*ham-2*::*hph A*	FGSC
I-1-83	*ad-3A his-3 A*	Gift from A. J. Griffiths
AS2-4	*his-3*; *ham-3*::*hph a*	FGSC 11300 × FGSC 6103
AS1-40	*his-3 ham-4*::*hph A*	FGSC 12080 × FGSC 6103
AS3-1	*his-3*; *ham-2*::*hph a*	FGSC 12091 × FGSC6103
CR16-7	*ad-3A his-3 ham-4*^{RIP1} *a*	FGSC 988 × I-1-83 (*ham-4*^{RIP1})
CR16-8	*ham-4*^{RIP2} *a*	FGSC 988 × I-1-83 (*ham-4*^{RIP2})
CR16-16	*ad-3A his-3 ham-4*^{RIP3} *A*	FGSC 988 × I-1-83 (*ham-4*^{RIP3})
CR1-10	*pyr-4*; *ham-2*^{RIP1} *A*	Xiang et al. (2002)
CR3-17	*ham-2*^{RIP} *A*	Xiang et al. (2002)
CR65-1	*vps39*::*hph A*	*mus-51* (*NCU01539*::*hph*) × FGSC 988
P1-54	*his-3 Sad-1*^{RIP78} *mep A*	Gift from P. Shiu
P1-68	*his-3 Sad-1*^{RIP141} *mep a*	Gift from P. Shiu
R11-03	*H1*::*GFP A*	Gift from D. Jacobson
R12-60	*H1*::*GFP a*	Gift from D. Jacobson