Fungal Genetics and Biology 45 (2008) 963–972

Contents lists available at ScienceDirect

Fungal Genetics and Biology

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

Aspergillus nidulans ChiA is a glycosylphosphatidylinositol (GPI)-anchored chitinase specifically localized at polarized growth sites

Harutake Yamazaki [1], Aya Tanaka, Jun-ichi Kaneko, Akinori Ohta, Hiroyuki Horiuchi *

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

ARTICLE INFO

Article history:
Received 26 November 2007
Accepted 25 February 2008
Available online 10 March 2008

Keywords:
Chitinase
Aspergillus nidulans
GPI-anchor
Cell wall
O-Glycosylation

ABSTRACT

It is believed that chitinases play important physiological roles in filamentous fungi since chitin is one of the major cell wall components in these organisms. In this paper we investigated a chitinase gene, *chiA*, of *Aspergillus nidulans* and found that the gene product of *chiA* consists of a signal sequence, a region including chitinase consensus motifs, a Ser/Thr/Pro-rich region and a glycosylphosphatidylinositol (GPI)-anchor attachment motif. Phosphatidylinositol-specific phospholipase C treatment of the fusion protein of ChiA and enhanced green fluorescent protein (EGFP)—ChiA–EGFP—caused a change in its hydrophobicity, indicating that ChiA is a GPI-anchored protein. ChiA–EGFP localized at the germ tubes of conidia, at hyphal branching sites and hyphal tips. *chiA* expression was specifically high during conidia germination and in the marginal growth regions of colonies. These results suggest that ChiA functions as a GPI-anchored chitinase at the sites where cell wall remodeling and/or cell wall maturation actively take place.

1. Introduction

The cell wall of filamentous fungi is a complex structure mainly composed of polysaccharides, chitin and glucans. It is very important in fungal morphogenesis and in protection from diverse environmental stresses. Although the fungal cell wall has been considered to be an inert organelle, recent analyses revealed that it is a dynamic organelle in which constituent polymers are continuously synthesized, degraded, and chemically modified, and their structures are rearranged (Bernard and Latgé, 2001; Popolo et al., 2001). In the model fungus *Aspergillus nidulans*, chitin—a homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine—is one of the major cell wall components. Six chitin synthase-encoding genes have been cloned and characterized in *A. nidulans*, demonstrating that chitin synthases play critical roles in hyphal growth and conidiophore development (Borgia et al., 1996; Fujiwara et al., 2000; Horiuchi et al., 1999; Ichinomiya et al., 2005; Motoyama et al., 1997; Takeshita et al., 2005, 2006; Yanai et al., 1994).

Chitinases (EC 3.2.1.14), which hydrolyze the β-1,4-glycosidic linkage of chitin, are widely distributed in living organisms. Recent analyses of the whole genome sequence of ascomycete filamentous fungi revealed that there are more than 10 putative chitinase-encoding genes in the genomes of these organisms (Pusztahelyi et al., 2006; Seidl et al., 2005; Taib et al., 2005).

However, the functions of most of these chitinases are currently not well understood. All of the chitinase genes analyzed thus far from yeast and fungi encode chitinases belonging to glycosyl hydrolase family 18 (Henrissat, 1991). These chitinases are further divided into two subclasses, class III and class V. In filamentous fungi, chitinases are considered to function in processes that include cell-wall degradation and modification, such as spore germination, tip growth and branching of hyphae, spore differentiation, autolysis and mycoparasitism (Adams, 2004; Gooday et al., 1992). Deletion of the genes *chiB1* of *Aspergillus fumigatus* or the *Cts1* of *Coccidioides immitis* that encode class V chitinases had no effect on the growth or morphogenesis of these organisms (Jaques et al., 2003; Reichard et al., 2000). A class V chitinase from the zygomycete *Rhizopus oligosporus* was suggested to function in the growth and morphogenesis of this fungus (Takaya et al., 1998b). Recently, we showed that a class V chitinase, ChiB, was induced by carbon starvation and functioned in the autolytic process of *A. nidulans* (Yamazaki et al., 2007). Induction of *chiB* expression under these conditions was also reported (Emri et al., 2006; Pusztahelyi et al., 2006). Class III chitinases are required for cell separation in *Saccharomyces cerevisiae* and *Candida albicans* (Dünkler et al., 2005; Kuranda and Robbins, 1991). In *R. oligosporus*, two class III chitinases were suggested to be primarily involved in autolysis (Takaya et al., 1998b; Yanai et al., 1992). We have previously reported that an *A. nidulans* deletion mutant of *chiA*—a class III chitinase-encoding gene—showed defects in spore germination and hyphal growth (Takaya et al., 1998a). However,

* Corresponding author. Fax: +81 3 5841 8015.
E-mail address: ahhoriu@mail.ecc.u-tokyo.ac.jp (H. Horiuchi).
[1] Present address: Department of Gene Mechanisms, Graduate School of Biostudies, Kyoto University Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan.

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doi:10.1016/j.fgb.2008.02.008

these defective phenotypes were probably caused by a cryptic mutation(s) at a site other than the *chiA* locus because newly generated *chiA* deletion mutants did not show any phenotypic defects (Yamazaki et al., 2007).

In the present study, we show that (i) ChiA is synthesized as a GPI-anchored protein and modified by *O*-linked glycosylation, (ii) the expression level of *chiA* is very high during germination and in the marginal growth regions of colonieis and (iii) ChiA tagged with EGFP (enhanced green fluorescent protein) is specifically localized at polarized growth sites.

2. Materials and methods

2.1. Strains, cultures and media

Aspergillus nidulans strains used in this study are shown in Table 1. Minimal medium containing 1% glucose as a sole carbon source (MMG) and YG medium (0.5% yeast extract, 1% glucose, and 0.1% trace elements) for *A. nidulans* were used (Rowlands and Turner, 1973). To induce expression of genes under the control of *alcA* promoter (*alcA*(p)), we used 100 mM threonine and 0.1% fructose as carbon sources instead of glucose (MMTF or YTF). Media were supplemented with 0.2 mg/ml arginine, 0.02 mg/ml biotin, 0.5 mg/ml pyridoxine, 2.44 mg/ml uridine and 1.22 mg/ml uracil when necessary, and each supplement added was indicated by a single lowercase letter after the medium name. Strains were grown at 37 °C. Genetic transformation of *A. nidulans* was done by the method of Rasmussen et al. (1990).

2.2. Constructions of plasmids and A. nidulans strains

The 1.4-kb EcoRV–SacI fragment of pBP4 containing the *chiA* gene of *A. nidulans* (Takaya et al., 1998a) was ligated into HincII site of pUC19, to yield pES. For insertion of NotI site just upstream of the coding sequence of the Ser/Thr/Pro-rich region, polymerase chain reaction (PCR) was done for pES by using TaKaRa LA PCR in vitro Mutagenesis Kit (TaKaRa) with the primer 5′-AGGGGAGGTGGTGCGGCCGCGATGGTTGGGGTC-3′ (the under lined sequence represents the NotI recognition site), and the resulting DNA fragment digested with BamHI–SphI was ligated into pUC19 digested with BamHI–SphI, to yield pS13. A primer set of 5′-GGGCGGCCGCATGGTGAGCAAGGGCGAG-3′ and 5′-GGGCGGCCGCTCTTGTACAGCTCGTCCATG-3′ (the under lined sequences represent the NotI recognition sites) was used to amplify an EGFP-encoding sequence of pEGFP (Clontech). The 0.7-kb PCR-amplified product digested with NotI was ligated into pS13 digested with NotI, to yield pS13EGFP. The 1.8-kb StuI–SphI fragment of pBP4 and pS13EGFP digested with StuI–SmaI were ligated, blunted and ligated, to yield pS13EGFPC. The 1.7-kb XbaI–SphI fragment containing the *argB* gene of *A. nidulans* form pSS1 (Motoyama et al., 1994) was ligated into pS13EGFPC digested with SphI, to yield pCHIAEGFPARGB. The ChiA–EGFP strain was constructed by transformation of the strain ABPUS14 (Yamada et al., 2005) with NruI-digested pCHIAEGFPARGB. Arginine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which *chiA* was replaced by *chiA–egfp* (Fig. 1A).

A primer set of GATGCGGCCGCGTGAGCAAGGGCGAGG and GTCGCGGCCGCTTTAC (the under lined sequences represent the NotI recognition sites) was used to amplify an EGFP-encoding sequence of pEGFP. The 0.7-kb PCR-amplified product digested with NotI was ligated into NotI-digested pBluescript SK$^+$, to yield pEGFP-Not. The 3.2-kb PstI–XbaI fragment containing the *sC* gene of *A. nidulans* from pUSC (Yamada et al., 1997) was ligated into pEGFP-Not digested with BamHI–PstI, to yield pEGFP–sC. pEGFP–sC was partially digested with NotI and self-ligated for deletion of the NotI site between *egfp* and *sC*, to yield pEGFP–sCNX. The 3.9-kb NotI–BssHII fragment of pEGFP–sCNX containing *egfp* and *sC* and pS13 digested with NotI were ligated, blunted and ligated, to yield pSTPEGFPsC. The ChiAn–EGFP strain was constructed by transformation of the strain ABPUS14 with the 5.3-kb PstI–StuI fragment of pSTPEGFPsC. Methionine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which *chiA* was replaced by *chiAn–egfp* (Fig. 1A).

The 0.4-kb EcoRI–KpnI fragment containing the alcohol dehydrogenase gene promoter (*alcA*(p)) of *A. nidulans* from pAL3 (Waring et al., 1989) was ligated into pBluescript II digested with XbaI, to yield pBSalc. The 0.6-kb BamHI–PflMI fragment of pB3 containing the 5′-region of *chiA* gene of *A. nidulans* (Takaya et al., 1998a) and pBSalc digested with BamHI were ligated, blunted and ligated, to yield pNalc. The 3.2-kb PstI–XbaI fragment of pUSC containing *sC* and the 1.7-kb XbaI–SphI fragment of pSS1 containing *argB* were ligated into pNalc digested with SpeI, to yield pNalcsC and pNalcarg, respectively. The 2.5-kb MseI fragment of pBP4 was ligated into pNalcsC and pNalcarg digested with NotI, to yield pchiAalcsC and pchiAalcarg, respectively.

alc-ChiA–EGFP strain was constructed by transformation of the strain ChiA–EGFP with the 6.4-kb BamHI fragment of pchiAalcsC. Methionine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which ChiA–EGFP fusion protein was expressed under the control of *alcA*(p) at *chiA* locus.

alc-ChiAn–EGFP strain was constructed by transformation of the strain ChiAn–EGFP with the 3.2-kb BamHI–BglI fragment of pchiAalcarg. Arginine-prototrophic transformants were selected, and their total DNAs were used for Southern blot analysis to obtain clones in which ChiAn–EGFP fusion protein was expressed under the control of *alcA*(p) at *chiA* locus.

alc-ChiA strain was constructed by transforming ABPUS14 with the 6.4-kb BamHI fragment of pchiAalcsC. Methionine-prototro-

Table 1
Aspergillus nidulans strains used in this study

Strain	Genotype	Reference
A26	*biA1*	FGSC*
ABPUS14	*biA1 pyrG89; wA3*; *argB2 sC114*; *pyroA4*	Yamada et al. (2005)
ABPUS/sC-2	*biA1 pyrG89; wA3*; *argB2 sC114*::*sC*; *pyroA4*	Yamada et al. (2005)
ABPUS/sC-2/argB8, 9	*biA1 pyrG89; wA3*; *argB2*::*argB sC114*::*sC*; *pyroA4*	This study
ABPUS/sC-2/argB9/pyroA17	*biA1 pyrG89; wA3*; *argB2*::*argB sC114*::*sC*; *pyroA4*::*pyroA*	This study
ABPUS/sC-2/argB9/pyroA17/pyrG20	*biA1 pyrG89*::*pyrG; wA3*; *argB2*::*argB sC114*::*sC*; *pyroA4*::*pyroA*	This study
alc-ChiA	*biA1 pyrG89; ΔchiA*::*sC-alcA*(p)-*chiA wA3*; *argB2 sC114*; *pyroA4*	This study
alc-ChiA/argB	*biA1 pyrG89; ΔchiA*::*sC-alcA*(p)-*chiA wA3*; *argB2*::*argB sC114*; *pyroA4*	This study
ChiA–EGFP	*biA1 pyrG89; ΔchiA*::*chiA-egfp-argB wA3*; *argB2 sC114*; *pyroA4*	This study
ChiAn–EGFP	*biA1 pyrG89; ΔchiA*::*chiAn-egfp-argB wA3*; *argB2 sC114*; *pyroA4*	This study
alc-ChiA–EGFP	*biA1 pyrG89; ΔchiA*::*sC-alcA*(p)-*chiA-egfp-argB wA3*; *argB2 sC114*; *pyroA4*	This study
alc-ChiAn–EGFP	*biA1 pyrG89; ΔchiA*::*argB-alcA*(p)-*chiAn-egfp-sC wA3*; *argB2 sC114*; *pyroA4*	This study

* Fungal Genetic Stock Center (University of Missouri, Kansas City).