Physiological Significance of Glycolipid Catabolism in *Cryptococcus neoformans*

Takashi Watanabe; Yohei Ishibashi; and Makoto Ito

Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University,
6–10–1 Hakozaki, Higashi-ku, Fukuoka 812–8581, Japan
FAX: +81–92–642–2907, E-mail: makotoi@agr.kyushu-u.ac.jp

(Received on March 26, 2015, accepted on April 13, 2015)

Key Words: glycolipid catabolism, glucosylceramide quality control, sterylglucoside, *Cryptococcus neoformans*, capsule, vacuole

Abstract

The pathogenic fungus *Cryptococcus neoformans* causes cryptococcosis, an opportunistic infectious disease resulting in 600,000 deaths per year. The two major glycolipids in *C. neoformans* are glucosylceramide (GlcCer) with a fungus-specific ceramide (methyl d18:2/h18:0) and ergosteryl β -glucoside (EG); however, the catabolic pathway of these glycolipids has not yet been uncovered. We found two homologues of endoglycoceramidase (EGCase, EC 3.2.1.123) in *C. neoformans*, designated Endoglycoceramidase-related Protein 1 and 2 (EGCrP1 and EGCrP2). EGCase hydrolyzes the *O*-glycosidic linkage between oligosaccharides and ceramides in various glycosphingolipids. However, EGCrP1 and EGCrP2 show completely different specificities; that is, EGCrP1 is a neutral glucocerebrosidase specific to GlcCer, whereas EGCrP2 is an acid β -glucosidase capable of hydrolyzing not only GlcCer but also various β -glucosides, including *p*NP β -glucoside and EG. Using each disruption mutant of *egcrp1* and *egcrp2*, we elucidated that EGCrP1 plays an integral role in quality control of the fungus-specific GlcCer by eliminating immature GlcCer, which are by-products of the GlcCer synthesis pathway, whereas EGCrP2 is involved in the catabolism of EG in the vacuoles of *C. neoformans*. The analysis of *egcrp1*-disrupted mutants also revealed that the quality control of fungus-specific GlcCer is strongly linked to the formation of the polysaccharide capsule, an important virulence factor. On the other hand, the disruption of EG catabolism resulted in growth arrest, dysfunction in cell budding, and abnormal vacuole morphology. These results indicate that catabolism of two different glycolipids plays different physiological roles in *C. neoformans* and strongly suggest EGCrP1 and EGCrP2 as targets for anti-cryptococcal drugs with a new mechanism of action.

A. Introduction

The most characteristic feature of *Cryptococcus neoformans* is a thick polysaccharide capsule that surrounds the cell body (Fig. 1) (1). The capsule allows *C. neoformans* to invade and remain in hosts because it protects against phagocytosis by macrophages (2). The capsule also allows the fungi to grow in phagocytic cells by protecting against attacks by host cells (3, 4). In addition, the polysaccharide capsule was demonstrated to be an important virulence factor that allows *C. neoformans* to infect humans. Typical symp-

toms of cryptococcosis are meningitis and pneumonia, which occur frequently in immunodeficient patients, such as those with AIDS. It is estimated that cryptococcosis causes one million infections and 600,000 deaths per year; it is especially prevalent in Africa (5, 6). *C. neoformans* is primarily an opportunistic pathogen; in contrast, *C. gattii* causes cryptococcosis in immunocompetent individuals (7). Although *C. neoformans* is distributed worldwide, *C. gattii* is restricted to tropical areas. In the early 2000s, *C. gattii* gained attention following an outbreak on Vancouver Island in Canada and the United States, which may have occurred due to global warming (8–11).

Cryptococcosis is classified as a deep-seated mycosis. Several antifungal drugs have been developed for the treatment of this fungal disease. Amphotericin B, which binds ergosterol and forms pores in the fungal membrane, is frequently used to treat mycoses, including cryptococcosis. Other drugs used against cryptococcosis include fluconazole and flucytosine; the former inhibits ergosterol synthesis, and the latter, DNA synthesis (Fig. 9). However, these drugs cause severe side effects and have resulted in the appearance of a highly drug-resistant form of *C. neoformans* (12). Thus, the development of new anti-cryptococcal drugs is urgently required.

The major glycolipids in *C. neoformans* are glucosylceramide (GlcCer) and ergosteryl β -glucosides (EG) (13–15); however,

Fig. 1. Polysaccharide capsule surrounding *C. neoformans*. Left panel, wildtype; right panel, *egcrp1*-disrupted mutant (*egcrp1Δ*). A thick polysaccharide capsule is the most characteristic feature of *C. neoformans* and deeply related to the pathogenicity of the fungus. The disruption of *egcrp1* leads to a thinner polysaccharide capsule than that of the wildtype.

the catabolic pathways of these glycolipids were unknown until we discovered EGCrP1 and EGCrP2 (described in chapter B-2), GlcCer-degrading and EG-degrading enzymes, respectively. EGCrP1 and EGCrP2 were first identified as homologues of endoglycoceramidase (EGCase), which is an enzyme capable of hydrolyzing the *O*-glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids (GSLs). In this review, we describe the historical background, physiological significance, and future direction of EGCrP1 and EGCrP2. We emphasize the physiological significance of catabolic pathways of glycolipids driven by these enzymes in *C. neoformans*, and finally discuss the possibility of the development of anti-cryptococcal drugs with these fungal glycosidases as target molecules.

B. Quality Control of Fungus-specific GlcCer with EGCrP1 in *C. neoformans*

B-1. Synthesis Pathway of Fungus-specific GlcCer

A large number of fungi, including *C. neoformans*, synthesize a fungus-specific GlcCer with unique structural features in its ceramide moiety: two double bonds at C4 and C8 in the *trans* conformation, and a methyl substituent at C9 in the sphingoid base (methyl d18:2/h18:0) (Fig. 2). The synthesis pathway of fungus-specific GlcCer has been elucidated. Dihydrosphingosine is acylated by ceramide synthase to form ceramide (d18:0/h18:0) (16), which is sequentially converted to ceramide (d18:0/h18:0) by fatty acid 2-hydroxylase (17), to ceramide (d18:1/h18:0) by sphingolipid delta 4-desaturase (18), to ceramide (d18:2/h18:0) by sphingolipid delta 8-desaturase (19), and finally to fungus-specific ceramide (methyl d18:2/h18:0) by sphingolipid 9-methyltransferase (Fig. 2) (20, 21). The genes encoding delta 8-desaturase

Fig. 2. Fungus-specific GlcCer synthesis pathway in *C. neoformans*, including ceramide maturation and GlcCer quality control processes. *C. neoformans* possesses GlcCer as a sole glycosphingolipid (GSL). In *C. neoformans*, dihydroceramide (d18:0/h18:0) is converted to fungus-specific ceramide (methyl d18:2/h18:0) by the action of several ceramide modification enzymes. This process is designated 'ceramide maturation process' in this review. Finally, fungus-specific ceramide is converted to fungus-specific GlcCer by GCS1; however, immature ceramide is also converted to immature GlcCer by GCS1 due to the loose specificity of GCS1. EGCrP1 is a glucocerebrosidase responsible for bringing immature ceramides back into the 'ceramide maturation process' by degrading immature GlcCer. This function of EGCrP1, designated 'quality control of GlcCer' in this review, is integral for the efficient production of fungus-specific GlcCer and capsule formation.

and sphingolipid 9-methyltransferase were found in fungi but not in mammals. In this review, this ceramide synthesis (modification) pathway is designated ‘ceramide maturation process,’ and a mature form of ceramide (methyl d18:2/h18:0) and a mature form ceramide-containing GlcCer are designated ‘mature ceramide’ and ‘mature GlcCer,’ respectively (Fig. 2). In contrast, ‘immature ceramide’ indicates ceramide composed en route to sphingosine and fatty acid by a ceramide maturation process, and ‘immature GlcCer’ indicates GlcCer containing immature ceramide. Interestingly, disruption of these ceramide maturation enzymes resulted in a lack of mature GlcCer and the attenuation of virulence of the pathogenic fungi *C. neoformans* and *Candida albicans*, indicating that the ceramide maturation process is essential for virulence (21, 22). Immature GlcCer possessing d18:1/h18:0, d18:2/h18:0 could not compensate for the function of mature GlcCer (19, 21–23). GlcCer is synthesized by the transfer of glucose from UDP-glucose to ceramide by GlcCer synthase (GCS1, EC 2.4.1.80) in *C. neoformans* (24) (Fig. 2). GCS1 adopts not only mature ceramide but also immature ceramide as a substrate (25); therefore, immature GlcCer could also be generated during the process of GlcCer synthesis *in vivo* (Fig. 2). Importantly, once immature ceramide is converted to immature GlcCer by GCS1, maturation of the ceramide moiety is halted and immature GlcCer accumulates because enzymes involved in maturation process of ceramide, such as sphingolipid delta 4-desaturase and 9-methyltransferase, utilize a ceramide but not GlcCer as a substrate (20, 26). However, almost all GlcCer in *C. neoformans* is of the mature form under normal conditions (21, 24). The molecular mechanism underlying the elimination of immature GlcCer *in vivo* was not clarified until EGCrP1 was discovered.

B-2. Discovery of EGCrP1 as the First Identified Glucocerebrosidase in Fungi

GlcCer is degraded by the detachment of glucose from GlcCer by glucocerebrosidase (GCase, EC 3.2.1.45). In mammals, catabolism of GlcCer mainly takes place in lysosomes and is mediated by acid-GCase (GBA1) but can also be mediated by the cytosolic GCases GBA2 and GBA3 (27–29). In contrast to its synthesis, little is known about the catabolism of GlcCer because the genes encoding known GCases, including GBA1, 2, and 3, had not been identified in fungi. This suggests that fungi have novel GCase(s).

Endoglycoceramidase (EGCase, EC 3.2.1.123) is an enzyme capable of cleaving the *O*-glycosidic linkage between oligosaccharide and ceramide in various glycosphingolipids (GSLs) to release an intact oligosaccharide and ceramide (30–32). However, GlcCer is barely hydrolyzed by EGCase, and the minimum structure required for hydrolysis of GSLs is a disaccharide, such as lactose. We found that homologues of EGCase, designated EGCase-related

proteins (EGCrPs), are widely distributed in the fungus kingdom (14, 15). Two different homologues, EGCrP1 and EGCrP2, are found in the genome database of *C. neoformans*; however, the distribution of EGCrP1 is restricted to *C. neoformans* and a few other fungi, whereas EGCrP2 is ubiquitously distributed throughout the fungus kingdom (Fig. 3). The substrate specificity, optimum pH and physiological functions of EGCrP1 are markedly different from those of EGCrP2. In this chapter, we describe the identification and characterization of EGCrP1 as the first GCase in the fungi. EGCrP2 will be described in the following chapter C.

To clarify the specificity of EGCrP1, the EGCrP1 gene was cloned from complementary DNA of *C. neoformans* and expressed in *Escherichia coli* to synthesize recombinant enzyme. We found that recombinant EGCrP1 hydrolyzed GlcCer efficiently but did not hydrolyze any other GSLs. No *p*NP glycosides including *p*NP β -glucoside were hydrolyzed by EGCrP1. Maximal activity of EGCrP1 was observed at around pH 7.5 when C6-NBD-GlcCer was used as a substrate. These results clearly indicate that EGCrP1 is a neutral GCase in fungi (14).

B-3. Accumulation of an Immature GlcCer in *egcrp1*-disrupted *C. neoformans* Mutant

To verify whether EGCrP1 is involved in the catabolism of GlcCer *in vivo*, an *egcrp1*-deficient mutant (*egcrp1* Δ) of *C. neoformans* was generated by gene-targeting homologous recombination. GCase activity in the *egcrp1* Δ mutant decreased in the membrane (microsomal) fraction at neutral but not acidic pH (Fig. 4A), suggesting that EGCrP1 functions as a neutral GCase at the membrane. Some other GCase(s), which have a lower optimum pH, probably exist in *C. neoformans* in addition to EGCrP1 (14).

GlcCer levels in the wildtype and *egcrp1* Δ were determined by HPLC-based chromatography (33). As expected, the amount of fungus-specific GlcCer containing mature ceramide (methyl d18:2/h18:0) was increased in *egcrp1* Δ compared to that in the wildtype. In addition, a new peak emerged just before the peak of fungus-specific GlcCer in *egcrp1* Δ but not in the wildtype (14). To clarify the structure of accumulated GlcCer in *egcrp1* Δ , GlcCer in wildtype and mutants fractions were subjected to ESI-MS/MS analysis. Peaks at *m/z* 778 were observed in both strains and were identified to be a fungus-specific mature GlcCer. Meanwhile, three new peaks were observed in only the *egcrp1* Δ and were determined by MS/MS analysis to be immature GlcCer composed of d18:0/h18:0 (*m/z* 768), d18:1/h18:0 (*m/z* 766), and d18:2/h18:0 (*m/z* 764), respectively (Fig. 4B). This result indicates that immature GlcCer accumulated in *egcrp1* Δ but not in the wildtype. These immature GlcCer are byproducts generated by the GlcCer synthesis pathway due to the broad specificity of GCS1 toward ceramide species (Fig. 2). In the wildtype, immature GlcCer is converted to

Fig. 3. Phylogenetic tree of EGCase and its homologues EGCrP1 and EGCrP2. EGCases are distributed from procaryotes to invertebrates such as jellyfish, hydra, leech, earthworm, short-necked clam but not in fungus. Since EGCases from leech, earthworm, short-necked clam have not yet been cloned, they are not involved in this phylogenetic tree. EGCrP1 is restrictively conserved in *C. neoformans* and a few other fungi, whereas EGCrP2 is widely distributed throughout fungi, including four major deep-seated mycoses, such as *C. neoformans*, *Aspergillus fumigatus*, *Candida albicans*, and *Rhizopus delemar*. Very few fungi such as *C. neoformans* have both EGCrP1 and EGCrP2. Cited from ref. 15 with modification.

immature ceramide by EGCrP1, and the products are again modified to fungal-specific ceramide by the maturation of ceramide. Namely, EGCrP1 functions as a GCase to perform quality control of GlcCer, and assists the generation of fungus-specific GlcCer. It is worth noting that fungus-specific GlcCer is biologically important and that immature GlcCer is unable to compensate for the functions of fungus-specific GlcCer, as described in the section B-1.

B-4. Dysfunction of Polysaccharide Capsule Formation in *egcrp1Δ*

To estimate the biological functions of EGCrP1 *in vivo*, the growth of *egcrp1Δ* was compared to that of the wildtype under oxidative stress (hydrogen peroxide), high-salt stress, detergent stress, and different pH. However, no significant difference was observed between the wildtype and *egcrp1Δ* under these conditions. On the other hand, the polysaccharide capsule, an important virulence factor in *C. neoformans*, was smaller in *egcrp1Δ* under capsule-inducing conditions (Fig. 1). Although the precise mechanism under-

Fig. 4. Physiological roles of EGCrP1 in *C. neoformans*. A, GlcCer-degrading activity of wildtype and *egcrp1*-disrupted mutant (*egcrp1Δ*) *C. neoformans*. n.d., not detected. B, MS analysis of GlcCer from wildtype and *egcrp1Δ*. C, Putative functions of EGCrP1 in the quality control of GlcCer, which is related to polysaccharide capsule formation. Cited from ref. 14 with modification.

lying this phenotype is unclear, quality control of GlcCer could be related to the transport of polysaccharide-containing vesicles to the outside of the cell, which triggers capsule formation because the vesicles are mainly made of GlcCer and sterols (34) (Fig. 4C). In contrast, the polysaccharide capsule in a *gcs1*-deficient *C. neoformans* mutant was almost the same size as that of the wildtype (24). Therefore, we estimate that the accumulation of immature GlcCer in *egcrp1Δ* may lead the abnormal formation and/or transport of vesicles (Fig. 4C).

C. Sterylglucoside Metabolism and Vacuole Formation in *C. neoformans*

C-1. Synthesis and Function of Sterylglucosides

Sterylglucoside (SG), a glycolipid composed of sterol and

glucose, is among the most abundant sterol derivatives in many organisms (35). SG is found in bacteria, fungi, plants and mammals, including humans. According to the structure of the sterol moiety, SG is subdivided into the groups cholesteryl glucoside (36), sitosteryl glucoside (37), and ergosteryl glucoside (EG) (38). EG is the major SG type found in fungi and yeasts. β -Glucose is usually bound to sterol moieties; however, α -glucose is bound to cholesterol in *Helicobacter pylori* (39).

In fungi and plants, SG is synthesized by a sterol 3- β -glucosyltransferase (SGT, EC 2.4.1.173), which catalyzes the transfer of glucose from UDP-glucose to sterol (Fig. 5) (40). In *C. neoformans*, a homologue of *Saccharomyces cerevisiae* SGT (Ugt51/Atg26) was found to synthesize EG (unpublished data). In mammals, no homologue of SGT was found; alternatively,

Fig. 5. Synthesis pathway of steryl β -glucoside (SG). Synthesis pathway of SG in bacteria, fungi, and plants is completely different from that in mammals. In the former, sterol glucosyltransferase transfers glucose from UDP-Glc to sterols (40), while acid glucocerebrosidase GBA1 transfers glucose from GlcCer to sterols in mammals (41).

SG seems to be synthesized by transferring glucose from GlcCer to cholesterol, and this reaction is likely to be catalyzed by acid GCase (GBA1) (Fig. 5) (41).

SG levels have been shown to increase in human fibroblasts upon heat shock from 37 to 42°C for 15–30 min (36). Because SG can induce the expression of heat shock protein 70 (HSP70) in fibroblasts (42), SG is considered to be HSP-inducible as well as stress-induced in mammals. Thus, it is suggested that SG is a lipid mediator of the stress-responsive signal. Heat shock also induced the accumulation of EG, a major SG in lower eukaryotes, slime mold and fungi (38, 43). In the slime mold *Physarum polycephalum*, SG was detected following exposure from 24 to 40°C for 5 min (43). In the methanotrophic yeast *Pichia pastoris*, SG accumulated following ethanol stress as well as heat shock (38). However, the molecular mechanism underlying how heat stress induces the expression and accumulation of SG or how SG induces the expression of HSP is not understood.

It has been reported that EG is related to a type of autophagy (pexophagy) in *P. pastoris* because pexophagy did not occur when SGT gene was disrupted (44). In contrast, disruption of *UGT51/ATG26* did not affect the process of pexophagy in *S. cerevisiae* and *Yarrowia lipolytica*, possibly due to the lower amount of EG in these two yeasts (45, 46).

C-2. Identification of EGCrP2 as a Steryl- β -glucosidase, the Missing Link of SG Metabolism

As described in chapter C-1, SGT-dependent SG synthesis and its biological significance have been extensively investigated; however, no clear picture about the catabolism of SG has emerged because the SG-degrading enzyme (steryl- β -glucosidase, EC 3.2.1.104) responsible for SG catabolism *in vivo* was unknown (Fig. 6). Nevertheless, there are a few reports available describing the enzymatic hydrolysis of SG. Homogenates of *Sinapis alba* seedlings hydrolyzed SG under acidic conditions (47). The β -glucosidase (LacS) of archaea, *Sulfolobus solfataricus*, hydrolyzed SG and was shown to be biotechnologically useful for eliminating

Fig. 6. EGCrP2 is a missing link in fungus SG metabolic pathway. EGCrP2 is the missing link of SG metabolism in fungi. The discovery of EGCrP2 shed light on SG catabolism, which had long been a mystery.

Table 1. Substrate specificity and optimum pH for EGCase and its homologues, EGCrP1 and EGCrP2.

	EGCrP1	EGCrP2	EGCase II
Optimum pH	7.0–7.5	5–6	5–6
GM1a	×	×	○
Asialo GM1	×	×	○
Gb3Cer	×	×	○
LacCer	×	×	○
C6-NBD-GlcCer	○	○	×
GlcCer (fungus)	○	○	×
pNP β -glucoside	×	○	×
4MU β -glucoside	×	○	×
Resorufin β -glucoside	×	○	×
Ergosteryl β -glucoside	×	○	×
Sitosteryl β -glucoside	×	○	×
n-Octyl β -glucoside	×	○	×
Indoxyl β -glucoside	×	○	×
Salicin	×	○	×

○, Degraded; ×, not degraded.

The substrate specificity of EGCase is completely different from that of EGCrP1 and EGCrP2. EGCase hydrolyzes the oligosaccharide-linked GSLs but hardly hydrolyze GlcCer, which is a favorite substrate for EGCrP1 and EGCrP2. EGCrP1 is specific to GlcCer, while EGCrP2 hydrolyzes not only GlcCer but also various steryl β -glucosides and other β -glucosides, indicating that EGCrP1 is a β -glucocerebrosidase specific to GlcCer and EGCrP2, a β -glucosidase having broad aglycone specificities.

SG from biodiesel (48). However, it was unclear whether LacS is responsible for SG catabolism in archaea.

We identified EGCrP2 as one of the homologues of EGCase in *C. neoformans* (15). EGCrP2 is an acid β -glucosidase with a wide specificity for the aglycone moiety; it hydrolyzes not only

Fig. 7. Accumulation of ergosteryl β -glucoside (EG) in an *egcrp2*-disrupted *C. neoformans* mutant (*egcrp2* Δ). A, Decrease of β -glucosidase activity in *egcrp2* Δ . B, TLC showing the accumulation of EG in *egcrp2* Δ . Lane 1, sitosteryl β -glucoside (standard); lane 2, wildtype; lane 3, *egcrp1* Δ ; lane 4, *egcrp2* Δ ; lane 5, *egcrp1* Δ /*egcrp2* Δ double disrupted mutant. Arrow indicated the accumulated EG in *egcrp2* Δ and *egcrp1* Δ /*egcrp2* Δ double disrupted mutant. C, Structure of the glycolipid accumulated in *egcrp2* Δ . The structure of the glycolipid accumulated in *egcrp2* Δ was determined by 2D-NMR to be ergosteryl 3- β -glucoside. Cited from ref. 15 with modification.

GlcCer but also various SGs including EG and artificial β -glucosides, such as *p*NP β -glucoside and 4MU β -glucoside. In contrast to EGCrP2, EGCrP1 is specific to GlcCer, whereas EGCase reacts with all GSLs except GlcCer. The specificities of EGCase and its homologues are summarized in Table 1.

To elucidate the physiological substrates and functions of EGCrP2, EGCrP2-disrupted mutants (*egcrp2* Δ) were generated from *C. neoformans*. The β -glucosidase activity decreased in the cell lysate of *egcrp2* Δ at pH 5.0 but not at pH 7.3 as measured by C6-NBD-GlcCer, indicating that EGCrP2 functions as an acid β -glucosidase *in vivo* (Fig. 7A). TLC analysis of cell extracts showed that the accumulation of GlcCer was not significant in *egcrp2* Δ ; alternatively, an unknown lipid, whose *R_f* was similar to that of SG, accumulated at high levels in *egcrp2* Δ (Fig. 7B). The accumulated lipid was determined to be ergosteryl β -glucoside (EG), a major SG in fungi, by mass spectrometry and 2D-NMR analysis (Fig. 7C). These results clearly indicated that EGCrP2 functions as an acid β -glucosidase responsible for catabolism of EG in *C. neoformans* and that EGCrP2 is the missing link of SG metabolism in fungi. The discovery of EGCrP2 shed light on SG catabolism, which had long been a mystery (Fig. 6).

C-3. Physiological Significance of EG Catabolism in *C. neoformans*

The disruption of *egcrp2* but not *egcrp1* resulted in the arrest of cell growth from the middle log phase to the early stationary phase (Fig. 8A). The *egcrp2* Δ were larger than wildtype, possibly due to dysfunction in the budding process (Fig. 8B). The average cell volumes of *egcrp2* Δ were 5 to 6-fold larger than those of wildtype and *egcrp1* Δ as estimated by flow cytometry (Fig. 8C). The most striking difference between intracellular organelles in wildtype and *egcrp2* Δ was observed for the vacuoles. The vacuoles of *egcrp2* Δ were enlarged compared to those of the wildtype (Fig. 8D). However, the abnormal morphology of vacuoles was not detected in *egcrp1* Δ , suggesting that EG but not GlcCer catabolism is related to vacuole formation.

EGCrP2 is an acid β -glucosidase, and the mutant *egcrp2* Δ showed an abnormal morphology of vacuoles; thus, we hypothesized that EG may be catabolized by EGCrP2 in acidic compartments in the cell, such as the vacuoles. Thus, the vacuole-enriched fraction was biochemically isolated from wildtype and *egcrp2* Δ and was subjected to analyses of β -glucosidase activity and EG content. The specific activity of β -glucosidase at pH 5.0 was significantly lower in the vacuole fraction of *egcrp2* Δ than that of wildtype. EG was detected in the vacuole-enriched fraction of *egcrp2* Δ

Fig. 8. Phenotype analyses of *egcrp2Δ* of *C. neoformans*. A, Growth curve of wildtype (open circles), *egcrp1Δ* (open squares), *egcrp2Δ* (closed circles), and *egcrp1Δ/egcrp2Δ* double disrupted mutant (closed squares). B, Cells observed under a differential interference-contrast microscope. C, Flow cytometric analysis showing the average volume of cells. D, Vacuoles stained with carboxy-DCFDA of wildtype and *egcrp2Δ*. *egcrp2Δ* showed the distinct growth arrest, a dysfunction in cell budding, and enlargement of vacuole. Cited from ref. 15 with modification.

but not in that of wildtype. These results strongly suggested that EGCrP2 catabolizes EG in the vacuoles of *C. neoformans*.

D. Discussion

D-1. The Structures and Specificities of EGCase and Its Homologues EGCrP1, 2

EGCase and its two homologues belong to the glycoside hydrolase (GH) family 5 glycosidase, with which β -(1,4)-glucanases (cellulases), β -(1,4)-mannanases, and β -(1,4)-xylanases are involved. Among the glycosidases involved with the GH family 5, only EGCase and its homologues can hydrolyze glycolipids; however, the substrate specificity is completely different between proteins, as shown in Table 1. EGCase hydrolyzes the *O*-glycosidic linkage between the oligosaccharide and ceramide. On the other hand, EGCrP1 and EGCrP2 hydrolyze the *O*-glycosidic linkage between

glucose and ceramide. EGCrP2 can hydrolyze EG; however, this glycolipid is completely resistant to hydrolysis by EGCrP1 and EGCase. The X-ray crystal structure of EGCase revealed that the active site is split into a wide polar cavity and a narrow hydrophobic tunnel; the former is to bound the polyhydroxylated oligosaccharide and the latter to ceramide lipid chains (49). An X-ray crystal structures of EGCrPs would provide structural insights for understanding the different substrate specificities of these GH family 5 glycosidases.

D-2. Physiological Roles of EGCase and Its Homologues

Actinomycetes *Rhodococcus equi* (50) and hydra *Hydra magnipapillata* (51) produce EGCase; however, they possess no GSLs, which are substrates for EGCase. Hydra EGCase was strongly expressed in digestive cells; thus, it presumably plays a nutrition-

Fig. 9. EGCrP1, 2 as new targets for the development of anti-fungal drugs. Several types of anti-fungal drugs, e.g. 5-Fluorocytosine which inhibits the synthesis of DNA and RNA, Amphotericin B which binds to ergosterol to penetrate the cell membranes, and Fluconazole which inhibits the synthesis of ergosterols, are currently used for the treatment of deep mycoses; however, the development of new drugs targeting deep-seated mycoses is urgently required because these drugs have caused serious side effects and have resulted in the appearance of antibiotic-resistant fungi. Homologous sequences of EGCrP1 and -2 have not been found in the human genome database and the reaction mechanisms to inhibit the cell growth and capsule formation are different from those of known anti-fungal drugs, and, thus, these glycosidases are thought to be promising targets for the development of new drugs with few side effects.

al role in the organism. GM1 ganglioside injected into the gastric cavity was incorporated and then directly catabolized by EGCase to GM1-oligosaccharide and ceramide, which were further degraded by exoglycosidases and ceramidase, respectively. Fatty acid generated by the action of ceramidase was quickly incorporated into cells and metabolized to phospholipids and triacylglyceride (51). On the other hand, the major glycolipids of *C. neoformans* are GlcCer and EG, which are the endogenous substrates of EGCrP1 and EGCrP2, respectively. The intracellular localization patterns of EGCrP1 and EGCrP2 could differ; EGCrP1 seems to localize in the ER/Golgi apparatus, where it participates in quality control of GlcCer coupled with GCS1, whereas EGCrP2 probably localizes in the vacuoles, where it is involved in the catabolism of EG. The dysfunction of GlcCer quality control in *egcrp1Δ* results in formation of a smaller polysaccharide capsule, and loss of EG catabolism in *egcrp2Δ* leads to a growth defect accompanied by vacuole enlargement. These results indicate the different biological roles of the two EGCase homologues in *C. neoformans*.

D-3. EGCrPs as a New Target for the Development of Anti-fungal Drugs

The number of patients with deep-seated mycoses, such as

cryptococcosis, candidiasis, aspergillosis, and mucormycosis, is increasing worldwide, and Japan is no exception to this trend. Several types of anti-fungal drugs are used for the treatment of deep mycoses; however, these drugs have caused serious side effects and have resulted in the appearance of antibiotic-resistant fungi. Thus, the development of new drugs targeting deep-seated mycoses, including cryptococcosis, is urgently required (Fig. 9). Because homologous sequences of EGCrP1 and -2 have not been found in the human genome database, these glycosidases are thought to be promising targets for the development of anti-fungal drugs with few side effects. EGCrP1 is conserved in *C. neoformans*, whereas EGCrP2 is widely distributed in deep-seated mycoses. Therefore, EGCrP1 could be a potent target for anti-cryptococcal drugs and EGCrP2, anti-fungal drugs with a wide spectrum.

The reader may have doubts as to whether the inhibitors for glycosidase(s) could become powerful antibiotics. Yes, it is possible. Oseltamivir (commercial name, Tamiflu), a potent drug targeting the influenza virus, is an inhibitor of viral neuramidase (EC 3.2.1.18).

Acknowledgments

The authors thank Mr. Tomoharu Ito, Ms. Mika Fujita, Dr. Hatsumi Goda, Dr. Keishi Sakaguchi, and Dr. Nozomu Okino at Kyushu University for technical support and valuable suggestions. We also appreciate the help of Dr. Tomofumi Miyamoto at Kyushu

University with NMR analysis and Dr. Kazutaka Ikeda at Keio University (Riken at present) and Dr. Ryo Taguchi at University of Tokyo (Chubu University at present) with MS analysis. This work was supported by JSPS Grant-in Aid for Scientific Research B (24380058).

References

1. Doering, T. L. (2009) *Annu. Rev. Microbiol.* **63**, 223–247.
2. Kozel, T. R., and Gotschlich, E. C. (1982) *J. Immunol.* **129**, 1675–1680.
3. Feldmesser, M., Kress, Y., Novikoff, P., and Casadevall, A. (2000) *Infect. Immun.* **68**, 4225–4237.
4. Lee, S. C., Kress, Y., Zhao, M. L., Dickson, D. W., and Casadevall, A. (1995) *Lab. Invest.* **73**, 871–879.
5. Park, B. J., Wannemuehler, K. A., Marston, B. J., Govender, N., Pappas, P. G., and Chiller, T. M. (2009) *AIDS* **23**, 525–530.
6. Srikantha, D., Santiago-Tirado, F. H., and Doering, T. L. (2014) *Yeast* **31**, 47–60.
7. Sorrell, T. C. (2001) *Med. Mycol.* **39**, 155–168.
8. Fraser, J. A., Giles, S. S., Wenink, E. C., Geunes-Boyer, S. G., Wright, J. R., Diezmann, S., Allen, A., Stajich, J. E., Dietrich, F. S., Perfect, J. R., and Heitman, J. (2005) *Nature* **437**, 1360–1364.
9. Fyfe, M., MacDougall, L., Romney, M., Starr, M., Pearce, M., Mak, S., Mithani, S., and Kibsey, P. (2008) *Can. Commun. Dis. Rep.* **34**, 1–12.
10. Hoang, L. M., Maguire, J. A., Doyle, P., Fyfe, M., and Roscoe, D. L. (2004) *J. Med. Microbiol.* **53**, 935–940.
11. Byrnes, E. J. 3rd, Bildfell, R. J., Frank, S. A., Mitchell, T. G., Marr, K. A., and Heitman, J. (2009) *J. Infect. Dis.* **199**, 1081–1086.
12. Balkis, M. M., Leidich, S. D., Mukherjee, P. K., and Ghannoum, M. A. (2002) *Drugs* **62**, 1025–1040.
13. Rhome, R., McQuiston, T., Kechichian, T., Bielawska, A., Hennig, M., Drago, M., Morace, G., Luberto, C., and Del Poeta, M. (2007) *Eukaryot. Cell* **6**, 1715–1726.
14. Ishibashi, Y., Ikeda, K., Sakaguchi, K., Okino, N., Taguchi, R., and Ito, M. (2012) *J. Biol. Chem.* **287**, 368–381.
15. Watanabe, T., Ito, T., Goda, H. M., Ishibashi, Y., Miyamoto, T., Ikeda, K., Taguchi, R., Okino, N., and Ito, M. (2015) *J. Biol. Chem.* **290**, 1005–1019.
16. Ternes, P., Wobbe, T., Schwarz, M., Albrecht, S., Feussner, K., Riezman, I., Cregg, J. M., Heinz, E., Riezman, H., Feussner, I., and Warnecke, D. (2011) *J. Biol. Chem.* **286**, 11401–11414.
17. Haak, D., Gable, K., Beeler, T., and Dunn, T. (1997) *J. Biol. Chem.* **272**, 29704–29710.
18. Michaelson, L. V., Zauner, S., Markham, J. E., Haslam, R. P., Desikan, R., Mugford, S., Albrecht, S., Warnecke, D., Sperling, P., Heinz, E., and Napier, J. A. (2009) *Plant Physiol.* **149**, 487–498.
19. Oura, T., and Kajiwar, S. (2008) *Microbiology* **154**, 3795–3803.
20. Ternes, P., Sperling, P., Albrecht, S., Franke, S., Cregg, J. M., Warnecke, D., and Heinz, E. (2006) *J. Biol. Chem.* **281**, 5582–5592.
21. Singh, A., Wang, H., Silva, L. C., Na, C., Prieto, M., Futerman, A. H., Luberto, C., and Del Poeta, M. (2012) *Cell. Microbiol.* **14**, 500–516.
22. Noble, S. M., French, S., Kohn, L. A., Chen, V., and Johnson, A. D. (2010) *Nat. Genet.* **42**, 590–598.
23. Oura, T., and Kajiwar, S. (2010) *Microbiology* **156**, 1234–1243.
24. Rittershaus, P. C., Kechichian, T. B., Allegood, J. C., Merrill, A. H. Jr., Hennig, M., Luberto, C., and Del Poeta, M. (2006) *J. Clin. Invest.* **116**, 1651–1659.
25. Leipelt, M., Warnecke, D., Zahring, U., Ott, C., Muller, F., Hube, B., and Heinz, E. (2001) *J. Biol. Chem.* **276**, 33621–33629.
26. Michel, C., van Echten-Deckert, G., Rother, J., Sandhoff, K., Wang, E., and Merrill, A. H. Jr. (1997) *J. Biol. Chem.* **272**, 22432–22437.
27. Kolter, T., and Sandhoff, K. (2005) *Annu. Rev. Cell Dev. Biol.* **21**, 81–103.
28. Matern, H., Boermans, H., Lottspeich, F., and Matern, S. (2001) *J. Biol. Chem.* **276**, 37929–37933.
29. Hayashi, Y., Okino, N., Kakuta, Y., Shikanai, T., Tani, M., Narimatsu, H., and Ito, M. (2007) *J. Biol. Chem.* **282**, 30889–30900.
30. Izu, H., Izumi, Y., Kurome, Y., Sano, M., Kondo, A., Kato, I., and Ito, M. (1997) *J. Biol. Chem.* **272**, 19846–19850.
31. Ishibashi, Y., Nakasone, T., Kiyohara, M., Horibata, Y., Sakaguchi, K., Hijikata, A., Ichinose, S., Omori, A., Yasui, Y., Imamura, A., Ishida, H., Kiso, M., Okino, N., and Ito, M. (2007) *J. Biol. Chem.* **282**, 11386–11396.
32. Ishibashi, Y., Kobayashi, U., Hijikata, A., Sakaguchi, K., Goda, H. M., Tamura, T., Okino, N., and Ito, M. (2012) *J. Lipid Res.* **53**, 2242–2251.
33. Zama, K., Hayashi, Y., Ito, S., Hirabayashi, Y., Inoue, T., Ohno, K., Okino, N., and Ito, M. (2009) *Glycobiology* **19**, 767–775.
34. Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Frases, S., Miranda, K., Zaragoza, O., Alvarez, M., Nakouzi, A., Feldmesser, M., and Casadevall, A. (2007) *Eukaryot. Cell* **6**, 48–59.
35. Grille, S., Zaslowski, A., Thiele, S., Plat, J., and Warnecke, D. (2010) *Prog. Lipid Res.* **49**, 262–288.
36. Kunitomo, S., Kobayashi, T., Kobayashi, S., and Murakami-Murofushi, K. (2000) *Cell Stress Chaperones* **5**, 3–7.
37. Power, F. B., and Salway, A. H. (1913) *J. Chem. Soc.* **103**, 399–406.
38. Sakaki, T., Zahring, U., Warnecke, D. C., Fahl, A., Knogge, W., and Heinz, E. (2001) *Yeast* **18**, 679–695.
39. Hirai, Y., Haque, M., Yoshida, T., Yokota, K., Yasuda, T., and Oguma, K. (1995) *J. Bacteriol.* **177**, 5327–5333.
40. Warnecke, D., Erdmann, R., Fahl, A., Hube, B., Muller, F., Zank, T., Zahring, U., and Heinz, E. (1999) *J. Biol. Chem.* **274**, 13048–13059.
41. Akiyama, H., Kobayashi, S., Hirabayashi, Y., and Murakami-Murofushi, K. (2013) *Biochem. Biophys. Res. Commun.* **441**, 838–843.
42. Kunitomo, S., Murofushi, W., Kai, H., Ishida, Y., Uchiyama, A., Kobayashi, T., Kobayashi, S., Murofushi, H., and Murakami-Murofushi, K. (2002) *Cell Struct. Funct.* **27**, 157–162.
43. Murakami-Murofushi, K., Nishikawa, K., Hirakawa, E., and Murofushi, H. (1997) *J. Biol. Chem.* **272**, 486–489.

44. Oku, M., Warnecke, D., Noda, T., Muller, F., Heinz, E., Mukaiyama, H., Kato, N., and Sakai, Y. (2003) *EMBO J.* **22**, 3231–3241.
45. Cao, Y., and Klionsky, D. J. (2007) *Autophagy* **3**, 17–20.
46. Stasyk, O. V., Nazarko, T. Y., Stasyk, O. G., Krasovska, O. S., Warnecke, D., Nicaud, J. M., Cregg, J. M., and Sibirny, A. A. (2003) *Cell Biol. Int.* **27**, 947–952.
47. Kalinowska, M., and Wojciechowski, Z. A. (1978) *Phytochemistry* **17**, 1533–1537.
48. Aguirre, A., Peiru, S., Eberhardt, F., Vetcher, L., Cabrera, R., and Menzella, H. (2014) *Appl. Microbiol. Biotechnol.* **98**, 4033–4040.
49. Caines, M. E. C., Vaughan, M. D., Tarling, C. A., Hancock, S. M., Warren, R. A. J., Withers, S. G., and Strynadka, N. C. J. (2007) *J. Biol. Chem.* **282**, 14300–14308.
50. Ito, M., and Yamagata, T. (1986) *J. Biol. Chem.* **261**, 14278–14282.
51. Horibata, Y., Sakaguchi, K., Okino, N., Iida, H., Inagaki, M., Fujisawa, T., Hama, Y., and Ito, M. (2004) *J. Biol. Chem.* **279**, 33379–33389.

Information of the Authors

Takashi Watanabe is a postdoctoral fellow of the Faculty of Agriculture at Kyushu University. He received his Ms. degree from the Graduate School of Engineering, Tokai University in 2012 (Prof. Junko Matsuda) and received his Ph.D. from the Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University in 2015 (Prof. Makoto Ito). His current interest is the glycolipid metabolism and its biological function in fungi.

Yohei Ishibashi obtained his Ph.D. from the Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University in 2009 under the supervision of Professor Makoto Ito. In 2008, he visited Dr. Rudolf Geyer Lab at University of Giessen, Germany, as a research student. From 2008 to 2010, he received Research Fellowship for Young Scientists (DC2, PD) from JSPS. From 2010 to 2013, he worked at the Laboratory of Molecular Membrane Neuroscience, RIKEN Brain Science, as a Special Postdoctoral Researcher (Yoshio Hirabayashi Team Leader). From 2014, he serves as an assistant professor, Faculty of Agriculture, Kyushu University. His major interest is metabolism and functions of glycolipids, sphingolipids, and sugar nucleotides in fungi, marine algae, and mammalian cells.

Makoto Ito received his Ph.D. in 1981 from Kyushu University, Fukuoka, Japan, under the supervision of Dr. Manabu Kitamikado. During his doctoral course, he spent 1 year at the Department of Biochemistry, Tulane Medical School, New Orleans, USA (Prof. Yu-Teh Li). Between 1982 and 1993, he worked at the glycoconjugate research laboratory (Dr. Tatsuya Yamagata) at the Mitsubishi Kasei Institute of Life Sciences first as a postdoctoral fellow, then as senior researcher. Dr. Ito received the Young Scientist Award for Biochemists from the Japanese Biochemical Society in 1991. He then returned to Kyushu University in 1993 as an associate professor and was appointed full professor in the Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University in 2002. He additionally serves as the professor at the Bio-Architecture Center, Kyushu University, from 2005. His research has focused on the structures, functions and metabolism of glycoconjugates and sphingolipids. His current research interests include glycolipid metabolism and its associated enzymes.