Biochemical and Biophysical Research Communications 410 (2011) 786–791

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Sphingolipid synthesis is involved in autophagy in *Saccharomyces cerevisiae*

Maki Yamagata, Keisuke Obara, Akio Kihara *

Laboratory of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12-jo, Nishi 6-choume, Kita-ku, Sapporo 060-0812, Japan

ARTICLE INFO

Article history:
Received 30 May 2011
Available online 14 June 2011

Keywords:
Autophagy
Lipid
Sphingolipid

ABSTRACT

In eukaryotes, autophagy is a conserved protein degradation system that degrades cytoplasmic components by encompassing them with double-membrane structures, called autophagosomes, and delivering them to the lytic compartments of vacuoles/lysosomes. Certain Atg proteins are known to be involved in autophagy, yet the identity and function of lipid molecules involved remain largely unknown. We investigated the involvement of sphingolipids in autophagy using *Saccharomyces cerevisiae*. Inhibiting synthesis of the simplest complex sphingolipid, inositol phosphorylceramide (IPC), resulted in reduced autophagic activities. Similar results were obtained using myriocin, an inhibitor of the first step in sphingolipid synthesis. Our results indicate that sphingolipids, especially IPC, are required for autophagy. Inhibition of sphingolipid synthesis had no effect on formation of Atg12-Atg5 or Atg8-phosphatidylethanolamine conjugates, on maturation of vacuolar proteases, or on formation of the pre-autophagosomal structure (PAS). These results suggest that sphingolipids are not involved in the cellular signaling that leads to formation of the PAS, but may be involved in the process of autophagosome formation.

1. Introduction

In autophagy, a bulk protein degradation system, cytoplasmic components are delivered to degradation compartments (vacuoles in yeast and lysosomes in mammals) and degraded. This system is conserved in eukaryotes. One well-defined physiological role of autophagy is to recycle amino acids, especially under starvation. A growing number of other physiological roles have been revealed by recent studies. Newly identified roles include removal of protein aggregates that might otherwise cause neuronal disorders such as Huntington's disease, extirpation of pathogenic bacteria, and antigen presentation [1].

Electron microscopic analyses of nutrient-deprived yeast have revealed details of the autophagic process [2,3]. A cup-shaped isolated membrane first appears in the cytosol, then extends, and finally encompasses a portion of cytoplasm, thereby generating a double-membraned structure called an autophagosome. The outer membrane of the autophagosome fuses with the vacuole membrane, releasing a single-membrane autophagic body, into the lumen of the vacuole. The hydrolases within the vacuole degrade the autophagic body and its contents to recycle their lipids and amino acids.

Over 30 autophagy-related proteins (Atg proteins) have been identified, 18 of which are essential for autophagosome formation [4–7]. Atg12 and Atg8 are ubiquitin-like molecules, and each covalently conjugates with a specific partner, Atg5 or phosphatidylethanolamine (PE), respectively [8–10]. Like the ubiquitin conjugation system, which utilizes three proteins (E1, E2, and E3) to attach ubiquitin to target proteins, Atg12 conjugation to Atg5 is mediated by the E1-like protein Atg7, and the E2-like protein Atg10. During Atg8-PE conjugation, the C-terminal Gly residue of Atg8 is cleaved by the protease Atg4, then the exposed C-terminus is conjugated to PE through successive actions of Atg7 and the E2-like Atg3 and the E3-like Atg12-Atg5 conjugate. The formation of the Atg8-PE conjugate is stimulated under nutrient-starvation, i.e. during induction of autophagy, and the conjugate is localized both on the extending isolation membranes and the autophagosome membranes [11,12].

The Atg proteins functioning in autophagy have been analyzed extensively, yet with the exception of PE and phosphatidylinositol 3-phosphate [10,13], little is known about the lipid molecules that form autophagosomes. Sphingolipids are abundant lipid components of eukaryotic plasma membranes that function in a wide range of biological processes including proliferation, apoptosis, adhesion, skin barrier formation, and trafficking of immune cells [14]. These traits make sphingolipids strong candidates for study as possible autophagosomal lipids.

Abbreviations: AbA, aureobasidin A; ALP, alkaline phosphatase; ApeI, aminopeptidase I; Cer, ceramide; CPY, carboxypeptidase Y; Cvt, cytoplasm to vacuole targeting; DHS, dihydrosphingosine; IPC, inositol phosphorylceramide; MIPC, mannosylinositol phosphorylceramide; $M(IP)_2C$, mannosyldiinositol phosphorylceramide; Myr, myriocin; PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PHS, phytosphingosine; SC, synthetic complete; S(-NC), synthetic medium deprived nitrogen and carbon sources.

* Corresponding author. Fax: +81 11 706 4900.
E-mail address: kihara@pharm.hokudai.ac.jp (A. Kihara).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.06.061

The backbone of sphingolipids, ceramide (Cer), is composed of a long-chain base attached to a fatty acid via an amide bond. The biosynthesis of sphingolipids begins with the condensation of serine with palmitoyl-CoA to form 3-ketodihydrosphingosine, which is then reduced to dihydrosphingosine (DHS) and acylated to dihydroceramide. Alternatively, a hydroxy group is attached to the 4-position of DHS, generating phytosphingosine (PHS). PHS and some DHS are then converted to Cers (dihydroceramide and phytoceramide) and further to inositol-containing complex sphingolipids, specifically inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC), and mannosyldiinositol phosphorylceramide ($M(IP)_2C$) (Fig. 1) [14].

Cer and the sphingolipid metabolite sphingosine 1-phosphate reportedly induce autophagy in mammals, yet the causal molecular mechanisms are unclear. In yeast, a model organism in which autophagy has been extensively analyzed, certain autophagy process can be monitored or blocked by appropriate *atg* mutations. However, the functions of sphingolipids in yeast autophagy have rarely been studied. A single report described a slight increase (~1.2-fold) in autophagic activity in the absence of $M(IP)_2C$, caused by a double deletion of the *IPT1* gene, which encodes inositolphosphotransferase, and the *SKN1* gene, which is also involved in $M(IP)_2C$ synthesis through an unknown mechanism [15]. However, a single deletion mutation of the *IPT1* gene had no effect [15].

To address this nearly total lack of information regarding autophagosomal lipid biology in yeast, we investigated the involvement of sphingolipids using mutants of sphingolipid biosynthesis genes and inhibitors of sphingolipid synthesis. We found that blocking 3-ketodihydrosphingosine synthesis, the first step of sphingolipid synthesis, or IPC synthesis results in reduced autophagic activity, whereas inhibition of MIPC or $M(IP)_2C$ synthesis has no effect. This study affirms that sphingolipid synthesis is indeed required for normal progression of autophagy.

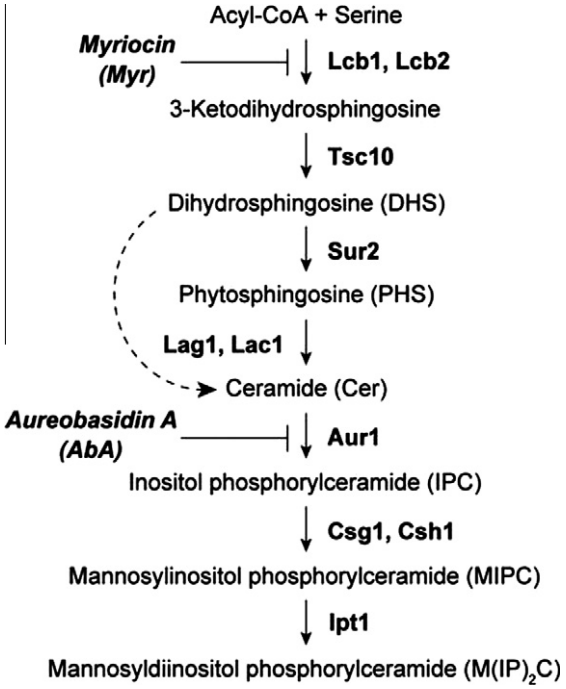

Fig. 1. The sphingolipid synthesis pathway of yeast. The pathway and responsible enzymes for yeast sphingolipid synthesis are shown. Processes inhibited by Myr and AbA are indicated. In Cer synthesis, a very long-chain fatty acid is conjugated to DHS or PHS via an amide bond.

2. Materials and methods

2.1. Yeast strain and media

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Cells were grown in either YPD medium (1% yeast extract, 2% peptone, and 2% D-glucose) or synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids (Sigma, St. Louis, MO), 2% D-glucose, 0.5% casamino acid, 20 mg/l tryptophan, 20 mg/l adenine, and 20 mg/l uracil) as a rich medium. Nitrogen- and carbon-deprived synthetic medium (S(-NC); 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Wako, Osaka, Japan)) was used to induce starvation. Aureobasidin A (AbA) and myriocin (Myr) were purchased from Takara Bio (Sigma, Japan) and Sigma, respectively.

Gene disruption of the *CSG1*, *CSH1*, or *IPT1* gene was performed as follows. *S. cerevisiae* mutants 2771 (*csg1Δ::KanMX4*), 3300 (*csh1Δ::KanMX4*), and 4007 (*ipt1Δ::KanMX4*) were obtained from Open Biosystems (Huntsville, AL). Gene fragments *csg1Δ::KanMX4*, *csh1Δ::KanMX4*, and *ipt1Δ::KanMX4*, which each encompass both 5′- and 3′-untranslated regions with more than 200 bp, were amplified from genomic DNA prepared from their respective mutant cells, and were used for homologous recombination to disrupt each gene under desired genetic backgrounds.

2.2. Assays for autophagy

Detection of autophagy was performed by microscopic observation of autophagic bodies accumulated in vacuoles, or by alkaline phosphatase (ALP) assays [2,16]. For observation of autophagic bodies, the vacuolar protease-deficient yeast strains BJ2168 and BJ3505 and their derivatives were used. Cells grown in rich medium to log-phase ($OD_{600} \geqslant 1$) were transferred to S(-NC) medium. After incubation for 5 h at 30 °C, cells were observed using a phase-contrast microscope IX-81 (Olympus, Tokyo, Japan). A minimum of 400 cells for each mutant strain were examined, and cells accumulating autophagic bodies were counted. ALP assays were performed as described previously [17], using yeast strains derived from KVY55, which expresses a mutant ALP lacking a signal sequence.

2.3. Immunoblotting

Total cell lysates were prepared by the alkaline/trichloroacetic acid method as described previously [17]. Lysates equivalent to 0.3 OD_{600} were subjected to SDS–PAGE. For detection of Atg8,

Table 1
Yeast strains.

Strains	Genotype	Reference
SEY6210	*MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	[27]
GSY115	SEY6210, *atg14Δ::LEU2*	[24]
ORY1700	SEY6210, *atg17Δ::ATG17-GFP-KanMX4*	[25]
KVY55	SEY6210, *pho8Δ60*	[11]
MYY56	KVY55, *atg14Δ::KanMX4*	This study
MYY230	KVY55, *csg1Δ::natNT2 csh1Δ::KanMX4*	This study
MYY226	KVY55, *ipt1Δ::KanMX4*	This study
BJ2168	***MATa*** *prb1-1122 prc1-407 pep4-3 leu2 trp1 ura3-52*	Y.G.S.C.
MYY46	BJ2168, *atg14Δ::KanMX4*	This study
MYY100	BJ2168, *csg1Δ::natNT2 csh1Δ::KanMX4*	This study
BJ3505	***MATa*** *pep4::HIS3 prb1-1, 6R HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can1*	Y.G.S.C.
MYY47	BJ3505, *atg14Δ::KanMX4*	This study
MYY228	BJ3505, *csg1Δ::natNT2 csh1Δ::KanMX4*	This study
MYY224	BJ3505, *ipt1Δ::KanMX4*	This study

Y.G.S.C. Yeast Genetic Stock Center.