RESEARCH LETTER

Skn1 and Ipt1 negatively regulate autophagy in *Saccharomyces cerevisiae*

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Abstract

We demonstrated that a yeast deletion mutant in *IPT1* and *SKN1*, encoding proteins involved in the biosynthesis of mannosyldiinositolphosphoryl ceramides, is characterized by increased autophagy and DNA fragmentation upon nitrogen (N) starvation as compared with the single deletion mutants or wild type (WT). Apoptotic features were not significantly different between single and double deletion mutants upon N starvation, pointing to increased autophagy in the double Δ*ipt1* Δ*skn1* deletion mutant independent of apoptosis. We observed increased basal levels of phytosphingosine in membranes of the double Δ*ipt1* Δ*skn1* deletion mutant as compared with the single deletion mutants or WT. These data point to a negative regulation of autophagy by both Ipt1 and Skn1 in yeast, with a putative involvement of phytosphingosine in this process.

Introduction

We previously demonstrated that biosynthesis of the sphingolipid class of mannosyldiinositolphosphoryl ceramides [$M(IP)_2C$] in yeast depends on the nutrient conditions (Im *et al.*, 2003; Thevissen *et al.*, 2005). Skn1 and Ipt1 in yeast are both involved in the biosynthesis of $M(IP)_2C$ (Dickson *et al.*, 1997; Thevissen *et al.*, 2005). When grown in nutrient-rich media, Δ*ipt1* and Δ*skn1* single and double deletion mutants are characterized by membranes devoid of $M(IP)_2C$ (Dickson *et al.*, 1997; Thevissen *et al.*, 2005). However, when grown under nutrient limitation in half-strength potato dextrose broth (PDB), the single deletion mutants Δ*ipt1* and Δ*skn1* show reappearance of $M(IP)_2C$ in their membranes, whereas $M(IP)_2C$ is completely absent in membranes of the double Δ*ipt1* Δ*skn1* deletion mutant grown under these conditions (Im *et al.*, 2003; Thevissen *et al.*, 2005). These data point to *SKN1*- or *IPT1*-dependent $M(IP)_2C$ biosynthesis in a Δ*ipt1* or a Δ*skn1* deletion mutant, respectively, upon nutrient depletion, whereas in the double Δ*ipt1* Δ*skn1* deletion mutant, $M(IP)_2C$ biosynthesis is blocked independent of medium composition. Hence, nutrient conditions influence the biosynthesis of $M(IP)_2C$ in yeast.

Autophagy is a catabolic membrane-trafficking phenomenon that occurs in response to drastic changes in the nutrients available to yeast cells, for example during starvation for nitrogen (N) or carbon (Abeliovich & Klionsky, 2001). Although both autophagy and the $M(IP)_2C$ content of yeast membranes seem to be responsive to nutritional stress, a direct link between these processes has not been investigated in yeast to date. Hence, the question arises as to whether Δ*ipt1* or Δ*skn1* single and double deletion mutants are characterized by an altered autophagic response as compared with the corresponding wild type (WT). Therefore, in this study, we used N starvation to assess differences in the autophagic response of the different Δ*ipt1* and/or Δ*skn1* deletion mutants as compared with WT, as well as their sphingolipid profiles and putative induction of apoptosis, which has previously been linked to autophagy (Maiuri *et al.*, 2007; Scott *et al.*, 2007). Because overexpression of autophagy-related

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protein 1, Atg1, in *Drosophila* was previously shown to induce autophagy and to cause cell death accompanied by increased DNA fragmentation (Scott *et al.*, 2007), we further assessed DNA fragmentation upon N starvation in all mutants and WT.

Materials and methods

Materials and microorganisms

The yeast strains used are *Saccharomyces cerevisiae* BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and the corresponding Δ*ipt1*, Δ*skn1* (Invitrogen, Carlsbad, CA) mutants and the double Δ*ipt1* Δ*skn1* deletion mutant (Thevissen *et al.*, 2005), the pho8Δ60::pho8 pho13Δ::kan-lox strain (WT, YTS158) (Noda *et al.*, 1995) and the corresponding Δ*atg1*, Δ*ipt1*, Δ*skn1* and Δ*ipt1* Δ*skn1* mutants.

Challenge with N starvation medium

Overnight cultures in YPD medium (1% yeast extract; 2% peptone, 2% glucose) were transferred to SD medium [0.8 g L^{-1} complete amino acid supplement mixture (Bio 101 Systems); 6.5 g L^{-1} yeast nitrogen base (YNB); 20 g L^{-1} glucose] at a start $OD_{600\,nm} = 0.2$, grown to the exponential phase till $OD_{600\,nm} = 0.8$, washed twice with SD-N medium (0.17% YNB w/o ammonium sulfate and amino acids, 2% glucose) and shifted to SD-N medium for 4 h. As a control, cells were also shifted to SD medium after reaching the exponential phase.

Assay for monitoring autophagy

For monitoring bulk autophagy, the alkaline phosphatase activity of Pho8Δ60 was carried out as described previously (Noda *et al.*, 1995; Klionsky, 2007). The percentage of autophagy of the different mutants was relative to the WT autophagy level under the different conditions.

Assay for reactive oxygen species (ROS) induction and phosphatidylserine externalization

After challenge with SD-N medium, cell numbers were measured (using CASY cell counter), ROS levels were determined (via staining with dihydroethidium) and phosphatidylserine externalization of the yeast cultures (via staining with fluorescein isothiocyanate-labeled annexin V in combination with propidium iodide) was quantified using flow cytometry and BD FACSDIVA software (Madeo *et al.*, 1997; Büttner *et al.*, 2007). Experiments were repeated at least three times.

Assay for DNA fragmentation

DNA fragmentation following a shift to SD-N medium was quantified using flow cytometry and BD FACSDIVA software after terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (Madeo *et al.*, 1997; Büttner *et al.*, 2007).

Sphingolipid analysis

For sphingolipid labeling, yeast cultures were incubated in SD-N-inositol containing [^{3}H]myo-inositol [1 μCi mL^{-1}; American Radiolabelled Chemicals (St. Louis, MO)], after which sphingolipids were extracted and analyzed (Thevissen *et al.*, 2005). Ceramide and sphingoid base analysis was performed by Lipidomics CORE at Medical University of South Carolina as described previously using a sphingolipidomics approach (Bielawski *et al.*, 2006; Aerts *et al.*, 2008). For each condition, experiments were performed twice at least in duplicate.

Statistical analysis

Statistical analysis was performed using a paired *t*-test.

Results

Δ*ipt1* Δ*skn1* mutant is characterized by increased autophagy under N starvation

To determine whether induction of autophagy is affected in the Δ*ipt1* Δ*skn1* mutant as compared with the single deletion mutants and WT, we used the Pho8Δ60 assay (Klionsky, 2007). Pho8Δ60 is a truncated variant of the vacuolar alkaline phosphatase Pho8, which lacks the N-terminal transmembrane region that normally allows entry into the endoplasmic reticulum, resulting in accumulation of the mutant protein in the cytosol (Noda *et al.*, 1995). Cytosolic Pho8Δ60 is sequestered as a nonspecific cargo within autophagosomes upon induction of autophagy and delivered into the vacuole, where it is processed into an enzymatically active form due to removal of a C-terminal propeptide. Therefore, the alkaline phosphatase activity of Pho8Δ60 reflects the magnitude of autophagic cargo delivery. To this end, *SKN1* and/or *IPT1* were deleted in a Pho8Δ60 yeast strain background. Upon challenge with N starvation medium, Δ*ipt1* Δ*skn1* cells showed a significant 10–20% increase in Pho8Δ60 activity as compared with the single deletion mutants or the corresponding Pho8Δ60 WT (Fig. 1), indicating increased autophagy upon deletion of both *IPT1* and *SKN1*. This is in contrast to the single deletion mutants in *IPT1* or *SKN1*, which did not show significantly increased autophagy as compared with the corresponding WT under conditions of N starvation (Fig. 1). Deletion of *ATG1*, encoding a protein serine/threonine kinase required for autophagy (Matsuura *et al.*, 1997),

served as a negative control in this experiment and showed essentially no increase in Pho8Δ60-dependent alkaline phosphatase activity upon starvation.

N starvation of single and double Δ*ipt1* Δ*skn1* deletion mutants leads to induction of typical cell death markers

A functional cross-talk exists between autophagy and apoptosis (Maiuri *et al.*, 2007). Upon challenge with N starvation medium, we observed a slight, but significant, increase in the death rate (10–15%) of all the deletion mutant strains as compared with WT (Fig. 2a), while no difference was observed when shifting to a rich medium (SD), ruling out a survival defect of the mutant strains (data not shown). Additionally, all mutant strains displayed an enhancement of ROS accumulation (threefold) as compared with WT (Fig. 2b) upon challenge with N starvation medium, as well as a slight overall increase in the number of early apoptotic (AnnexinV positive, PI negative), late apoptotic (AnnexinV positive, PI positive) and necrotic (only PI positive) cells (Fig. 2c). Thus, the single and double Δ*ipt1* Δ*skn1* deletion mutants show comparable death rates upon N starvation.

We next assessed the level of DNA fragmentation, a further phenotypic marker of apoptosis in yeast (Madeo *et al.*, 1997). All deletion mutants consistently showed enhanced DNA fragmentation as compared with WT (Fig. 2d). However, the increase in DNA fragmentation obtained for the double Δ*ipt1* Δ*skn1* deletion mutant (fourfold increase) was markedly higher than for the single deletion mutants (1.5–2-fold increase). This surplus DNA fragmentation may therefore be of nonapoptotic origin and points to a link between autophagy and increased DNA fragmentation, as demonstrated previously in *Drosophila* upon overexpression of Atg1, where autophagy is induced and causes cell death accompanied by DNA fragmentation (Scott *et al.*, 2007).

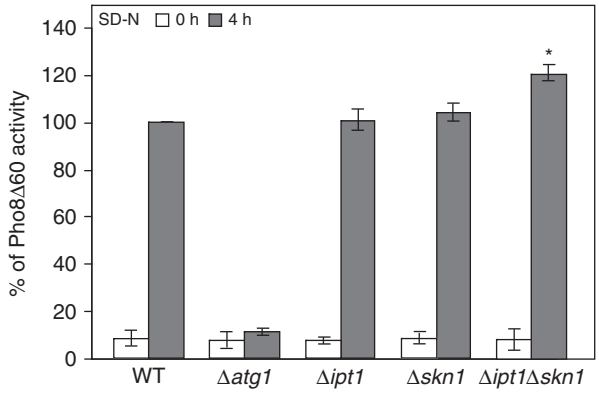

Fig. 1. The Δ*ipt1* Δ*skn1* double mutant is characterized by increased autophagy during N starvation. WT (YTS158), Δ*atg1*, Δ*ipt1*, Δ*skn1* and Δ*ipt1* Δ*skn1* strains expressing Pho8Δ60 were grown in YPD medium and shifted to SD-N for 4 h. Samples at the indicated time points were collected, and the cell lysates were assayed for alkaline phosphatase activity. The results represent the mean of three separate experiments, and the error bars indicate the SD. The value for the WT strain at 4 h of starvation was normalized to 100%. $^{*}P < 0.05$.

Δ*ipt1* Δ*skn1* mutant has increased levels of sphingoid bases

Nutrient conditions influence the biosynthesis of $M(IP)_2C$ in yeast (Im *et al.*, 2003; Thevissen *et al.*, 2005). Therefore,

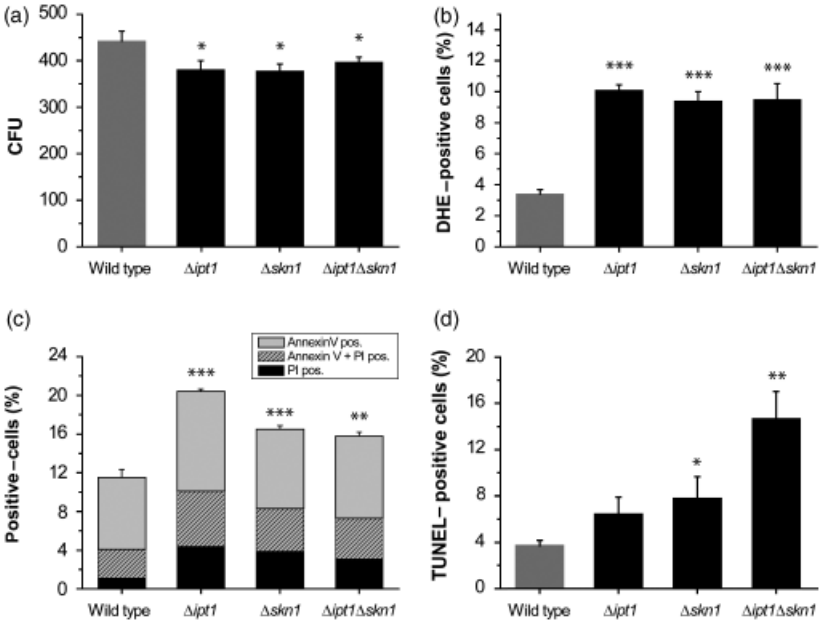

Fig. 2. The double Δ*ipt1* Δ*skn1* deletion mutant exhibits enhanced DNA fragmentation under N starvation. (a) Survival determined by clonogenicity of WT cells, the Δ*ipt1* and Δ*skn1* single mutants or the double Δ*ipt1* Δ*skn1* deletion mutant after N starvation for 4 h during early exponential growth. Data represent mean ± SEM of four independent experiments. $^{*}P < 0.05$. (b) Quantification (FACS analysis) of ROS accumulation using dihydroethidium (DHE) staining for the experiment shown in (a). $^{***}P < 0.001$. (c) Quantification (FACS analysis) of phosphatidylserine externalization and loss of membrane integrity using annexin V/PI containing for the experiment shown in (a). (d) Quantification (FACS analysis) of DNA fragmentation using terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) staining for the experiment shown in (a). $^{*}P < 0.05$; $^{**}P < 0.01$. In all experiments, 30 000 cells were evaluated. Data represent mean ± SEM of four independent experiments.

we analyzed the levels of complex sphingolipids, namely $M(IP)_2C$, mannosylinositolphosphoryl ceramides (MIPC) and inositolphosphoryl ceramides (IPC), in membranes of the single and double Δ*ipt1* Δ*skn1* deletion mutants and WT under N starvation. Unlike when grown in half-strength PDB, there was no detectable $M(IP)_2C$ in any of the mutants upon challenge with N starvation medium, whereas the content of MIPC was increased in all mutants as compared with WT (data not shown), as demonstrated previously when these mutants were grown in a rich medium (Thevissen *et al.*, 2005). Hence, based on the detection limits of our system, membranes of the single and double deletion mutants were not characterized by different contents of complex sphingolipids upon N starvation.

Next, we determined the levels of sphingolipid metabolites including α-hydroxy-phytoceramides, dihydroceramides, phytoceramides, dihydrosphingosine, phytosphingosine and corresponding sphingoid base phosphates via the sphingolipidomics approach in all mutants and WT upon N starvation (Fig. 3). Although LC/MS analysis of sphingolipid metabolites did not reveal significant differences between the double Δ*ipt1* Δ*skn1* deletion mutant and the single mutants or WT upon N starvation, it was observed that higher basal levels (without starvation) of phytosphingosine were present in membranes of the double Δ*ipt1* Δ*skn1* deletion mutant (Fig. 3a) as compared with the single deletion mutants or WT. In addition, the double Δ*ipt1* Δ*skn*1, single Δ*skn1* deletion mutants and WT showed significantly increased levels of α-hydroxy-C18:1-phytoceramides upon N starvation as compared with growth without starvation (Fig. 3b), while levels of phytosphingosine-1-phosphate were decreased upon N starvation (Fig. 3c). In conclusion, membranes of the double Δ*ipt1* Δ*skn1* deletion mutant are characterized by increased basal levels of phytosphingosine.

Discussion

This study reports on the increased induction of autophagy upon N starvation in a double Δ*ipt1* Δ*skn1* deletion mutant of yeast as compared with the single deletion mutants or WT. Apoptotic features were slightly increased in the single and double Δ*ipt1* Δ*skn1* deletion mutants as compared with WT upon N starvation, but there was no significant difference between single and double deletion mutants in this regard, pointing to increased autophagy in the double Δ*ipt1* Δ*skn1* deletion mutant independent of apoptosis. The double Δ*ipt1* Δ*skn1* deletion mutant was further characterized by increased DNA fragmentation upon N starvation as compared with the single deletion mutants or WT. This surplus DNA fragmentation seems to be of nonapoptotic origin because apoptotic features of the double Δ*ipt1* Δ*skn1* deletion mutant were not significantly different from those of single mutants upon N starvation. Hence, these data

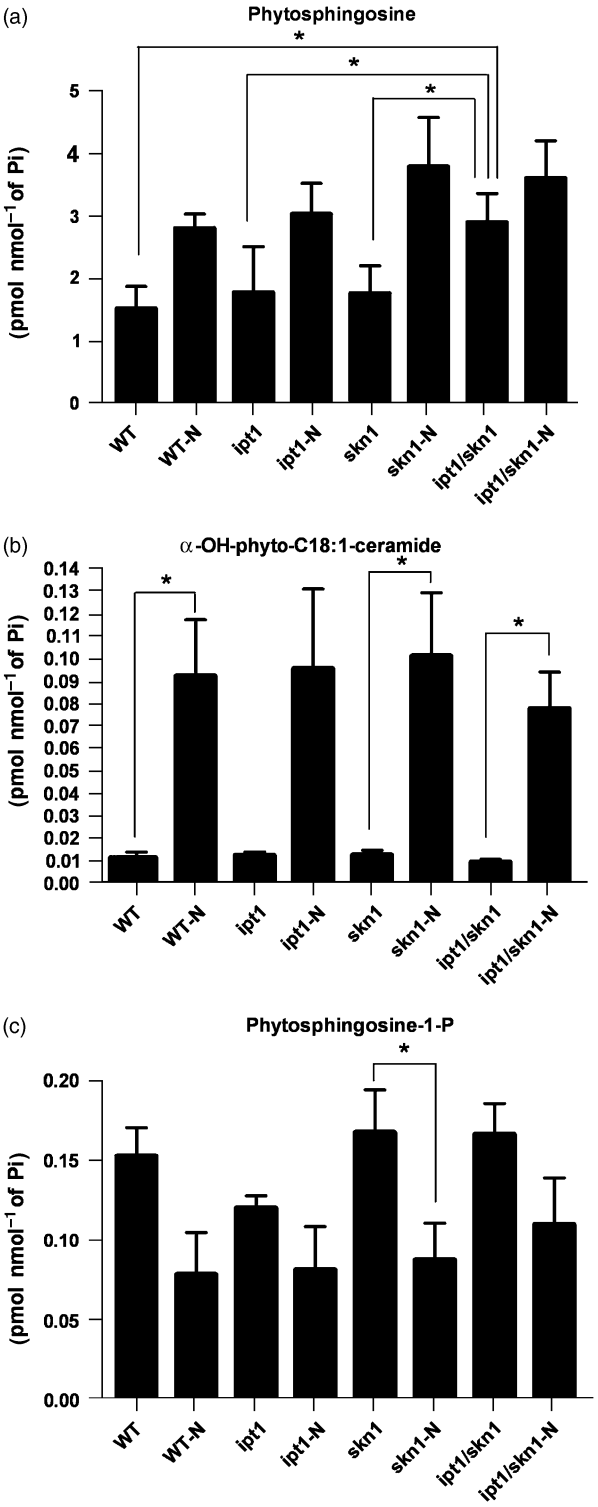

Fig. 3. Sphingolipidomics analysis. Profiles of representative sphingolipid metabolites in WT, the single and double Δ*ipt1* Δ*skn1* deletion mutants under N starvation (−N) or without N starvation. Levels of (a) phytosphingosine, (b) α-OH-phyto-C18:1-ceramide and (c) phytosphingosine-1-P were normalized to total phosphate. $^*P < 0.05$.

point to a link between autophagy and increased DNA fragmentation, as demonstrated previously in *Drosophila* upon overexpression of Atg1 (Scott *et al.*, 2007).

To gain more mechanistic insight into the increased autophagy and DNA fragmentation in the double Δ*ipt1* Δ*skn1* deletion mutant as compared with the single deletion mutants and WT, we focused on putative differences in complex sphingolipids and sphingolipid metabolites in the different yeast strains upon N starvation. In contrast to previous observations for nutrient starvation in half-strength PDB media, which induced the presence of $M(IP)_2C$ in Δ*ipt1* and Δ*skn1* single deletion mutants (Im *et al.*, 2003; Thevissen *et al.*, 2005), N starvation did not lead to detectable differences in the levels of complex sphingolipids or sphingolipid metabolites in the double Δ*ipt1* Δ*skn1* deletion mutant as compared with the single deletion mutants or WT. Interestingly, higher basal levels of the sphingoid base phytosphingosine were observed in the double Δ*ipt1* Δ*skn1* mutant as compared with the single deletion mutants or WT. Treatment of Pho8 Δ60 yeast cells with the ceramide synthase inhibitor fumonisin B_1, resulting in the accumulation of sphingoid bases, resulted in a slight, but reproducible increase in alkaline phosphatase activity under starvation conditions (data not shown). All these data point to a putative role for sphingoid bases in the induction of autophagy and/or DNA fragmentation in yeast. Up till now, there are no reports on a link between sphingolipids or sphingolipid metabolism and autophagy or DNA fragmentation in yeast. In mammals, however, few reports highlight the link between the sphingolipid rheostat and autophagy (Lavieu *et al.*, 2007, 2008). The sphingolipid rheostat in mammals is composed of the relative levels of sphingolipids and their metabolites, namely ceramide (Cer), sphingosine (Sph) and sphingosine-1-phosphate (S1P). In mammalian cells, both ceramide and S1P stimulate autophagy (Lavieu *et al.*, 2008), whereas in yeast, it seems that nonphosphorylated sphingoid bases might stimulate autophagy as documented in this study.

An important signaling pathway involved in the regulation of autophagy is the Ras/PKA pathway (Budovskaya *et al.*, 2004). Inactivation of the Ras/PKA pathway, by overexpression of a dominant-negative allele of *RAS2*, known as $RAS2^{ala22}$, resulted in increased induction of autophagy as compared with WT. However, additional inactivation of the genes encoding the PKA catalytic subunits, *TPK1*, *TPK2* and *TPK3*, in the double Δ*ipt1* Δ*skn1* deletion mutant did not result in an enhanced autophagy phenotype (data not shown) as compared with the double Δ*ipt1* Δ*skn1* deletion mutant, indicating that Skn1, together with Ipt1, might act in the same pathway as Ras/PKA regarding induction/regulation of autophagy. Moreover, PKA and Sch9 signaling pathways are known to regulate autophagy cooperatively in yeast (Yorimitsu *et al.*, 2007). Long-chain bases including phytosphingosine are recognized as regulators of AGC-type protein kinase (where AGC stands for protein kinases A, G and C) Pkh1 and Pkh2, which are homologues of mammalian phosphoinositide-dependent protein kinase 1 (Sun *et al.*, 2000). Based on *in vitro* data, Liu *et al.* (2005a, b) demonstrated that phytosphingosine stimulates Pkh1 to activate additional downstream kinases including Ypk1, Ypk2 and Sch9, and additionally, that phytosphingosine can directly activate Ypk1, Ypk2 and Sch9. In conclusion, it could be that the higher basal levels of phytosphingosine, which we observed in the double Δ*ipt1* Δ*skn1* mutant, affect Sch9 function directly or indirectly, and concomitantly, the authophagy response. Hence, future research will be directed towards determining whether Sch9 or other kinases are part of the link between sphingolipids and autophagy in yeast.

In conclusion, all the data obtained in this study point to a negative regulation of autophagy by both Ipt1 and Skn1 in yeast, which could be mediated by sphingoid bases and might act in the same pathway as the Ras/PKA signaling pathway. Apparently, Ipt1 and Skn1 can functionally complement each other under nutrient limitation, not only regarding synthesis of the complex sphingolipid $M(IP)_2C$ upon nutrient limitation in half-strength PDB (Thevissen *et al.*, 2005) but also regarding the negative regulation of autophagy under N starvation, as demonstrated in this study.

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