Activation and Localization of Inositol Phosphosphingolipid Phospholipase C, Isc1p, to the Mitochondria during Growth of Saccharomyces cerevisiae*

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Sphingomyelinases (SMases) generate ceramides, which are known to regulate cell cycle and growth. Only one enzyme that belongs to the extended family of SMases is present in S. cerevisiae, Isc1p; however, little is known about its regulation or physiologic function. Deletion of ISCI in S. cerevisiae resulted in a growth defect, and the slow growth phenotype was rescued by plasmid-borne expression of Isc1, confirming its role in growth. The levels of phytoceramide exhibited an Isc1p-dependent increase of 4-fold after 24 h of growth. In addition, the specific activity of Isc1p was significantly elevated (3-fold) between the early logarithmic and the late logarithmic/start of stationary phases of growth. The activation of the enzyme was not associated with increased levels of the protein, indicating that the mechanism is independent of transcription/translation. Interestingly, this activation was lost upon delipidation of the enzyme, raising the possibility of regulation by associated lipids. Confocal microscopy revealed that the enzyme was predominantly in the ER during early growth but became associated with mitochondria in late logarithmic growth. These results were also supported by differential centrifugation and isolation of mitochondria and further confirmed in mitochondria purified using sucrose gradients at the different stages of growth. These results reveal that the activity and localization of Isc1p are regulated in a growth-dependent manner. A novel mechanism for activation of Isc1p through localization to mitochondria is proposed. The results also suggest a role for Isc1p-generated ceramides in optimal regulation of growth.

In mammalian cells, the breakdown of sphingomyelin (SM) results in the formation of ceramide, a bioactive lipid that plays roles in regulating cell responses to a variety of extracellular signals through the modulation of the activity of downstream effectors, which in turn control basic cellular functions such as cell growth, cell cycle arrest, apoptosis, and senescence (1–6).

Sphingolipids are also important regulatory molecules in yeast, where they are known to be required for viability (7), for optimal life span (8), and for the regulation of responses of yeast cells to stress (1, 9). Indeed, recent studies have disclosed important roles for yeast sphingoid bases in the regulation of cell cycle arrest (10), degradation of nutrient permeases (11), and endocytosis (12). Yeast ceramides have also been shown to regulate cell cycle progression, possibly through activation of trimeric protein phosphatases composed of Tpd3, Cdc55, and Sit4 (13), analogous to the role of ceramide and ceramide-activated protein phosphatases in regulating mammalian cell cycle and apoptotic responses (14, 15).

Sphingomyelinases (SMases) hydrolyze the phosphodiester linkage of SM, producing ceramide and phosphorylcholine. Such activity was found to exist in organisms ranging from bacteria to mammals, including S. cerevisiae, and SMases and SMase-like enzymes have been recently cloned from mice, humans, Saccharomyces cerevisiae, and Schizosaccharomyces pombe (16–19).

 yer019w was recently identified as the S. cerevisiae gene that encodes for an inositol phosphosphingolipid phospholipase C, named ISCI (18). Comparison of recent reports on Isc1p (18, 20) and human neutral SMase 2 (nSMase 2) (17, 21) shows that these enzymes share many common features. They both (a) hydrolyze SM; (b) require Mg2⁺ for optimal activity; (c) demonstrate a neutral pH optimum; (d) are activated by anionic phospholipids; (e) present ~90% identity; and (f) contain a newly discovered domain that is conserved in the entire family of SMases, the P-loop-like domain (20), which appears to be important for substrate binding and/or catalysis.

Indeed, Isc1p has emerged as the leading enzyme in the study of the mechanisms of this emerging family of sphingo-phospholipid phosphodiesterases. A recent study demonstrated that the second transmembrane domain and the C terminus of Isc1p are required for binding of Isc1p to anionic phospholipids, especially CL, PG, and PS, phospholipids that are potent and essential activators of the enzyme in vitro (22). It was also shown that the C terminus interacts with the remainder of the enzyme, and it was proposed that this interaction plays a critical role in enzyme function through a novel tethering mechanism of enzyme activation by lipid cofactors (22).

Since mammalian SMases play roles in cell growth and the molecular mechanisms for the regulation of these enzymes are not well understood, we became interested in determining whether Isc1p plays a role in cellular growth of S. cerevisiae, in which case the regulation of Isc1p may become an important model. Although deletion of ISCI rendered viable cells, these cells have growth defects, and a recent study showed that Css1p, the S. pombe homologue of Isc1p, is required for viability, that it regulates the coordination of cell wall formation, and...
that its function can be complemented by Isc1p (19). Here we report that Isc1p is required for optimal growth of S. cerevisiae and that the enzyme is activated during growth. Overexpression of ISCI under the control of a heterologous promoter and analysis of microsomal fractions suggested that this increase in specific activity was not due to transcriptional/translation activation. Interestingly and instead, data from confocal microscopy in combination with biochemical experiments using isolated and purified mitochondria demonstrated that Isc1p translocates/localizes to the mitochondria during the postdiauxic phase of growth. Therefore, these results show that Isc1p is regulated (activated and translocated) during growth and suggest a novel mechanism for the activation of Isc1p that Isc1p translocates/localizes to the mitochondria during the late logarithmic and postdiauxic phases of S. cerevisiae growth.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal mouse anti-FLAG M2 antibody was obtained from Sigma. Monoclonal mouse anti-protin and mouse anti-dolichol phosphate mannose synthase (Dpm1) were obtained from Molecular Probes, Inc. (Eugene, OR), and polyclonal goat anti-Kar2p was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-mouse and donkey anti-goat horseradish peroxidase-conjugated antibodies and donkey anti-rabbit rhodamine-conjugated antibody were acquired from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Anti-donkey anti-rabbit rhodamine-conjugated antibody were acquired from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Goat anti-mouse and anti-rabbit antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-mouse and donkey anti-goat horseradish peroxidase-conjugated antibodies and donkey anti-rabbit rhodamine-conjugated antibody were acquired from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Anti-donkey anti-rabbit rhodamine-conjugated antibody were acquired from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

Preparation of Yeast Lysates—Yeast cells were suspended in buffer containing 25 mM Tris-Cl (pH 7.4), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 4 mM/ml each chymotain, leupeptin, antipain, and pepstatin A. Spheroplasts were prepared according to previously published procedures (25, 26). Cells were pretreated with 0.1% Triton X-100, 0.5 mM DTT, 10 μM MG132, 10 μM MG142, and 0.1% protease inhibitor cocktail (Roche Applied Science). Spheroplasts were harvested by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatant was then washed and resuspended in buffer containing 120 mM NaCl, 20 mM sodium phosphate, and Zymolase 100T was added for 30 min. After the incubation, 0.1 M of chloroform, 0.5 mM of methanol, and 0.2 M of water were added according to the method of Polch et al. (27), and the radioactivity in a portion (400 μl) of the upper phase was mixed with Safeguard and microplate scintillation counting.

Preparation of Sandwich Hybridization Membranes—To obtain microsomal fractions, cell lysates were centrifuged at 100,000 × g for 1 h to obtain the microsomal and cytosolic fractions. Microsomal membranes were decontaminated by washing in lysis buffer in the presence of 1% Triton X-100 and incubated for 30 min at 4 °C. The suspensions were centrifuged at 100,000 × g for 90 min, and aliquots from the supernatants were used for enzymatic determinations under the conditions described above for the Isc1p activity assay. For differential centrifugation experiments, cell lysates were centrifuged at 13,000 × g for 20 min to obtain the P10 pellets. The supernatants were transferred and centrifuged at 100,000 × g for 1 h to obtain the P10 pellets. Plots of isolation efficiency were based on published procedures (26, 29, 30) that reported that microsomes associated with mitochondrial surface can be removed in part by a decrease in the pH value of the isolation buffer (25). Spheroplasts were prepared in buffer A (20 mM potassium phosphate, pH 7.4, 1.2 M sorbitol) and resuspended in buffer B containing 10 mM K-mes, pH 8.0, 0.8 M sorbitol, 0.5 mM EDTA. After the addition of 5 μM phenylmethylsulfonyl fluoride, the cellular homogenate was prepared, and microsomal preparations were obtained as described by Glick et al. (29).

Isolation of Purified Mitochondria—Crude mitochondria were isolated as described above, suspended in buffer C (0.6 M sorbitol, 20 mM potassium phosphate, pH 7.4, 0.8 M sorbitol, 0.5 mM EDTA). The addition of 5 μM phenylmethylsulfonyl fluoride, the cellular homogenate was prepared, and crude mitochondrial preparations were obtained as described by Glick et al. (29).

Identification of the different Isc1p constructs developed in this study

| Name               | PCR primers                           | Region of Isc1 gene |
|--------------------|---------------------------------------|---------------------|
| GFP-Isc1           | F: CGG GGT ACC ATG TAC AAG AGA AAA AGC AGA GAT TCT CTT TTT TCA GAA AGT TGG C | Entire coding sequence |
|                    | R: GCC GTC GCT CAA GAA GAT TTT GTC C |                     |
| Isc1EndoProm       | R: GCC GTC GCT CAA GAA GAT TTT GTC C | 0.5-kb promoter region |
|                    | F: GCC AGG GAC AAG TCT GGC TGC CTA GAT ACA TAC |                     |
| FLAG-Isc1          | R: GCC GTC GCT CAA GAA GAT TTT GTC C | Entire coding sequence |
|                    | F: GCC AGG GAC AAG TCT GGC TGC CTA GAT AAC |                     |
|                    | R: GCC GTC GCT CAA GAA GAT TTT GTC C |                     |

Table 1

The nomenclature of recombinant Isc1p proteins, the PCR primers utilized to create the proteins, the fusion tags present, and the regions of Isc1p encoded by the proteins are shown. F and R refer to the forward and reverse PCR primers, respectively. All primer sequences are oriented 5′-3′.
were grown for the indicated number of hours, and the $A_{600}$ was determined. The results are averages of triplicate experiments. The results are representative of at least three independent experiments.

**Measurements of Mass Levels of Phytoceramide**—Cells were harvested and washed with water, and lipids were extracted following the method of Bligh and Dyer (32). The chloroform organ phase was divided into aliquots, dried down, and processed for inorganic phosphorus determination or phytoceramide measurements using the *Escherichia coli* diacylglycerol kinase assay. Phytoceramide was quantitated using external standards and normalized for phosphorous content (33).

**Confocal Microscopy**—Cells were examined under a confocal laser-scanning microscope (Olympus IX70) with a Plan Apo × 60 oil objective (numerical aperture 1.4), and images were captured using PerkinElmer Ultraview software that was set at the spinning disc mode.

**Cell Fixation and Fluorescence Staining**—Mitochondria were stained as described (34). In brief, cells were collected by centrifugation and gently suspended in growth medium containing 500 nM dye MitoTracker® Red CM-H2XRos (Molecular Probes), incubated for 30 min in the dark at 30 °C, and washed three times with phosphate-buffered saline. Cells were fixed for 2 h with 3.7% formaldehyde in the medium and washed three times with phosphate-buffered saline, and spheroplasts were obtained as described above. For immunofluorescence, spheroplasts were loaded on a glass slide precoated with poly-l-lysine. Washing, blocking, and antibody incubations were performed in a humid chamber as described (35), and the cells were covered with a coverslip for microscopic visualization. The excitation wavelength for GFP was 488 nm, and 525/550-nm emission was detected. The excitation wavelength for MitoTracker Red was 568, and the emission detection was >590 nm. No optical cross-talk between the emission channels was observed. For quantification of the colocalization of GFP-Isc1 with intracellular markers, the number of cells showing colocalization was expressed as a percentage of the total number of evaluable cells. Double-labeling experiments were performed using MitoTracker Red and Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes) as described above. To invert the fluorophores, MitoTracker Green FM (Molecular Probes) and Alexa Fluor 633-conjugated antibodies (Molecular Probes) were also used.

**RESULTS**

Deletion of *ISC1* (*isc1*) caused cells to grow slowly. The defect in growth was readily detectable at late log and stationary phases when compared with the parental wild type strain (Fig. 1). The optical density (OD) values for *isc1* strain (ko) grown for 72 h were comparable with the values for the 24-h wild type cultures, and, at most, *isc1* reached −70% of the wild type stationary phase growth. The *isc1* slow growth phenotype was rescued by plasmid-borne expression of *ISC1* (Fig. 1), confirming that the slow growth was indeed caused by loss of *ISC1*. Thus, Isc1p is required for optimal cellular growth.

The above results suggested the hypothesis that Isc1p enzyme activity may be regulated differentially during different phases of yeast growth, and two approaches were undertaken in order to determine whether Isc1p is selectively activated. First, the levels of phytoceramide were measured in extracts prepared from wild type or *isc1* cells, grown for 4, 12, 24, and 48 h after dilution of the culture to 0.1 $A_{600}$ units, as described under “Experimental Procedures.” Results are averages ± S.D. of three different experiments.

**Fig. 2. ISC1-dependent changes of phytoceramide levels during growth.** The levels of phytoceramide in wild type (wt) or *isc1* mutant (ko) cells cultured for the indicated times were determined by the diacylglycerol kinase assay as described under “Experimental Procedures.” Results are averages ± S.D. of three different experiments.
formed on equal loading of total protein, did not reveal an increase in the expression levels of Isc1p at 24 h compared with 4 h, and actually a decrease was reproducibly observed. Thus, it is concluded that the increase in specific activity of Isc1p is not due to an increase in the relative expression levels of Isc1p. Since these results argue against a transcriptional/translational mechanism of Isc1p regulation, other mechanisms of activation were investigated.

In order to perform additional biochemical experiments, a higher amount of Isc1 protein was required. For this purpose, FLAG-Isc1 was overexpressed under the control of a promoter inducible by galactose, in the background of ISC1 deletion strain. This approach also allowed further investigation to determine whether regulation of Isc1p activity was at the level of transcription, since these studies employed a heterologous promoter. Initially, we determined whether this strain exhibited the same features that the wild type did. Again, an increase (−4–5-fold) of specific activity at 24 versus 4 h was reproducibly obtained (Fig. 3c). Importantly, these results demonstrate that the increase in specific activity is promoter-independent and validate the use of this construct to investigate the basis of the activation of Isc1p during cell growth. Also, immunoblotting did not reveal an increase in the expression levels of Isc1p −55-kDa band at 24 h compared with 4 h (Fig. 3d, upper panel). To control for the effect of the galactose promoter, we monitored in parallel the expression levels of another FLAG-tagged protein, mammalian nSMase 2, also expressed under the control of galactose-inducible promoter. Expression pattern analysis revealed a slightly stronger band of the expected size (−78 kDa) at 4 compared with 24 h (Fig 3d, middle panel), indicating that indeed, expression of FLAG proteins under the GAL1 promoter do not increase during the time frame of analysis. Also, immunoblotting for an endogenous yeast protein, Kar2p, showed no increase in its expression (Fig. 3d, lower panel). Thus, these results provide further evidence against transcriptional/translational activation of Isc1p.

Next, specific activity was measured in microsomes obtained from cells overexpressing FLAG-Isc1 at 4 or 24 h, in parallel to total lysates as a control. Microsomes showed only 1.3-fold of activation and did not parallel the activation (−4–5-fold) found in total lysates (Table II). These results suggested the involvement of a regulator (loss of activator or gain of inhibitor) in the microsomal fractions containing Isc1p as opposed to total lysates. Importantly, these results clearly negate significant regulation of Isc1p activity at the transcriptional or translational level as the cause for the enzyme activation, and they even argue against most post-translational covalent modifications, since there was no change in the chromatographic behavior of Isc1p on SDS gel electrophoresis and since the activation was not retained upon further purification of the enzyme.

![FIG. 3](http://www.jbc.org/)

**Fig. 3. Activation of Isc1p during growth.** A, activation of endogenous Isc1p. Wild type (wt) and Δisc1 (ko) cells were grown for the indicated times, and cell lysates were prepared as described under “Experimental Procedures.” Activity was determined using 30 μg of total protein. Results are averages ± S.D. of three different experiments. B, activation of FLAG-Isc1 (lower panel). FLAG-Isc1 was expressed under the control of the endogenous ISC1 promoter, and activity was measured as described above. Protein (30 μg) was subjected to immunoblotting using anti-FLAG antibody (upper panel). The results shown are from one experiment representative of three separate experiments. C, FLAG-Isc1 was overexpressed under the control of galactose promoter, and activity was measured as described above, except that 0.5 μg of total protein was assayed. D, 5 μg of protein were subjected to immunoblotting using mouse anti-FLAG or goat anti-Kar2p antibodies. The results shown are from one experiment, representative of three experiments.
In studies on mammalian nSMase, it was noted that the enzyme was tightly associated with activating lipids (21) and that delipidation of microsomes rendered the enzyme totally dependent on anionic phospholipids (such as PS). Since Isc1p shares with nSMase a strict dependence on anionic phospholipids for activation (18), we investigated the effects of delipidation (36) on the observed activation of Isc1p. For these studies, microsomal fractions obtained from cells grown for 4 or 24 h were incubated in the presence of 1% Triton X-100 and centrifuged, and aliquots from the supernatants obtained were assayed for Isc1p in the presence of constant amounts of detergent and activating phospholipids. The results showed clearly that following delipidation of microsomes, the activation was completely lost (Table II).

The above results suggested the presence of activating lipids associated with Isc1p in the lysates of late stage cells and not in early growth phase. This raised the possibility that the environment of Isc1p during late logarithmic growth phase may be different from early cells. Therefore, it became important to determine the intracellular localization of Isc1p in different phases of growth. To define the in vivo localization of Isc1p, a construct was engineered in which GFP was fused to the N-terminus of Isc1p. This chimera was biochemically similar to Isc1 compartments was observed, and at a much lower (23%) quantification of the merged fluorescence showed 82% colocalization. At 24 h, a close but distinct separation of the Kar2p and Isc1p compartments was observed, and at a much lower (23%) localization.

"eyelash-shaped" segments were primarily observed at the 4-h growth stage (Fig. 4a, third panel). On the other hand, a different shape with the structure of "tubular branched networks" was mainly observed for GFP-Isc1p in 24-h cultured cells (Fig. 4a, bottom left). The patterns of GFP-Isc1 were indistinguishable whether the cells were fixed or not (data not shown). These tubular branched network structures are well established in yeast microscopy as mitochondria (37–39) and frequently contain multiple branching points, thus forming the network, which is partially acquired in a confocal image. In contrast, the eyelash segment structures lack branching points and seem to correspond to the smaller and brighter circle of the yeast ER. Segment and tubular network GFP-Isc1 structures coexisted during growth; however, their frequencies varied significantly at 4 and 24 h. These two Isc1-containing structures were counted in 100 cells, and the segment/tubular network ratio was calculated for each time point (Fig. 4a). A marked decrease in this ratio from 4.5 to 0.4 was found between the 4- and 24-h time points, respectively. These results suggested a major change in the subcellular localization of GFP-Isc1p during the early and late phases of growth.

Although these distinct patterns for ER and mitochondria are widely accepted, it was still important to define this localization more accurately, especially given the change in localization. Therefore, the intracellular distribution of GFP-Isc1 was visualized in colocalization studies in which fluorescent dye staining or indirect immunofluorescence were followed by confocal microscopy. To test whether Isc1p localized to the ER, cells from 4- and 24-h cultures were fixed and subjected to immunofluorescence. A resident ER protein, Kar2p, was used as a marker for this compartment. No differences were observed in the pattern of Kar2p in GFP-Isc1-overexpressing and control cells at 4 or 24 h (Fig. 4, b and d), demonstrating that the ER does not change shape during growth and indicating lack of effect of Isc1p on ER morphology. At 4 h, colocalization between Kar2p staining and GFP-Isc1p was observed, and...
dependent experiments. Results are representative of at least three in-
homogenate for that time point. Results are averages and plotted as the percentage of total activity in the unfractionated

frequency. This partial colocalization and the reduction in the percentage of association with ER seem to indicate that Isc1p localized to another compartment close to ER such as mito-

dochondria at 24 h, as suggested by the morphology of Isc1

change in the intracellular localization of Isc1p during growth. Altogether, these data demonstrate a significant

observed, as previously reported (34). These results indicate a change in the localization of Isc1p, suggesting that during the logarithmic phase of growth the enzyme colocalizes with the ER and that it may move to the mitochondria at 24 h of growth, also remaining close to the ER. Thus, taken together, the morphology studies and the co-localization results demonstrate significant differences in the localization of GFP-Isc1p between the early and late phases of growth.

Biochemical fractionation studies were employed next to pro-
vide an independent confirmation for a change in localization of Isc1p during the different growth phases. Total lysates were prepared from cells overexpressing FLAG-Isc1 cultured for 4 and 24 h and then subjected to differential centrifugation to obtain a 13,000 × g mitochondria-rich fraction (P13) and a 100,000 × g microscope-rich fraction (P100). At both time points, Isc1p activity in each fraction was determined and plotted as a percentage of total activity present in the unfrac-

tional homogenate from the same time point (Fig. 5a). Importantly, the distribution of Isc1p was significantly and dra-

matically changed during these phases of growth. About 75% of the activity present at 4 h precipitated with the P100 fraction.

In contrast, the majority of the activity (>90%) present in the 24-h extracts fractionated into the P13 pellet, consistent with a change in localization from ER to mitochondria (26, 40).

Immunoblot analysis provided another confirmation for the change in the localization of Isc1p (Fig. 5b). When equal amounts of protein were loaded, the analysis of the fractions (P13 and P100) obtained from the 4-h total lysate indicated that Isc1p distributed primarily to the P100 at the logarithmic phase, in agreement with the activity and the colocalization results. Instead, at 24 h, most of the FLAG-Isc1 fractionated to the P13 fraction, also paralleling the activity and microscopical observations. Altogether, these data demonstrate a significant change in the intracellular localization of Isc1p during growth of S. cerevisiae.

Next, crude mitochondrial fractions were prepared from 4- and 24-h cultures as previously described (29) and analyzed by immunoblotting for Isc1p and other mitochondrial and ER-

resident proteins. As with the previous result, some Isc1p was detected in the P13 fraction at 4 h of growth, but this expres-

sion was lost in the further purified mitochondrial fraction (Fig.

6). Also, both porin (mitochondrial protein) and Dpm1p (ER protein) were detected in the P13 crude fraction, but only porin became enriched in the mitochondrial fraction. Thus, the partial sedimentation of Isc1p in the P13 fraction at 4 h probably resembles the general sedimentation behavior of ER proteins (such as Dpm1), which is in agreement with previous observations that show partial sedimentation of ER proteins in the P13 fraction (41). Importantly, and in stark contrast, whereas Isc1p
was almost absent from mitochondria obtained at 4 h, the mitochondria isolated at 24 h were enriched in Isc1p (Fig. 6). These results provide further evidence for a change in localization of Isc1p from ER to mitochondria.

Finally, pure mitochondria were obtained by further purification of crude mitochondria using sucrose gradients as described (30, 31). Step gradients from 30–60% sucrose were prepared to analyze pure mitochondria from 4 h (left) and 24 h (right). Porin was detected in the fractions from the lower third of the tube (see lane numbers). Membranes were stripped and reimmunoblotted using anti-FLAG antibodies. The results shown are from one experiment representative of two experiments.

The major conclusion from this study relates to the subcellular localization of Isc1p. Four lines of evidence support the change in the intracellular localization of Isc1 during growth.

(a) Morphology studies show that Isc1p is present in short curved and unbranched segments (part of the ER) at 4 h, whereas at 24 h it is predominantly in tubular branched networks (typical of mitochondria). (b) Colocalization studies show that Isc1p primarily co-localized with an ER marker at 4 h but not at 24 h, whereas it co-localized with mitochondrial markers, corresponding to the tubular network structures, at 24 h but not at 4 h. (c) Crude fractionation studies showed that Isc1p (activity and protein) sedimented with light membranes at 4 h of growth but sedimented with heavy membranes in the post-diauxic phase. (d) Isolated purified mitochondria were enriched in Isc1p at 24 h but not at 4 h.

In relation to the fractionation studies, it is worth noting that whereas most of the mitochondria are known to sediment with the P13 fraction, ER has a heterogeneous sedimentation profile, and ER proteins are found in P13, P30, P40, and P100 fractions, with 50–60% of the ER in the 13,000 × g spin (41). The ER marker Dpm1p has been shown to be present both in the P13 and the P100 fractions (42). Importantly, most of these studies have been performed using cultures entering the stationary phase, so care needs to be taken when making conclusions about organelle properties during the early log phase of growth. The differential centrifugation data (Fig. 5) revealed that at 4 h most of Isc1p was present in the P100 fraction (mostly ER) and some in the P13 (ER or mitochondria); however, the confocal data did not show colocalization of Isc1p with mitochondria at this time point, and thus the data suggest that the enzyme may be primarily in the ER rather than mitochondria during early log growth. This was further supported by further purification of the P13 fraction as shown in Fig. 6, where Isc1p became depleted from the mitochondrial fraction at 4 h. In contrast, it became evident that during the post-diauxic shift, Isc1p localized to mitochondria based on the microscopic and biochemical observations.

Importantly, this change in subcellular localization of Isc1p may provide a mechanism for activation of the enzyme. There was no evidence of change in the levels of the protein or any major post-translational modification as judged by Western blotting; however, more subtle changes such as phosphorylation without change in mobility on SDS-PAGE cannot be ruled out. Nevertheless, such stable post-translational modifications cannot be sufficient for activation, since activation was lost upon delipidation. Therefore, it is tempting to speculate that the enzyme becomes activated once it localizes to the mitochondria. Therefore, it is tempting to speculate that the enzyme becomes activated once it localizes to the mitochondria.
Activation and Localization of Isc1p during Growth

Indeed, the enzyme is nearly totally dependent on anionic phospholipids, especially PG, PS, and CL for activity in vitro (18), and we have previously proposed a novel "tether-and-pull" in vitro model for activation of Isc1p whereby the enzyme requires interaction of its carboxyl terminus with anionic phospholipids (22). This results in tethering the carboxyl terminus to the membrane, which in turn pulls the catalytic site close to its substrates in membranes. Notably, mitochondria are rich in PG and CL, which then may act as "activators" of Isc1p. According to this hypothesis, Isc1p, during early log phase, is located in a compartment (in the ER or close to the ER such as mitochondrial associated membranes (MAMs)) where concentrations of lipid activators/cofactors are low. Following the diauxic shift, the enzyme localizes to an environment rich in anionic phospholipids, namely the mitochondria.

The localization of Isc1p to mitochondria raises questions as to localization of its substrates. In yeast, the major sphingolipids inositol phosphorylceramide/mannosyl inositol phosphorylceramide/mannosyl diinositol phosphorylceramides function as substrates for Isc1p, and they have been suggested to be enriched at the plasma membrane; however, pools of these three lipids were also detected in mitochondria (25), raising the possibility that Isc1p may act directly on the inositol phosphorylceramide/mannosyl inositol phosphorylceramide/mannosyl diinositol phosphorylceramides in mitochondria following the diauxic shift. In mammalian cells, SM is enriched in the plasma membrane. However, two pieces of evidence favor the diauxic shift. In mammalian cells, SM is enriched in the amide/mannosyl inositol phosphorylceramide/mannosyl diinositol phosphorylceramides function as targeting of its substrates. In yeast, the major sphingolipid lipids PG and CL, raises the possibility of Isc1 to mitochondria.

The data on Isc1p presented here represent the first evidence for the in vivo mitochondrial localization of a yeast enzyme of the sphingolipid metabolism. In this regard, the growth phase-dependent localization of Isc1p to the mitochondria may be important for the regulation of growth. In addition, the regulated mitochondrial localization of Isc1p may also support a key role for the generation of mitochondrial ceramide in growth. Finally, because of its regulated localization, Isc1p may serve as a model to study the molecular mechanisms of regulation for the mammalian members of the family of neutral SMases as well as to understand the effects of the compartmentalized generation of ceramides. Further investigation is required to define the mechanisms that regulate the localization of Isc1p, its activation, and the mechanisms by which the generated ceramides regulate cell growth.

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