Distinct roles for *de novo* versus hydrolytic pathways of sphingolipid biosynthesis in *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae produces the sphingolipid ceramide by *de novo* synthesis as well as by hydrolysis of complex sphingolipids by Isc1p (inositolphosphoceramide-phospholipase C), which is homologous with the mammalian neutral sphingomyelinases. Though the roles of sphingolipids in yeast stress responses are well characterized, it has been unclear whether Isc1p contributes to stress-induced sphingolipids. The present study was undertaken in order to distinguish the relative roles of *de novo* sphingolipid biosynthesis versus Isc1p-mediated sphingolipid production in the heat-stress response. Ceramide production was measured at normal and increased temperature in an *ISC1* deletion and its parental strain (*ISC1* being the gene that codes for Isc1p). The results showed that Isc1p contributes specifically to the formation of the C₂₄-, C_{24:1}- and C₂₆-dihydroceramide species. The interaction between these two pathways of sphingolipid production was confirmed by the finding that

ISC1 deletion is synthetically lethal with the *lcb1-100* mutation. Interestingly, Isc1p did not contribute significantly to transient cell-cycle arrest or growth at elevated temperature, responses known to be regulated by the *de novo* pathway. In order to define specific contributions of *ISC1*, microarray hybridizations were performed, and analyses showed misregulation of genes involved in carbon source utilization and sexual reproduction, which was corroborated by defining a sporulation defect of the *isc1* Δ strain. These results indicate that the two pathways of ceramide production in yeast interact, but differ in their regulation of ceramides of distinct molecular species and serve distinct cellular functions.

Key words: ceramide, *de novo* synthesis, inositolphosphoceramide-phospholipase C (Isc1p), sphingomyelinase (SMase), sporulation.

INTRODUCTION

In recent years, bioactive sphingolipids, including ceramide, have emerged as key regulators of eukaryotic cell processes including stress responses, cell growth, differentiation and apoptosis [1–3]. Ceramide is generated via *de novo* synthesis from serine and fatty acyl-CoA, forming a sphingoid base which is then acylated to form ceramide. Ceramide is incorporated into complex sphingolipids, and thus can also be generated from their catabolism [4]. Considerable research has focused on the hydrolytic pathway of ceramide formation in mammalian cells by SMases (sphingomyelinases). Production of ceramide by SMases results from stimulation with pro-inflammatory cytokines and hormones and also from treatment with various inducers of apoptosis or stress, including chemotherapeutic agents (reviewed in [5]). However, other studies have demonstrated the role of *de novo*-synthesized ceramide in many of these responses [6–8] and, therefore, an ongoing issue in sphingolipid research has been the relative importance and roles of *de novo*-synthesized versus hydrolytically derived ceramides. Subsequently, a central theme in sphingolipid research has been to identify the respective sources of ceramide produced in response to various stimuli.

Yeast models have been invaluable for the identification of enzymes of sphingolipid metabolism. Though yeast do not contain sphingomyelin, they do form IPC (inositolphosphoceramide), which differs from sphingomyelin primarily in that it contains an inositol moiety instead of the choline group of sphingomyelin. Interestingly, yeast cell homogenate was demonstrated to have SMase activity against exogenous sphingomyelin [9], which led

to the discovery that the enzyme responsible for this activity is an IPC-phospholipase C (Isc1p) highly selective for IPC and its mannosylated derivatives and inactive toward other yeast phospholipids [9]. Overexpression of this enzyme conferred increased SMase activity to yeast cells [9], and deletion of the *ISC1* gene (which codes for Isc1p) eliminated the SMase and IPCase activities of yeast cell homogenate [9]. The gene product ISC1 bears significant sequence homology with both the bacterial SMase and the mammalian neutral SMases 1 and 2 [9] and requires Mg²⁺ as well as anionic phospholipids for activity [9]. A model for its activation by anionic-phospholipid binding has been recently proposed [10].

Though a significant amount of functional information is available for mammalian neutral SMase, including its activation by cytokines and chemotherapeutic agents and its role in generating ceramide in response to these agents, there is very little functional information for Isc1p. Furthermore, though many studies demonstrate key roles for *de novo*-produced sphingolipids in yeast biology, particularly in the HSR (heat-stress response), major functions for ceramides and other sphingolipids derived from the hydrolysis of complex sphingolipids in yeast remain to be discovered. A recent study demonstrated a role for Isc1p in the regulation of the ENA2 cation transporter and subsequent Na⁺ tolerance [11], and a study in *Schizosaccharomyces pombe* indicated a potential role for the *S. pombe* Isc1p homologue, CSS1, in cell-wall organization [12]. A recent study identified growth defects for an *ISC1* deletion strain on various non-fermentable carbon sources which was related to decreased levels of mitochondrial cytochrome *c* oxidase subunits [13].

Abbreviations used: HSR, heat-stress response; IPC, inositolphosphoceramide; Isc1p, inositolphosphoceramide-phospholipase C; N-SMase, neutral sphingomyelinase; ORF, open reading frame; RMA, robust multichip average; SMase, sphingomyelinase; YPD, yeast/peptone/dextrose.

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Other than what has been stated above, little is known of the role of Isc1p in yeast biology. Therefore, we sought to investigate potential roles for Isc1p in the yeast *Saccharomyces cerevisiae*. We adopted a combined approach, utilizing biochemical, genetic and microarray-gene-profiling studies in an attempt to define specific functions of Isc1p. The results indicate the involvement of Isc1p in ceramide accumulation in response to heat; however, the ceramide produced was distinct from *de novo*-synthesized ceramide with regard to chain length. Furthermore, gene-regulation studies provided insights into the osmotic-stress and carbon-source-utilization phenotypes and, importantly, led us to investigate and identify a specific role for Isc1p in facilitating sporulation.

MATERIALS AND METHODS

Materials

The generation of the *ISC1* deletion mutant in the laboratory strain JK93d α (*MAT α trp1 leu2-3 his4 ura3 ade2 rme1*) has been described previously [10]. The *lcb1-100* (*MAT α leu2 trp1 ura3 bar1 lcb1 temperature-sensitive*) strain and its wild-type isogenic background (RH406: *MAT α leu2 trp1 ura3 bar1*) were generously provided by Dr Howard Riezman (Department of Biochemistry, University of Geneva, Geneva, Switzerland).

Sphingolipid measurements

Diacylglycerol kinase assay

Cultures were grown to mid-exponential phase and then treated as indicated. Lipids were extracted using the procedure of Bligh and Dyer [13a], subjected to mild base hydrolysis, and ceramides were phosphorylated with *Escherichia coli* diacylglycerol kinase as described in [14]. Products of this reaction were purified by TLC and quantified as previously described [15].

Liquid chromatography–MS measurements

Cells were cultured to mid-exponential phase, treated as necessary, then collected by centrifugation at 2800 g. Cell pellets were snap-frozen in a methanol/solid CO₂ bath and stored at –80°C. Internal standards were added to frozen pellets, and sphingolipids were extracted in a one-phase neutral organic solvent (propan-2-ol/water/ethyl acetate, 30:10:60, by vol.) as described in [16]. A fraction of total extract from each sample was reserved for phosphate determination, and the remaining samples were then analysed by a Surveyor/TSQ 7000 liquid chromatography–MS system. Lipids were qualitatively defined by parent-ion scanning for known fragments characteristic for a specific sphingolipid class, including sphingoid bases, sphingoid base phosphates and ceramides, as described in [16]. Samples were quantitatively analysed on the basis of calibration curves generated with synthetic standards. The mass of each species was normalized by organic-phosphate determination. It should be noted that this method is capable of detecting dihydroceramides and phytoceramides, but not their α -hydroxylated derivatives.

Flow cytometry

Cells were grown at 30°C to mid-exponential phase and then heat-stressed for the indicated times. Since culture density can affect cell division, some samples were reserved at 30°C to serve as time-matched controls. At each time point, cells were harvested, stained with propidium iodide, and sorted on the basis of fluorescence as previously described [17].

RNA preparation

Yeast cells were cultured in rich medium at 30°C to mid-exponential phase and then transferred to 39°C for the indicated times in the heat-stress experiments. Cultures were centrifuged at 3000 g at 4°C, and RNA was isolated from cell pellets using the RNeasy RNA Isolation Kit from Qiagen following the enzymatic lysis protocol.

Microarray hybridization and analysis

All microarray procedures were described previously [18] and are essentially the same as recommended in the Affymetrix (Santa Clara, CA, U.S.A.) technical manual. Arrays were performed in duplicate and subjected to two types of analysis as follows.

Microarray Suite 5.0 analysis

Initial analysis was initiated with selection of misregulated genes using the Affymetrix MAS 5.0 software, starting with selecting those probe sets having a difference in signal log ratio (log₂ of the signal ratio for each strain) of > 1 or < –1 between the wild-type and *ISC1* deletion strain (which corresponds to a difference of at least 2-fold in the signal ratios). Of the probe sets that did not meet this criterion, those which had a different change call between mutant and wild-type by MAS 5.0 (change call indicates an 'increase', 'decrease', 'moderate increase', 'moderate decrease', or 'no change' relative to basal levels) and where in one experimental situation (i.e. at either time point in either strain) demonstrated a signal of at least 100 and changed at least 2-fold were combined with the genes selected on the basis of signal log ratio. This analysis was performed independently on each data set. Microsoft Access was then used to identify probe sets which met the above criteria in both experiments, and this subset was further restricted to include only those probe sets which behaved consistently in both experiments (e.g., repressed in the mutant in both experiments or induced in the mutant in both experiments). For these genes, fold change was calculated as (signal after heat stress)/(signal at normal temperature) and means and S.E.M. values were calculated using Microsoft Excel.

RMA (robust multichip average)

A more stringent and statistically rigorous method for selection of misregulated genes was carried out by normalizing the data using multi-array analysis method (RMA), which is implemented and available from the open source software package Bioconductor. To identify significantly differentially expressed genes, the empirical Bayes moderated *t* test was used. Since analysis requires large numbers of *t* tests to be performed, data were refined by first determining the *Q*-value (false-positive rate) according to the method of Storey and Tibshirani, also available in the Bioconductor package, and *Q*-value threshold was determined empirically by including transcripts known to be regulated in the wild-type data set.

Sporulation assay

The sporulation assay was performed as described previously [19]. In brief, diploid cells were plated on to sporulation medium [YPD (yeast/peptone/dextrose; 10 g of yeast extract, 20 g of peptone, 20 g of glucose/litre of distilled water, pH 6.5), including 1% potassium acetate] and incubated at 30°C for 7 days. At the end of this incubation, well-formed asci containing three or four spores were counted and expressed as a percentage of total cells on the plate.

Figure 1 Ceramide levels during heat stress in wild-type (Jk93d) and *isc1Δ* strains

Cells were heat-stressed, lipids were extracted and separated by TLC as described in the Materials and methods section and the levels of phytoceramide (A) and dihydroceramide (B) were quantified and normalized to total lipid phosphate. Results are presented as the average fold change of each time point with respect to basal levels (0 min time point) from three independent experiments. Results are means $\pm$ S.E.M.

RESULTS

Isc1p-mediated ceramide formation after heat stress

Several previous studies demonstrated an acute up-regulation of sphingolipid metabolism following heat stress [15,20–23]. Within 5–10 min of heat stress, sphingoid bases increase 10-fold, followed by an increase in phytoceramides by 30 min [15,22]. The initial rise in sphingoid bases is accomplished via *de novo* synthesis [15,23], and some ceramide production is probably also achieved through the *de novo*-synthesis pathway, which is supported by the partial inhibition of ceramide accumulation by fumonisins B1, an inhibitor of ceramide synthase (sphingoid base N-acyltransferase) [15]. However, it is also feasible that a component of the increase in phyto- and dihydro-ceramides is attained by Isc1p-mediated hydrolysis of IPC to generate ceramides.

Therefore, in order to determine the contribution of Isc1p to the rise in phytoceramide and dihydroceramide upon heat stress, ceramide levels were measured in the *ISC1* deletion strain (*isc1Δ*) and its isogenic background strain, JK93d α , over a time course of heat stress. Whereas the parental strain showed a 2-fold increase in phytoceramide detectable after 30 min of heat stress, followed by continued increase up to 8-fold by 2 h, the *isc1Δ* strain showed a moderate increase and a failure to accumulate phytoceramide at later time points (Figure 1A). Additionally, measurements of

Figure 2 Liquid-chromatography–MS identification of ceramide species deficient in the *lcb1-100* mutant strains

Cells were grown at 30 °C to mid-exponential phase and aliquots then incubated at 39 °C. Measurements were performed as described in the Materials and methods section. (A) Dihydroceramide, *isc1Δ* and its parental strain (wt) were incubated at 39 °C for 60 min. Results are means $\pm$ S.E.M. for two experiments. (B) Phytoceramide, *lcb1-100* and its parental strain (wt) were incubated at 30 °C and then switched to 39 °C for 60 min. Phytoceramide species of C₁₆, C₁₈, C_{18:1} and C₂₀ were below limits of detection in all strains.

dihydroceramide in the parental strain over the same time course demonstrated a 4-fold increase in dihydroceramide by 30 min, and these levels were sustained up to 2 h; however, in the *isc1Δ* strain, dihydroceramide increased only about 2-fold (Figure 1B). As the *de novo*-synthesis pathway is intact in this mutant, the lack of an appropriate ceramide increase indicates that Isc1p contributes to ceramide production in response to heat stress and thus may play a role in the HSR.

Ceramide species produced by Isc1p differ from those produced *de novo* during heat stress

We next wondered whether the two pools of ceramide, generated *de novo* or by Isc1p, may be biochemically different. Using liquid chromatography–MS as described in the Materials and methods section, non-hydroxy phyto- and dihydro-ceramide molecular species were determined before and after heat stress in the *lcb1-100* and *isc1Δ* strains and in their parental background strains (RH406 and JK93d α respectively). Prior to heat stress, all species measured were present in essentially similar levels among the strains. However, sphingolipid measurements taken after 1 h of heat stress revealed that, interestingly, deletion of *ISC1* did not decrease sphingoid bases or sphingoid base phosphates measured (results not shown). Significant decreases occurred, however, in dihydroceramide species of C₁₆, C₂₄, C_{24:1}, and C₂₆ chain length in the *isc1Δ* mutant as compared with the parental strain (Figure 2A). This suggests that the pool of complex sphingolipids hydrolysed by Isc1p comprises C₁₆ and very-long-chain dihydro-ceramide species, as these were the main sphingolipids whose

increased levels after heat stress were blunted by deletion of *ISC1*. On the other hand, the *lcb1-100* mutant showed severe loss of C₁₈ phyto- and dihydro-sphingosine relative to its parental strain, RH406, in agreement with previously published data [17]; however, this mutant also showed a major decrease in heat-mediated production of C₂₄, C₂₆ and C_{26:1} phytoceramides, which was not observed in the *isc1Δ* strain (Figure 2B and results not shown). Interestingly, however, blocking *de novo* synthesis did not impact dihydroceramide production during heat stress, as levels of dihydroceramides were comparable in the *lcb1-100* and its parental strain (results not shown). Taken together these data indicate that *de novo* synthesis accounts for phyto- and dihydro-sphingosine production and most phytoceramide production during heat stress, and that heat-induced dihydroceramide results from the action of *Isc1p*.

The *lcb1-100/isc1Δ* mutant is inviable

To investigate the role(s) of all heat-induced ceramide formation in yeast, i.e., *de novo* biosynthesis and *Isc1p*-mediated IPC hydrolysis, we attempted to generate a double mutant by transforming the *lcb1-100* strain with the previously described *ISC1* deletion construct [9]. Interestingly, after several transformation attempts, very few colonies were isolated. PCR screening of these few colonies indicated that these transformants were indeed deleted in *ISC1*; however, unlike the *lcb1-100* mutant strain, these putative double mutants were capable of the transient G₀/G₁-phase arrest associated with heat stress (results not shown). This led us to investigate whether these mutants had reverted from the *lcb1-100* mutation to wild-type in *LCB1* [*LCB* ('long-chain base) is a serine palmitoyltransferase component]. High-fidelity PCR of genomic DNA was used to clone the *LCB1* gene from two of the putative double mutants, the *lcb1-100* strain, and its wild-type background strain (RH406). The mutation causing the *lcb1-100* heat-sensitive phenotype was found to be due to a G-to-A mutation at nucleotide 1141 of the *LCB1* gene, which changes Ala³⁸¹ of the native protein to threonine, consistent with a recent report identifying the same mutation [24]. Interestingly, DNA sequencing revealed that neither putative double mutant contained the *lcb1-100* mutation, but rather contained the wild-type sequence for *LCB1*. Therefore it appears that these few colonies survived due to the reversion of the *lcb1-100* mutation, and thus, this mutation coupled with the *ISC1* deletion may be lethal.

This was further investigated by constructing an *isc1Δ/lcb1-100* double mutant by mating an *ISC1*-deleted α -type haploid strain with *lcb1-100* α -type haploid strain to generate a heterozygous diploid double mutant strain, followed by sporulation and asci dissection and screening. As the *ISC1* and *LCB1* genes are on separate yeast chromosomes (*Saccharomyces* Genome Database; <http://www.yeastgenome.org/>), one of four spores should contain both the *ISC1* deletion and the *lcb1-100* mutation. The *isc1Δ* strain was constructed by short flanking homology PCR to introduce the neomycin-resistance-gene ORF (open reading frame) into the *ISC1* locus [9]; thus, both the *isc1Δ* single mutant and *isc1Δ/lcb1-100* double mutant should grow on media containing G418. Furthermore, the *lcb1-100* mutation renders cells unable to grow at 37°C. Thus, an *lcb1-100/isc1Δ* double mutant should be able to grow on G418-containing media, but not at 37°C. This strategy was used to screen colonies resulting from asci dissection. Spores were grown on YPD and then replica-plated on to media containing G418. Screening of spores in 50 asci resulted in no spores which could grow on G418-containing media at 30°C, but not at 37°C. Therefore, we conclude that these two mutations are synthetically lethal.

Figure 3 Analysis of heat-induced cell-cycle arrest

Cells were grown to mid-exponential phase in YPD medium and aliquots were then placed at 30 or 39°C for 1 h. Sample preparation and analysis were as described in the Materials and methods section. Results are the means for two independent experiments shown with their range.

Sphingolipid-mediated heat-induced cell-cycle arrest

Previous studies have shown that yeast cells arrest at G₀/G₁-phase by 1 h of heat stress and resume a normal cell cycle by 2 h [17], that this arrest depends on sphingolipid formation [17], and that this cell cycle arrest is impaired in the *lcb1-100* mutant, in which heat-induced *de novo* sphingolipid biosynthesis is blocked [17]. To determine if ceramide obtained through IPC hydrolysis by *Isc1p* contributes to this transient arrest, flow cytometry of yeast cultures stained with propidium iodide was utilized to determine the percentage of S-phase cells upon a shift in temperature from 30 to 39°C in the strains. Because cell cycle is also regulated by culture density, cells were grown to mid-exponential phase and then aliquots were placed at either 30 or 39°C. Flow-cytometric analyses were performed for the 30 and 39°C cultures at 1 and 2 h, and results are presented as percentage of the time-matched-control value. As shown in Figure 3, comparing heat-treated cells with time-matched controls indicates a relative decrease in S-phase cells to 10%, or less than non-stressed cells, after 1 h for the parental strains (JK93dα and RH406), consistent with previous studies [17]. Furthermore, the *lcb1-100* strain showed a failure to arrest after 1 h of heat stress (approx. 20% of cells in S-phase, indicating exponential-phase growth), a finding also consistent with results obtained in previous studies [12]. In contrast with the *lcb1-100* mutant strain, the *isc1Δ* strain manifested a nearly identical arrest in cell-cycle arrest (Figure 3), demonstrating that sphingolipids generated by *Isc1p* do not contribute to cell cycle regulation in response to heat stress.

Microarray hybridization analysis of *Isc1p*-dependent heat-induced genes

We have previously demonstrated a major requirement for *de novo* sphingolipid synthesis in heat-induced gene regulation during the yeast HSR [18]. Though *Isc1p*-derived sphingolipids do not contribute to cell-cycle arrest during heat stress, it is possible that these sphingolipids may mediate specific gene regulation. In order to determine whether *Isc1p*-regulated sphingolipid metabolism contributes to gene regulation during the HSR and to determine the relative contributions of *Isc1p*-dependent sphingolipid production to heat-mediated gene regulation, microarray hybridization was used for analysis of gene-regulatory events in heat-stressed wild-type and *isc1Δ* yeast cultures. RNA was isolated from *isc1Δ* yeast and the JK93dα isogenic background-strain cultures grown at 30°C and then heat-stressed at 39°C for 15 min. RNA was processed as described in the Materials and methods section and

Figure 4 Microarray data predict defect in sexual reproduction

Genes showing significantly different regulation between the wt and the *isc1Δ* strain after heat stress were assigned to groups based on known function [complete data with average fold change is available in the online supplementary Table (<http://www.BiochemJ.org/bj/393/bj3930733add.htm>)].

used for hybridization to Affymetrix YG-S98 gene chips containing probes directed toward the entire yeast genome. The 15 min time point was chosen because mass measurements show modest *Isc1p*-dependent ceramide formation at early time points (i.e., 5 min; results not shown) and greater changes observable at the 30 min time point (Figures 1 and 2A). However, measuring transcript levels at later time points increases the likelihood of identifying genes which are not directly regulated by *Isc1p*-derived sphingolipid metabolites, but whose regulation results from downstream events. Furthermore, similar analyses performed at 30 and 60 min indicated differences between wild-type and mutant strains qualitatively similar to those at 15 min (results not shown).

Initial analysis of two independently derived microarray datasets according to the Affymetrix change calls and signal log ratios (as described in the Materials and methods section) revealed that, after 15 min of heat stress, 57 genes showed significant aberrant regulation in the *ISC1* deletion strain [see the supplementary Table (<http://www.BiochemJ.org/bj/393/bj3930733add.htm>)]. Individual research on each of these genes allowed them to be grouped based on known functions [as provided by the Gene Ontology Consortium and accessed through the Yeast Proteome Database (www.Incyte.com)] as well as known mechanisms of regulation (as compiled by the Yeast Proteome Database from all available microarray data in the literature).

Interestingly, according to information available for these genes in the Yeast Proteome Database, 12 of the 57 genes (21%) misregulated in the *isc1Δ* strain are thought to be regulated by Ste12p (Figure 4), a transcription factor required for much of the gene regulation after mating pheromone exposure [32]. Furthermore, another group of eight of the 57 (14%) genes in the dataset appear to be involved either in meiosis, as part of the sporulation program, and/or are known to function during processes of sexual reproduction (Figure 4). Grouping the genes involved in these processes, including the genes regulated by Ste12p, results in a dataset containing 20 genes, or roughly 35% of the misregulated genes. This finding strongly suggested that *Isc1p* may play a role in regulation of genes necessary for sexual reproduction in response to stress.

Another category emerging from this analysis is that of genes involved in carbon-source utilization. Several of these genes are known to be regulated by available carbon source, including *HSP12*, *SAC1*, *YJL175W*, *YHR173C*, *PDH1*, *SWR1*, and *CSR2* (according to information available in the Yeast Proteome Database and references cited therein). Interestingly, we also noted that 12 of the 57 (21%) of the genes misregulated during heat

Table 1 Genes showing altered exponential-phase expression in an *ISC1* deletion mutant according to RMA analysis

Analysis was performed as described in the Materials and methods section. 'Fold difference' is the ratio of mutant signal to wild-type signal.

ORF	Gene name	Function	Fold difference
YAR033W	<i>MST28</i>	Vesicle formation	0.2
YFL014W	<i>HSP12</i>	Heat-shock protein 12	4.9
YDR406W	<i>PDR15</i>	multidrug-resistance transporter (putative)	2.9
YNL160W	<i>YGP1</i>	gp37, a glycoprotein synthesized in response to nutrient limitation	3.1
YMR081C	<i>ISF1</i>	Regulation of mitochondrial biogenesis	3.4

stress in the *ISC1* mutant were shown, in a previous microarray study, to be regulated by LiCl on galactose as the sole carbon source, which impairs fermentation [25] (Figure 3), as lithium inhibits phosphoglucomutase, an enzyme essential for galactose metabolism. Interestingly, a recent study from our group identified a fermentation defect in the *isc1Δ* strain resulting in severe growth defects on glycerol and ethanol [13], a finding that is consistent with, and may be partially explained by, the present results. Thus the overlap between the present data and those obtained in previous studies suggests a potential role for *Isc1p* in the regulation of carbon-source utilization.

Sporulation defect in *isc1Δ* strain

Previous microarray studies had demonstrated the induction of *Isc1p* in response to exposure to low-nanomolar concentrations of α -factor, showing up to a 4-fold induction at 48 min after exposure [*Saccharomyces* Genome Database (<http://www.yeastgenome.org/>), viewed with the Global Microarray Viewer]. This finding, coupled with the observation discussed above, that *Isc1p* contributes to the regulation of genes known to be regulated during sexual reproduction (Figure 4) indicated a potential role of *Isc1p* in the pheromone response or its downstream events, including meiosis and sporulation. Because the ultimate result of mating in *S. cerevisiae* is spore formation, and any alterations in processes from the initial pheromone response to spore formation might be detected as failure to form spores properly, we investigated spore formation resulting from mating α -type with a-type *ISC1* deletion strains. Mating of a- and α -type *isc1Δ* strains produced diploids that were plated on sporulation media. After 7 days, well-formed asci containing three to four clearly visible spores were counted, and this number was expressed as a percentage of total cells observed. The results showed that diploid *isc1Δ* mutants formed nearly 50% less asci, indicating a requirement for *Isc1p* in spore formation (results not shown).

RNA analysis

In addition to the Affymetrix analysis based on change calls and signal log ratios, a more statistically rigorous RMA analysis was undertaken, as described in the Materials and methods section, to evaluate and 'mine' the microarray data for information of biological function. On the basis of this analysis, the *isc1Δ* and parental strains showed a few statistically significant transcriptional differences at baseline (Table 1) and in response to heat (Table 2). Among those genes showing changes, transcripts of *ISF1*, *HSP12*, *YGP1* and *PDR15* were aberrantly induced by 3–5-fold in the *isc1Δ* strain at normal growth temperature (Table 1). Interestingly, each of these transcripts is subject to repression by

Table 2 Genes with altered regulation under heat stress in an *ISC1* deletion mutant according to RMA analysis

Analysis was performed as described in the Materials and methods section. 'Fold difference' is the ratio of mutant signal to wild type signal.

ORF	Gene name	Function	Fold difference
YKL109W	<i>HAP4</i>	Transcriptional activator protein of <i>CYC1</i> *	5.1
YIL160C	<i>POT1</i>	3-Oxoacyl-CoA thiolase	3.0
YGR211W	<i>ZPR1</i>	Zinc-finger protein	0.1
YBL109W	Hypothetical ORF		4.6
YLR465C	<i>BSC3</i>	Dubious ORF	3.8

* *CYC1* encodes yeast iso-1-cytochrome *c*.

glucose (Yeast Proteome Database, and references cited therein). Furthermore, whereas *MST28* is aberrantly repressed under normal growth conditions in the *isc1Δ* strain, it is induced by glucose. After heat, the deletion strain does not properly regulate several genes, including *HAP4*, which codes for a transcriptional activator of cytochrome *c* oxidase subunits [26].

DISCUSSION

The *S. cerevisiae* SMase homologue *Isc1p* provides a critical tool in probing the hydrolytic pathway of ceramide formation in eukaryotic biology, allowing the use of yeast molecular, biochemical and genetic approaches. During heat stress in *S. cerevisiae*, sphingoid bases, their phosphates and the ceramides dihydroceramide and phytoceramide increase via *de novo* synthesis [17,25,27]. The major goal of the present study was to determine whether complex sphingolipid hydrolysis also contributes to ceramide formation during heat stress, and whether the function of *Isc1p*-derived ceramide overlaps that of *de novo* sphingolipids for the overall HSR. The data do indicate that *Isc1p* contributes to ceramide formation during heat stress; however, *Isc1p* mediates the preferential production of long- and very-long-chain dihydroceramide species. Furthermore, these ceramides are functionally distinct from *de novo*-synthesized sphingolipids in that they are not required for cell-cycle arrest. Moreover, *Isc1p* contributes to a distinct programme of transcriptional regulation during heat stress that shows no overlap with that mediated by *de novo* sphingolipid synthesis [Tables 1 and 2 and the supplementary Table (<http://www.BiochemJ.org/bj/393/bj3930733add.htm>); [18]]. The *Isc1p*-mediated pathway is further distinguished from the *de novo*-synthesis pathway in that it plays specific roles in sporulation and in growth using ethanol as a primary carbon source.

The IPCase encoded by *ISC1* is homologous in both sequence and function with mammalian SMases. Intriguingly, the results show that the substrate specificities for mammalian neutral SMase2, the mammalian orthologue of *Isc1p*, and *Isc1p*, are quite similar, as the mammalian enzyme shows high selectivity to sphingomyelins containing C_{24} and $C_{24:1}$ fatty acyl chains and produces selectively very-long-chain (i.e. C_{24} and $C_{24:1}$) ceramides [28]. This indicates that a significant level of conservation exists between the mammalian and yeast isoforms in their target 'subpopulation' of substrates. In yeast, only very long-chain ceramides (C_{24} , $C_{24:1}$, C_{26} and $C_{26:1}$) are incorporated into complex sphingolipids [29], which may partially explain the finding that the very-long-chain species are affected by *ISC1* deletion. Whether substrate availability is also a partial determinant of neutral SMase product specificity remains to be determined.

Though formation of very-long-chain ceramides by *Isc1p* consumes complex sphingolipids, it is highly unlikely that this cleavage results in significant changes in the cell-membrane composition of complex sphingolipids, as the mass of ceramide generated (of the order of less than 5 pmol/10⁸ cells) is four orders of magnitude less than total membrane glycerol- and sphingophospholipid mass (around 35 nmol/10⁸ cells) (L. A. Cowart and Y. A. Hannun, unpublished work). Moreover, in a previous study, we showed that heat stress did not result in significant changes in complex sphingolipids [9] and thus any effect of *Isc1p* is likely to be a result of increased very-long-chain ceramides (or possibly downstream metabolites) rather than a decrease in complex sphingolipid pools.

Since *Isc1p*-derived sphingolipids do not mediate cell-cycle arrest, microarray hybridizations were performed in an attempt to identify potential contributions of this pathway to gene regulation both in exponential-phase growth and during heat stress. Because recent studies have demonstrated that microarray findings are highly influenced by the methods of data analysis [30,31], two separate means of data analysis were chosen in order to interpret the raw gene-expression data more thoroughly. Indeed, in a previous study, we utilized two distinct methods of microarray data analysis [18], and this approach revealed more information than using either analysis technique alone. The validity of using multiple analysis algorithms is substantiated by the fact that both of the analysis methods employed in the present study led us to the hypothesis that *Isc1p* functions in growth on ethanol, which was confirmed by another study [13], but only the less stringent of the two analyses led to the hypothesis of a sporulation defect or osmotic stress phenotypes, which have been confirmed by the present study (see below) and others [11]. Therefore, the ability to substantiate findings from multiple microarray analysis algorithms underscores the validity of taking multiple approaches to data analysis in order to produce the most new information from each set of microarray hybridization experiments.

In the *isc1Δ* mutant, many genes involved in mating, meiosis and/or sporulation were not properly regulated, including many genes regulated by the pheromone-responsive transcription factor *Ste12p*, indicating a potential role for *Isc1p*-derived sphingolipids in these processes. These data combined with results from a previous microarray study showing the induction of *Isc1p* in response to mating pheromone [32] strongly suggest a role for *Isc1p* in processes associated with sexual reproduction in *S. cerevisiae*. In support of this hypothesis, sporulation of homozygous *ISC1*-null diploids was found to be only around 50% as efficacious as sporulation of the isogenic wild-type background strain. Interestingly, additional data lend further support for a role for sphingolipids in yeast sexual reproduction. A recent study demonstrated that ergosterol and sphingolipid-rich membrane rafts facilitate accumulation of mating-tip-projection proteins and, furthermore, that a double mutant strain (*lcb1-100/lcb3Δ*) in *de novo* sphingolipid biosynthesis showed a mating defect [33]. In another study, the *SUR7* gene, which was originally cloned as a suppressor of *rvs161* and *rvs167* deletion phenotypes (which include defects in sporulation and response to nutrient limitation), was demonstrated to play a role in sporulation and to affect sphingolipid metabolism [19]. Furthermore, other *SUR* genes (which are also suppressors of *rvs161* and *rvs167* deletion phenotypes) are known to be involved in sphingolipid metabolism, suggesting some link between sphingolipid metabolism and sporulation [19,34,35]. Interestingly, Young and co-workers postulated that an as-yet-uncharacterized ceramide-based signalling pathway contributes to effective sporulation [19]. Further investigation is needed to determine if *Isc1p* mediates this putative pathway.

In addition to sexual reproduction, the present study revealed a role for *Isc1p* in the regulation of genes responsive to osmotic stress. Interestingly, and supporting our microarray findings, a previous report suggested that *Isc1p* may play a role in the regulation of the Na^{2+} transporter *ENA1* under salt stress [11]. *ENA1* is a member of a highly conserved family including *ENA2* and *ENA5*, which reside in close chromosomal proximity to that for *ENA1* (*Saccharomyces* Genome Database). Owing to the high degree of conservation of these genes (> 96%), nucleic acid hybridization techniques are not optimal for determining their expression levels; however, the Affymetrix gene chip contains two probe sets directed toward *ENA5* and one probe set directed toward *ENA2*, which is 100% similar to *ENA1*. Data from the latter probe set showed that heat stress induces expression of *ENA1* and/or *ENA2*, and this increased expression did not occur in the *ISC1* deletion strain, demonstrating a role for *Isc1p* in regulating *ENA1/2* response to heat stress. This extends the results of the previous study implicating *Isc1p* in regulation of *ENA1/2* in response to osmotic stress [11]. Interestingly, the probe sets directed toward *ENA5* showed no changes with heat stress in either the wild-type or the deletion strain. Because a few other genes involved in salt or osmotic stress were misregulated in the deletion strain [see the supplementary table (<http://www.BiochemJ.org/bj/393/bj3930733add.htm>); included under 'Other' in Figure 4], *Isc1p* may play a role in osmotic stress, of which *ENA1/2* regulation is a component.

The results presented here demonstrate the requirement of *Isc1p* for regulation of genes responsive to impaired fermentation, which generates a nutritional stress. The literature on yeast stress responses indicates a role for partially overlapping signalling pathways in various stress responses mediated through mitogen-activated protein kinase signalling cascades (reviewed in [36]). Furthermore, although sporulation is a downstream result of exposure to mating pheromone, both stress-responsive genes and sporulation genes are regulated by partially similar overlapping pathways [36,37]. Moreover, three of the five genes identified by RMA analysis as aberrantly regulated in the *isc1Δ* strain, *ISF1*, *HSP12* and *YGP1*, were recently described as induced by the N-terminal domain of *Sst2p*, a protein participating in G-protein-coupled signal transduction that functions in pheromone signalling and affects *Ste12p*-mediated transcription [19,38]. Of particular interest is the recent finding that the protein *Sst2p* functions not only as a component of signalling pathways in the yeast pheromone response, but also during the HSR and Hog-1-independent signalling during osmotic stress, three biological situations in which the present study found potential roles for *Isc1p* [19]. *Sst2p* is a member of the RGS (regulator of G-protein signalling) family of proteins widely conserved in eukaryotic systems whose membrane localization is regulated through a DEP domain (a conserved Dishevelled, *Egl-10* and *pleckstrin* domain) and functions as a signalling link between pheromone/stress responses and protein localization via vesicular trafficking, and a key role for it in protein sorting has been proposed [19]. Further supporting the likelihood of *Sst2p* in *Isc1p*-mediated processes is the observation that *Sst2p* is essential for appropriate regulation of *HSP12*, a heat-shock protein gene that is also regulated by mating pheromone and osmotic stress, *YGP1*, which encodes a glycoprotein induced in response to glucose, and *HSP26*, encoding a heat-shock protein, each of which was determined to depend on *Isc1p* for appropriate expression in the microarray studies [19]. Thus we propose that deletion of *ISC1* may cause aberrant regulation of localization and/or function of *Sst2p*, which would result in faulty reproduction, fermentation and heat- and osmotic-stress responses. Further studies will be required to determine the nature of the interaction between *Isc1p* and *Sst2p*.

Interestingly, ceramide formation in response to temperature elevation was previously described in mammalian NIH-WT-3T3 [39], Molt-4 [6] and HL-60 cells [39,40]. In the 3T3 cells, heat-mediated ceramide generation up-regulated the transcription of α B-crystallin [39]. A later study demonstrated heat-induced ceramide formation in HL-60 cells and determined that the source of the ceramide was associated with sphingomyelin hydrolysis [40]. In another haematopoietic cell line, Molt-4, *de novo* ceramide formation was shown to contribute a significant portion of heat-induced ceramide [6]. Thus, heat-mediated ceramide formation from both *de novo* synthesis and sphingomyelin hydrolysis is evolutionarily conserved from *S. cerevisiae* to mammalian cells. However, the relative and specific contributions of each of these sources of ceramide have to this point remained unclear.

Importantly, in the present work we have demonstrated the utilization of microarray data to probe biological phenotypes of gene deletion in *S. cerevisiae*. Though the entire genome of this model organism is now known, less than 50% of the detected transcripts have been functionally characterized. In an effort to identify biological functions, investigators often delete genes and search for phenotypes. The present study demonstrates that microarray studies in deletion mutants can be successfully utilized to formulate hypotheses regarding potential phenotypes, and these hypotheses can subsequently be tested using other methods.

The present study indicates that *de novo*-generated sphingolipids and IPC-derived sphingolipids are biochemically distinct (Figures 2A and 2B). Furthermore, they serve distinct roles, as the *isc1Δ* and *lcb1-100* mutants demonstrated no similarity in heat-mediated transcriptional defects (Tables 1 and 2 and the supplementary Table (<http://www.BiochemJ.org/bj/393/bj3930733add.htm>); [18]), and only the *lcb1-100* mutant shows a defect in heat-induced cell-cycle arrest (Figure 3). In contrast with the *lcb1-100* mutant, however, the *isc1Δ* deletion strain exhibited a sporulation defect. To our knowledge, this is the first study in yeast to examine the respective roles of *de novo*-synthesized versus *Isc1p* pathways of sphingolipid metabolism, and together, our results demonstrate the distinct biochemical and functional properties of ceramides produced by each of these pathways during heat stress, indicating that these pathways are not overlapping but play separate roles in yeast biology.

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