CHAPTER 2

ENLARGEMENT OF THE ENDOPLASMIC RETICULUM MEMBRANE IN SACCHAROMYCES CEREBISIAE IS NOT NECESSARILY LINKED TO THE UNFOLDED PROTEIN RESPONSE VIA IRE1P

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ABSTRACT

Conditions that stress the endoplasmic reticulum (ER) in Saccharomyces cerevisiae can elicit a combination of an unfolded protein response (UPR) and an inositol response (IR). This results in increased synthesis of ER protein-folding factors and of enzymes participating in phospholipid biosynthesis. It was suggested that in cells grown on glucose or galactose medium, the UPR and the IR are linked and controlled by the ER stress sensor Ire1p. However, our studies suggest that during growth on oleate the IR is controlled both by an Ire1p-dependent pathway and by an Ire1p-independent pathway.

INTRODUCTION

Saccharomyces cerevisiae is well equipped to cope with stressful conditions arising from the surroundings, such as heat shock, osmotic or oxidative stress (Hohmann and Mager, 1997). However, internal conditions can also change and cause difficulties that a yeast cell must try to surmount in order to prolong its life. This is, for instance, the case when proteins accumulate in the ER. Proteins of the endosomal compartment or proteins to be secreted are folded in the ER and sometimes oligomerized into multi-protein complexes. A number of folding factors assist in these processes, for instance Kar2p, Pdi1p, FKB2 and Eug1p, collectively called chaperones (Shamu et al., 1994). The success of folding and oligomerization is controlled in the ER by a 'quality control system'. When a protein fails the test, it is not allowed to continue its itinerary to the Golgi apparatus and subsequent compartments. Rather, it remains in the ER for a longer time to complete folding, or it is eventually degraded (Hammond and Helenius, 1995).

In response to the accumulation of proteins, the volume of the ER increases. This is achieved by induction of the 'unfolded protein response' (UPR), which results in enhanced synthesis of additional chaperones and in the 'inositol response' (IR), which stimulates synthesis of membrane lipids. The UPR pathway has been carefully dissected and its components have been characterized (Sidrauski et al., 1998). Here we mention two components.

A pivotal role is played by Ire1p (Nikawa and Yamashita, 1992), an integral membrane protein of the ER and the perinuclear membrane. Its N-terminal half, residing in the ER lumen, detects accumulation of
Figure 1: Schematic representation of Ire1p signalling, leading to the synthesis of ER proteins via the UPR as well as the synthesis of ER membranes via the IR.

proteins while the C-terminal half, reaching out into the nucleus, has a dual function. Ire1p is a protein kinase which undergoes autophosphorylation upon oligomerization; this is thought to activate its ribonuclease activity which initiates splicing of a precursor mRNA encoding Hac1p.

Hac1p is a transcription factor and the second component of the UPR (Cox and Walter, 1996; Kawahara et al., 1997; Shamu, 1998). It activates transcription of genes that share an unfolded protein response element (UPRE) in their promoters. Most of these genes code for chaperones and thus close the UPR regulatory loop. It was argued that the increase in enzymes required for enhanced phospholipid biosynthesis (via the IR) is also mediated by Ire1p and Hac1p (Cox et al., 1997). In this view, UPR and IR are bifurcations from the same stress sensor: i.e. Ire1p (Figure1).

The importance to the yeast cell of this combined response to intracellular stress is exemplified by the behaviour of an ire1 mutant. When cells were stressed by incubation with either tunicamycin (an inhibitor of N-linked glycosylation) or reducing agents (interfering with protein folding) ire1Δ cells died (Cox et al., 1993). Furthermore, ire1Δ cells proved to be inositol auxotrophs (Nikawa and Yamashita, 1992), suggesting that the IR is compromised in the ire1 mutant. Overexpression of the ER integral membrane protein HMG-CoA reductase 1 (Hmg1p) normally results in an increase in the ER compartment and in
accumulation of Hmg1p in stacked ER membranes called karmellae (Parrish et al., 1995; Wright et al., 1988). Interestingly, ire1Δ cells died upon overexpression of Hmg1p, probably because they were unable to induce both an UPR and an IR (Cox et al., 1997).

We tried to use this information in our studies on the biogenesis and function of peroxisomes. In one of our genetic screens we discovered PEX15 (Elgersma et al., 1993). PEX15 encodes an integral peroxisomal membrane protein and loss of its function results in mislocalization of peroxisomal matrix proteins to the cytoplasm and loss of the typical ovoid-shaped peroxisomal morphology as revealed by electron microscopy. Several observations suggested routing of Pex15p to peroxisomes via the ER. Expressed Pex15p (or Pex15p derivatives) accumulated in ER-like membranes, which were continuous with the perinuclear membrane. Furthermore, a fusion protein of Pex15p and invertase lacking its ER targeting signal was glycosylated and the stacked membranes resembled karmellae (Elgersma et al., 1997). Other groups also made observations fueling the idea that the ER might be involved in some aspects of peroxisome biogenesis. For instance, mutants in Yarrowia lipolytica that were defective in the secretion of proteins via ER and Golgi apparatus, also proved to be disturbed in the biogenesis of peroxisomes (Titorenko and Rachubinski, 1998). In addition, two peroxisomal integral membrane proteins were shown to be targeted to the ER and to be glycosylated before they reached the peroxisome (Titorenko and Rachubinski, 1998). Treatment of Hansenula polymorpha with Brefeldin A, an inhibitor of ER/Golgi vesicular traffic, resulted in accumulation of peroxisomal matrix and membrane proteins in an ER-like compartment (Salomons et al., 1997).

To further try to identify the ER as a possible link in the chain of events leading to the formation of peroxisomes, we wondered whether accumulation of Pex15p would be deleterious to a cell that cannot induce a UPR due to deletion of IRE1. Indeed, the ire1 mutant died when Pex15p was produced at elevated levels. However, when we overproduced a mitochondrial integral membrane protein as a control, to our surprise ire1 cells also died, which questions the specificity of the UPR and IR. Peroxisomes increase in volume and number when yeast grows on a fatty acid as sole carbon source (Veenhuis et al., 1987). When we repeated the expression of membrane proteins with ire1 cells and grew the cells on oleate instead of glucose or galactose, these did not die and karmellae-like membranes were still formed. In addition, cells grown on oleate were not
dependent on external inositol, in contrast to *ire1* cells grown on glucose or galactose. Here, we discuss some implications of our findings.

**MATERIALS AND METHODS**

The *S. cerevisiae* strains used were: 'wild type' JC104 and 'ire1Δ' CS173 (Cox et al., 1993, a kind gift from R. Chapman); 'wild type' BJ1991 (Mata; *leu2, trp1, ura3-251, prb1-1122, pep4-3*) and 'ire1Δ' (the same as BJ1991 except *ire1::URA3*), generated with disruption plasmid PCS135 (Cox et al., 1993, kindly provided by R. Chapman). *pip2Δ, oaf1Δ* and *pip2Δ/oaf1Δ* were also made in BJ1991, as described in (Rottensteiner et al., 1996). Synthetic minimal media with or without inositol were used as described by Sherman (1991). As carbon source we used either 0.3% or 2% (w/v) glucose, or 2% (w/v) galactose, or 0.1%(w/v) oleate/0.4%(w/v) Tween-40.

The plasmids used were based on YEplac112 (Gietz and Sugino, 1988), with either the gal1/10 or the catalase promoter (Elgersma, 1995) cloned between the EcoRI and SacI site, with the NH tag in SacI-Bam HI and with the PEX15, ACR1 or PEX14 ORFs in BamHI-HinDIII. The Hmg1p expression constructs were made by cloning a HMG1 fragment that was fused in-frame to GFP from plasmid pCR425 (a kind gift from R. Wright) and cut with BamHI and XhoI into the YEplac112 vector containing the gal1/10 promoter or the catalase promoter cut with BamHI and Sall. The inv-GFP-HDEL construct used was described in (Hettema et al., 1998).

ORFs and promoter sequences were amplified by PCR using specific primers introducing restriction sites directly before the translation-initiation site or directly after the stop codon. The INO1 ORE fragment was obtained by annealing oligonucleotides F-ORE AATTCACTGATCGGAAACGAGCT CTTTATCACCCTAG and R-ORE GATCTACGGTGATAAAGAGCTCGTTCCGATCAGTG; 1 pmol of each in 1x SSC was slowly cooled from 100 °C to room temperature. The FOX3 ORE used was described in (Einerhand et al., 1991; Sidrauski et al., 1998).

Techniques used were: DNA manipulations (Sambrook et al., 1989), yeast transformation (Ito et al., 1983) and bandshift assays (extract preparations, labelling and analysis, Rottensteiner et al., 1996). SAGE data were obtained from (Kal et al., 1999). Immunolabelling of ultra-thin cryo-sections with polyclonal antibodies against NH (Elgersma, 1995, a kind gift from P. van der Sluijs) and against GFP (Clontech) was performed according to Gould et al. (1990).
RESULTS

Pex15p induced karmellae formation

Northern blot and SAGE analysis indicated that PEX15 and other PEX genes were expressed at low steady-state levels (less than 1 mRNA copy per cell), more or less irrespective of the carbon source in the medium (Kal et al., 1999). Expression of PEX15 from an expression plasmid in cells grown on oleate induced karmellae-like structures which contained Pex15p as shown before (Figure 2A).

In order to test whether these membranes were ER-membranes, we developed a GFP derivative as ER-marker. The presequence of invertase was appended to the N-terminus of GFP to function as an ER-addressing signal and the yeast ER-retention signal (HDEL) was fused to the C-terminal end of GFP (Hettema et al., 1998). Expression of this fusion protein (preINV-GFP-HDEL) and inspection of the cells by immuno-gold electron microscopy showed membranes decorated with gold particles that had all the characteristics of the ER compartment: staining of the perinuclear membrane and membranes located at the periphery of the cell close to the plasma membrane (Figure 2B). Co-expression of Pex15p (small gold particles) and preINV-GFP-HDEL (large gold particles) showed exact co-localization of small and large gold particles, proving that under certain conditions Pex15p is indeed associated with the ER (Figure 2B, C and D).

Expression of membrane proteins in ire1Δ grown on galactose or oleate

Based on these findings, we hypothesised that yeast cells start the UPR/IR upon Pex15p overexpression and we expected that an ire1 mutant would die under these conditions. To test this hypothesis, we expressed Pex15p under control of the gal1/10 promoter (the experimental set-up of Cox et al. (1997). Indeed, the ire1Δ transformants died (Figure 3A). Although we initially interpreted this result as an additional suggestion for a possible functional relationship between peroxisomes and the ER, a number of disturbing

Figure 2: Electron microscopic analysis of the location of Pex15p in karmellae in oleate-grown wild-type cells transformed with: (A) NH-PEX15/2µ; (B) inv-GFP-HDEL/CEN; (C) NH-PEX15/2µ and inv-GFP-HDEL/CEN; (D) detail of C. Analysis of the formation of karmellae in oleate-grown wild-type cells (E) and ire1Δ cells (F), transformed with HMG1-GFP/2µ. Antibodies used were a-NH (A,C,D) and a-GFP (B,C,D,E,F). N= nucleus. Bar = 0.2 µm.
ER enlargements

A

B

C

D

E

F
observations were made when we carried out additional controls.

A protein that has been used in studies on karmellae formation and the UPR is Hmg1p. When we studied the UPR in cells grown on oleate-containing medium and used Hmg1p expressed from the catalase promoter to stress the ER, to our surprise both wild-type and ire1 mutant cells showed formation of karmellae-like membrane structures (Figure 2E and F) and ire1 cells did not die despite the compromised UPR/IR pathways (compare Figure 3A with Figure 3B). The same result was obtained when the integral peroxisomal membrane protein Pex15p was expressed on oleate in ire1 cells (Figure 3B).

Integral membrane proteins from other organelles were expressed at elevated levels as controls. A typical example shown here is Acr1p, an integral membrane protein of the mitochondrial inner membrane (Verleur, 1998). Contrary to expectation, ire1 cells died on galactose-containing medium due to Acr1p expression (Figure 3A). Wild-type cells expressing Acr1p do not show formation of karmellae and immuno-gold labelling indicated that Acr1p was only present in mitochondria. Western blot analysis indicated that the proteins (tagged versions of Hmg1p, Pex15p and Acr1p) were expressed at similar levels (not shown). On the other hand, production of the peripheral peroxisomal membrane protein Pex14p did not compromise the ire1 mutant (when grown on galactose, not shown). Again, ire1 cells expressing Acr1p survived on oleate-containing medium (Figure 3B). It is clear that the aspecificity of the UPR/IR, that we observed in our experiments does not allow us to draw conclusions with regard to a functional ER-to-peroxisome relationship. Despite this, we tried to explain the discrepancy between the phenotypes of ire1 cells grown on media containing different carbon sources.

Regulation of INO1 expression

Yeast cells with a non-functional Ire1p display auxotrophy to inositol (Greenberg and Lopes, 1996). Surprisingly, in our experiments addition of inositol to oleate-containing minimal plates was not necessary to allow growth of ire1 cells. Inositol is an essential precursor for the synthesis of phosphatidylinositols and is an important controller of phospholipid biosynthesis. It is produced from glucose-6-phosphate by the enzyme Ino1p (Greenberg and Lopes, 1996). Both the INO1 gene and the enzyme are caught in a delicate network of control. Well-known components of the
Figure 3: Growth of wild-type and ire1Δ cells transformed with expression constructs encoding integral membrane proteins under control of the galactose inducible gal1/10 promoter (A) or oleate inducible catalase promoter (B). Cells were transformed with HMG1-GFP/2μ, NH-PEX15/2μ or NH-ACR1/2μ. Growth is shown on selective glucose before induction (A+B) and selective galactose (A) or selective oleate (B) after induction.

transcriptional control circuit comprise Opi1p, a factor repressing transcription of the INO1 gene, and Ino2p and Ino4p, both promoting its transcription (Greenberg and Lopes, 1996; Nikoloff et al., 1992; Klig et al., 1988). However, additional components may be involved. In our studies on peroxisome biogenesis using genome-wide transcription analysis with
SAGE, we noticed that in cells grown on oleate the INO1 mRNA level was much higher than in a mutant lacking two important transcription factors (pip2 and oaf1) that are required for induction of genes coding for peroxisomal proteins (21 copies per cell versus 1, Kal et al., 1999). The Pip2p/Oaf1p heterodimer binds to a cis-acting DNA sequence called oleate response element (ORE) that is present in the promoters of such genes (Einerhand et al., 1993; Luo et al., 1996; Rottensteiner et al., 1996). Interestingly, the INO1 gene contained a putative ORE at position -368 to -347. To test whether this is a functional ORE, we carried out a DNA band-shift experiment. A DNA segment of 36 bp containing the INO1 ‘ORE’ was radio-labelled and incubated with protein extracts of wild-type and mutant cells (pip2Δ, oaf1Δ and the double mutant pip2Δ/oaf1Δ) grown on either glucose or oleate as carbon source. ORE DNA from the FOX3 promoter served as a control (Figure 4). The INO1 ORE fragment gave rise to a specific retardation complex similar to that obtained with FOX3 DNA. It was absent when INO1 DNA (or FOX3 DNA) was incubated with the mutant extracts, indicating that Pip2p and Oaf1p are responsible for the formation of this retarded complex. Although this suggests that the INO1 gene contains a functional ORE and could thus explain that INO1 is actively transcribed in cells grown on oleate, we have not yet been able to show that the INO1 ORE can activate a reporter gene on its own, as we reported for the ORE of the FOX3 gene (Einerhand et al., 1991).

DISCUSSION

We showed that in S. cerevisiae, overproduction of the peroxisomal integral membrane protein Pex15p resulted in karmellae formation and cell death in an ire1 genetic background, just like by overproduction of the integral ER membrane protein Hmg1p, when using a galactose induction system. Two explanations may be considered to understand this observation: i) Pex15p first targets to the ER before arriving in the peroxisomal membrane. This would be in line with recent suggestions that the ER could be involved in the biogenesis of peroxisomes. ii) The ER membranes readily take up hydrophobic proteins that do not reach their normal acceptor membrane in time. In that case the fact that an overproduced peroxisomal membrane protein was found in ER membranes does not provide an argument for a functional relationship
between ER and peroxisomes. We favour the second possibility, based on the fact that in \textit{ire1\Delta} production of the inner mitochondrial membrane protein Acr1p from an expression plasmid resulted in cell death too.

\textit{S. cerevisiae} with an \textit{ire1} mutation was dependent on the addition of inositol to a minimal glucose-based or galactose-based growth medium. The intracellular concentration of inositol functions as an important sensor to control the biosynthesis of phospholipids, both at the enzymatic level and at the transcriptional level (Greenberg and Lopes, 1996). The combined loss of the UPR (\textit{ire1}) and inositol prototrophy (IR) was worked out in further detail to support a model in which Ire1p and Hac1p coordinate the production of ER proteins as well as of enzymes involved in the synthesis of ER phospholipids (Cox et al., 1997). Although such a coordination might be the case under certain conditions of growth in which either glucose or galactose is provided as carbon source, our experiments show that in cells grown on oleate this link was not apparent. \textit{ire1\Delta} cells grew perfectly well in minimal medium with oleate as sole carbon source without addition of inositol. Remarkably, we still observed enlargement of the ER compartment (karmellae formation) upon overexpression of Pex15p or Hmg1p. In fact, electron microscopy revealed initial karmellae formation when \textit{ire1\Delta} cells were shifted to galactose medium to induce Pex15p or Hmg1p expression (results not shown).

Taken together, our results suggest that the IR is not necessarily linked to the UPR and that it can be independently controlled in additional ways.
(Figure 1). This is in line with another study in which IRE1 gene disruption did not prevent cytochrome-P450-induced ER proliferation (Menzel et al., 1997).

In a genome-wide analysis of transcription using serial analysis of gene expression (SAGE), we observed that INO1 mRNA is present at a relatively high copy number per cell (21) in cells grown on oleate. This would explain why an ire1 mutant displayed no inositol auxotrophy in a growth medium containing fatty-acids. Furthermore, the INO1 mRNA level dropped in the pip2/oaf1 double mutant lacking the transcription factors required for induction of genes coding for peroxisomal proteins in cells grown on oleate. Preliminary experiments on the INO1 promoter suggest that it indeed contains an ORE, to which Pip2p/Oaf1p can bind. This would be in addition to the UAS_{ina} served by the Ino2p/Ino4p factors, another positive mode of control of the expression of the INO1 gene. The expression of the INO1 gene should, we feel, be studied in more detail, since its product plays such an important role in the coordination of phospholipid biosynthesis in the cell.

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REFERENCES


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