Studies on peroxisome biogenesis
Stroobants, A.K.

Citation for published version (APA):

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ADDENDUM 2 TO CHAPTER 2

REGULATION OF INO1 EXPRESSION

INTRODUCTION
When *S. cerevisiae* is shifted from a glucose to an oleate containing medium, it becomes dependent on the presence of functional peroxisomes. In this situation the expression of certain proteins which have an ORE (oleate response element) upstream of the coding region in the genes encoding them is upregulated. So is INO1 which contains a putative ORE. Ino1p is one of the key enzymes in phospholipid synthesis. It is responsible for the conversion of glucose-6P to inositol-1P. Inositol-1P is used for the synthesis of phosphatidyl inositol (PI), one of the building blocks of bio-membranes. The interest in INO1 regulation is illustrated by many review articles which are written about this topic (Carman and Henry, 1999; Greenberg and Lopes, 1996; Henry and Patton-Vogt, 1998).

Several other enzymes which are important for the synthesis of phospholipids seem to be expressed in a similar manner as INO1. This is achieved (partially) via an UAS\textsubscript{INO} element in their promoter region. The level of INO1 expression can be used as an indirect measure for phospholipid synthesis. This means that upregulation of INO1 after a shift to oleate medium is an indication for upregulation of phospholipid synthesis and thus indirectly of membrane synthesis.

One of the conditions in which membrane synthesis is upregulated is during the unfolded protein response. Cox et al. (1997) suggested that the unfolded protein response (UPR) and inositol response (IR) go hand in hand and are controlled by Ire1p. In their view Ire1p is essential for INO1 expression. At least, this seems to be the case in yeast which is grown on glucose or galactose based media. However, observations made with yeast grown on oleate medium indicate that the actual situation is more complex (chapter 2). One of the conclusions that could be drawn is that the IR can take place in the absence of Ire1p.
Because of this discrepancy found when yeast is grown on different media, we studied inositol synthesis under various conditions to get more insight in the regulation of \textit{INO1} expression. Particularly because, as mentioned in chapter 2, there are indications that the transcription factor couple Pip2p and Oaf1p plays a role in \textit{INO1} expression upon oleate induction. At present not much is known about how the positive regulators of \textit{INO1} expression, Ino2p and Ino4p, and the negative regulator Opi1p act on different media. We investigated several components which are involved in \textit{INO1} regulation and as a consequence in inositol synthesis by studying growth of mutants on different media with and without inositol. Growth of wild type and mutant cells on media lacking inositol is used as a criterium for the ability of cells to induce inositol synthesis.

**MATERIALS AND METHODS**

See materials and methods section of chapter 2. \textit{ire1Δ, hac1Δ, ino1Δ, ino4Δ} and \textit{ire1Δ/opi1Δ} were in a \textit{JO 04} background and were gifts of R. Chapman. The double knock-outs \textit{pip2Δ/ire1Δ} and \textit{oaf1Δ/ire1Δ} were made the same way as \textit{ire1Δ} by disrupting \textit{IRE1} in the already existing \textit{pip2Δ} and \textit{oaf1Δ} strains. For growth experiments cells were precultured in 2\% glucose medium with inositol for 16 hours, than grown on 0.3\% glucose medium with inositol for 24 hours. The cells were washed with water once and then equal amounts of cells were distributed evenly over plates containing different media.

The micro-array experiment was performed by M. Groot-koerkamp, A. Mul, G. Hardy and H. Tabak (university of Amsterdam, the Netherlands) by an as yet unpublished method modified after Hoheisels method (Hauser et al., 1998).

The \textit{INO1} gene with the putative ORE was generated by PCR with primers forward: TTTGAATTCGATGAAGACGATGAGGCC and reverse: TTTAAGCTTATTACAACAATCTCTCTCTCG, the \textit{INO1} gene without ORE was generated the same way with forward primer: TTTGAATTCTAGTTCT AAATAACACATAGAG. The fragments were digested with EcoRI and HindIII and cloned into YCplac22 (Gietz and Sugino, 1988; Sambrook et al., 1989). The resulting plasmids were transformed to \textit{ino1Δ} (Ito et al., 1983).
RESULTS AND DISCUSSION

Results of a micro-array experiment in which expression of S. cerevisiae genes is followed over time after a shift from a growth medium containing glucose to one containing oleate as the sole carbon source, indicate that INO1 expression peaks at 25 minutes after a shift from glucose to oleate medium. Interestingly, this upregulation coincides with that of the peroxisomal matrix protein thiolase, indicating that phospholipid synthesis and induction of peroxisome proliferation take place simultaneously. Also expression of other proteins involved in phospholipid synthesis, Cho1p, Psd1p, Cho2p and Opi3p follow this peak (Figure 5 in chapter 1). The genes encoding these proteins all contain an UASINO. It is clear that a signal for upregulation is generated, however the expression levels vary. INO1 is the most strongly upregulated, reason enough to study its regulation in more detail.

In order to check the capacity of mutants to regulate INO1 expression, growth experiments were performed. Growth of equal amounts of cells on media with different carbon sources with and without inositol was tested on solid media. The inability of mutants to grow on medium lacking inositol indicates that the missing gene is essential for inositol synthesis and thus for INO1 expression in the tested circumstances. The carbon sources used were either glucose, galactose, glycerol or oleate. The result of these experiments is presented in Table I.

In the presence of inositol all mutants can grow on glucose, galactose and glycerol medium. On oleate medium containing inositol the only mutants that have a growth defect are pip2Δ, oaf1Δ, and double mutants with either one (ire1Δ/pip2Δ and ire1Δ/oaf1Δ). This can be easily explained because Pip2p and Oaf1p are necessary for induction of genes with an ORE, genes encoding proteins which are essential when the yeast is grown on oleate, like β-oxidation enzymes. The presence or absence of inositol is in this case not the cause of the growth defect.

Two S. cerevisiae strains were compared (because some mutants were made in another genetic background than others), wild type JC104 and BJ1991 and their ire1Δ derivatives. One difference in growth behaviour of these strains was observed. On galactose medium lacking inositol ire1Δ in JC104 has growth problems, while ire1Δ in BJ1991 grows normally. This is a trivial problem which only occurs when galactose is used in growth medium. Since BJ1991 has a positive result (growth on
Table I: Growth of wild type and mutants (of two strains) on minimal media containing different carbon sources with or without inositol.

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- (+) = only a few small colonies appear.
+/- = less colonies than on other media, normal colony size.

galactose) this strain is considered as the most reliable for interpretation. Unfortunately, Cox and colleagues used the JC104 strain and grew it on galactose medium for their studies on the UPR (Cox et al., 1997) which makes their results less easy to interpret. Besides this, their assumption that the use of glucose or galactose as carbon source in medium does not influence growth results is probably not valid: also in BJ1991 deletion of IRE1 has a lethal effect in the absence of inositol when the cells are grown on glucose, but on galactose based medium this effect is not observed. This implies that the growth defect of JC104 ire1Δ cells on galactose medium in which HMG-CoA reductase was overexpressed, does not necessarily represent the inability of these cells to induce the UPR.

It is clear from growth experiments with ire1Δ in both strains that the situation in yeast grown on glycerol or oleate based medium is different
Figure 1: Schematic representation of transcription of INO1 leading to inositol synthesis. The transcription factors Oaf1p and Pip2p bind to the ORE, whereas Ino2p and Ino4p regulate transcription via binding to UAS_{inO}.

from that in glucose grown cells. On glycerol and oleate media ire1Δ cells do not reveal a growth defect in the absence of inositol. This suggests that Ire1p is not part of a signalling chain leading to inositol production when glycerol or oleate are used as carbon source for yeast growth.

Other results presented in Table I are as expected:
(1) INO1 is essential for inositol synthesis in all conditions,
(2) Hac1p and Ire1p are in the same pathway leading to inositol synthesis,
(3) The absence of the negative regulator OPI1 is no problem for growth on any medium, (4) Pip2 and Oaf1 are not essential for growth in the absence of inositol when the cells are grown on carbon sources different from oleate.

However some results were not completely as anticipated. Interestingly, Ino4p as the positive regulator of INO1 expression is essential for INO1 expression in all circumstances tested. This means that Ino4p is also important for inositol synthesis when the Ire1p independent pathway leading to INO1 expression is followed.

The growth defect on glucose caused by the absence of IRE1 is completely rescued when OPI1 is deleted as well. This can only be explained when Ire1p and Opi1p are operating in different pathways (Figure 2). Ire1p contributes to INO1 upregulation (pathway 1), while Opi1p represses another route (pathway 2) to INO1 expression. Opi1p seems to be only operating or fully functional as a repressor when yeast is grown on glucose medium, indicating that it is involved in glucose repression of the IRE1 independent signalling pathway (pathway 2).
Figure 2: Model of different signalling pathways leading to INO1 transcription in yeast under inositol synthesis requiring conditions (extended model of Cox et al. (1997)).

We made double knock-outs of PIP2 or OAF1 with IRE1 with the idea that if ire1p is acting in a different signalling pathway than Pip2p and Oaf1p and these are the only pathways leading to inositol synthesis, these double mutants would not survive on medium without inositol regardless of which carbon source is used. The result of a growth experiment revealed that growth is still possible in such double mutants on galactose and also to some extent on glycerol containing media. This means that if pathways 1 and 2 are omitted, a third rescue route to INO1 expression should exist.

In chapter 2 we suggested that on oleate the Pip2/Oaf1 pathway was probably essential for INO1 expression. Here we studied this pathway in more detail and found that the situation is more complicated than we expected. INO1 expression in oleate induced cells was tested with different lengths of the promoter region preceding the ORF. One construct was made which included the putative ORE and another which started behind this ORE. Expression of INO1 with either of these two promoter regions was tested in a ino1Δ background on oleate plates with and without addition of inositol. Cells grew in all circumstances tested: the absence or presence of inositol and the absence or presence of the putative ORE on a construct with the INO1 gene gave the same result. This means that INO1 expression on oleate does not require the presence of its ORE.
Taken together these results are difficult to explain. Figure 2 provides the basis for the formulation of a hypothesis, but it remains hard to understand the complete network of INO1 regulation as yet. One difficulty is the question: what is the difference in INO1 regulation between a stress condition, in which yeast is completely dependent on inositol synthesis, and a non stress condition?

In conclusion, we can state that INO1 expression is regulated via a quite complex mechanism. By testing growth of mutants influencing INO1 expression on different carbon sources it became apparent that there are at least 2, but probably 3 routes leading to INO1 transcription. The assumptions made by Cox and colleagues were not valid, Ire1p is by far not the only signalling protein leading to inositol synthesis. Also in contradiction with what we suggested, expression of INO1 via the putative ORE in its promoter region seems to be not the only additional factor in oleate induced cells as compared to the regulation in cells which are grown on glucose medium.

Peroxisome biogenesis or proliferation is a matter of maintenance of both peroxisomal proteins and membranes. Membrane maintenance is dependent on the amount of phospholipid synthesis in the organelle or the capacity to retrieve phospholipids or membranes from other compartments. The latter is probably valid for peroxisomes, since they do not contain the necessary proteins to synthesise phospholipids themselves. The induction of INO1 expression when yeast is shifted from glucose to oleate medium and the same trend in expression of some other genes important for phospholipid synthesis are the first observations which provide insight in how membrane upregulation is achieved when circumstances require peroxisome biogenesis.

Acknowledgements

We thank Rowan Chapman for helpful discussions and strains.
REFERENCES


