Overexpression of HAP4 in glucose-derepressed yeast cells reveals respiratory control of glucose-regulated genes

Romeo Lascaris, Jan Piwowarski, Hans van der Spek, Joost Teixeira de Mattos, Les Grivell and Jolanda Blom

Swammerdam Institute for Life Sciences, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

A link between control of respiration and glucose repression in yeast is reported. The HAP4 gene was overexpressed in a Δmig1 deletion background, generating a mutant in which respiratory function is stimulated and glucose repression is diminished. Although this combination does not result in derepression of genes encoding proteins involved in respiratory function, it nevertheless generates resistance against 2-deoxyglucose and hence contributes to more derepressed growth characteristics. Unexpectedly, overexpression of HAP4 in the Δmig1 deletion strain causes strong repression of several target genes of the Mig1p repressor. Repression is not restricted to glucose growth conditions and does not require the glucose repressors Mig2p or Hxk2p. It was observed that expression of the SUC2 gene is transiently repressed after glucose is added to respiratory-growing Δmig1 cells. Additional overexpression of HAP4 prevents release from this novel repressed state. The data presented show that respiratory function controls transcription of genes required for the metabolism of alternative sugars. This respiratory feedback control is suggested to regulate the feed into glycolysis in derepressed conditions.

INTRODUCTION

It has been shown that the balance between respiratory and fermentative metabolism in Saccharomyces cerevisiae can be shifted towards respiration by increasing the expression of the HAP4 gene (Blom et al., 2000). Whole-genome expression profiling and fingerprinting of the regulatory activity network show that the changes due to overexpression of HAP4 lead to an increase in mitochondrial biogenesis (Lascaris et al., 2002). The Hap4p activator apparently is an important regulator for mitochondrial biogenesis and, hence, for mitochondrial function (Forsburg & Guarente, 1989; Grivell, 1995). Indeed, recently we made observations that place Hap4p high in the hierarchy of controlling factors (Lascaris et al., 2002).

The glucose repression pathway regulates a large number of genes, including those involved in metabolism of sugars other than glucose (SUC/MAL/GAL genes) and genes encoding proteins required for respiration (Gancedo, 1998). One of the downstream effectors of the glucose repression pathway is Mig1p, a transcriptional repressor that is translocated from the cytoplasm to the nucleus when glucose is added to the medium (De Vit et al., 1997). Mig2p is a homologue of Mig1p, which remains localized in the nucleus in response to glucose and which probably performs a less important role in glucose repression than Mig1p (Lutfiyya & Johnston, 1996). Another component of the glucose repression pathway is Hxk2p. This glycolytic enzyme also acts as a transcription regulatory protein by binding to the glucose-repressed SUC2 promoter as a complex with other proteins (Herrero et al., 1998).

Since overexpression of the HAP4 gene enables mitochondrial biogenesis to escape from glucose repression (Lascaris et al., 2002), the question arises whether an additional defect in the glucose repression pathway may modify the effect of HAP4 overexpression. Since carbon source control is for a large part mediated by the transcription repressor Mig1p – which is essential for a functional glucose repression pathway – we analysed whether deletion of the glucose repressor gene MIG1 could influence the effect of overexpression of HAP4.

To analyse the double mutant Δmig1HAP4 we used plate assays that provided information on cellular physiology. Subsequent analysis of the transcriptional changes did not show an effect of deletion of MIG1 on targets of Hap4p, but surprisingly demonstrated a strong feedback control of
HAP4 overexpression on several Mig1p-targeted genes. Our results indicate that metabolic pathways that feed glycolysis in glucose-repressed conditions are under the control of respiration.

METHODS

Strains. All strains used derive from the wild-type strain CEN.PK113 (MATa MAL2-8 s CUC2). HAP4-overexpressing strain 436 GH (MATa MAL2-8 s CUC2 spr3::TDH3p-HAP4) is described in van Maris et al. (2001). The Δmig1 deletion strain and Δmig1HAP4↑ were generated in strain CEN.PK113 and the HAP4-overexpressing strain 436 GH, respectively, by disruption of the Mig1 gene using a KanMX disruption cassette. This cassette was made by inserting a 1.5 kb Smal–SpeI fragment from the pFA6a plasmid (Wach et al., 1994), containing the KanMX module, in a pUC18 plasmid with a BamHI-inserted 1.4 kb PCR-derived fragment containing the Mig1 coding region from which an internal 783 bp BsrI–HindI–SpeI fragment was deleted. The resulting vector was used as template to amplify a 2.1 kb fragment containing the Mig1-KanMX disruption cassette with a forward primer annealing to position 101–126 downstream from the ATG and a reverse primer annealing to position 1491–1470 downstream from the ATG. The Δmig1Δmig2 double deletion and the Δhxl2 deletion strains were kindly provided by Birgitte Ronnow (Klein et al., 1999) and Leonie van Raamsdonk (Diderich et al., 2001; Raamsdonk et al., 2001). In both strains the HAP4 overexpression cassette was inserted as described in van Maris et al. (2001), generating the Δmig1Δmig2HAP4↑ and the Δhxl2HAP4↑ strains.

Growth conditions and 2-deoxyglucose (2-DOG) sensitivity plate assay. Cells were grown aerobically in YPD (1% yeast extract, 1% Bacto-peptone and 2 or 3% D-glucose), YPRaff (YP containing 3% raffinose) or YPEG (YP containing 2% ethanol and 2% glycerol). In all experiments cells were harvested at early exponential phase. In 2-DOG sensitivity plate assays, cells were spotted at different dilutions (10⁻¹⁰) on rich medium plates that contained a carbon source (2% glucose, 3% raffinose, 3% melibiose, 3% galactose or 2%/2% ethanol/glycerol) and 200 μg 2-DOG ml⁻¹ (50 μg ml⁻¹ for ethanol/glycerol plates).

Northern analysis and microarray experiments. RNA isolation, Northern blotting, (pre-)hybridizations, washing and visualization were performed as described previously (Lascaris et al., 2000). Probes to detect CYT1, SUC2 and the loading control ACT1 were obtained by ³²P[ATP] labelling of PCR-generated DNA fragments. The JEN1 probe was an 844 bp Ncol–PstI fragment from the pH3 (pUC18::JEN1) plasmid that was kindly provided by Raquel Pego de Andrade (Andrade & Casal, 2001). Other probes derived from DNA fragments are as described by van Maris et al. (2001).

RESULTS

Mig1p and Hap4p control complementary metabolic pathways

Since HAP4 overexpression enables escape from glucose repression, we analysed whether the changes that occur in this strain can be modified by additionally mutating part of the glucose repression pathway. For this reason the HAP4 gene was overexpressed in a Δmig1 deletion strain, resulting in the Δmig1HAP4↑ strain. It should be noted that the growth rates of the strains used in this analysis are not affected.

First, we analysed to what extent growth was affected by the glucose analogue 2-DOG (Zimmermann & Scheel, 1977). This glucose analogue triggers signalling of glucose repression, but cannot be metabolized via glycolysis, causing strong inhibition of growth on carbon sources other than glucose. A strain with a defect in the glucose repression system, i.e. a Δmig1 deletion strain, has been shown to be less sensitive to 2-DOG as confirmed by our assays (see Fig. 1). Similar to the Δmig1 deletion strain, overexpression of the HAP4 gene also enables cells to escape from glucose repression since they grow better than the corresponding wild-type cells on raffinose, melibiose (Fig. 1) and galactose (data not shown) medium containing 2-DOG. Importantly, cells in which both the Mig1p repressor is absent and the gene encoding the Hap4p activator is overexpressed grew best in the presence of 2-DOG. This shows that both mutations act additively to relieve glucose repression and indicates that both mutations affect different parts of the glucose repression system.

This was confirmed by addition of 2-DOG to cells growing on the non-fermentable carbon sources ethanol and glycerol (right panels of Fig. 1). In this case overexpression of HAP4 has a marked effect on growth, whereas the absence of MIG1 hardly enhanced growth in the presence...
of 2-DOG. These growth characteristics are consistent with independent action of Hap4p and Mig1p in carbon source metabolism: HAP4 overexpression enables escape from glucose repression by up-regulation of respiratory function and deletion of Δmig1 enables escape from glucose repression by derepression of genes involved in the metabolism of sugars other than glucose.

**Deletion of MIG1 and overproduction of HAP4 do not act additively on the respiratory chain reporters CYT1 and QCR8**

We looked at whether the combined deletion of MIG1 and the overexpression of HAP4 result in increased expression of respiratory chain components under glucose-growth conditions. The expression of the respiratory genes CYT1 (Fig. 2) and QCR8 (data not shown) was not strongly derepressed in the glucose-grown Δmig1 deletion strain itself. Minor derepression of CYT1, however, can be observed, which is likely to be due to a minor derepression of the endogenous HAP4 gene. Importantly, if the MIG1 gene is deleted in the HAP4 overexpressing strain, no additional increase in transcription can be observed.

The data presented thus far show that deletion of MIG1 and overexpression of HAP4 have unexpectedly well separated effects on carbon source control. Note that the endogenous HAP4 gene is strongly regulated in response to glucose, but is not strongly derepressed in the Δmig1 deletion strain (see Fig. 2). Hence, repressors other than Mig1p (e.g. Mig2p) mediate glucose repression of mitochondrial biogenesis.

**HAP4 overproduction causes strong repression of the derepressed SUC2 gene**

Although a Δmig1 deletion apparently does not contribute significantly in HAP4↑-mediated up-regulation of genes involved in mitochondrial function, the converse possibility was also investigated: does overexpression of HAP4 contribute to up-regulation/derepression of Mig1p-regulated genes that are involved in metabolism of sugars other than glucose? For this reason we analysed whether overexpression of HAP4 affects the expression of SUC2, one of the prime targets of Mig1p. As shown in Fig. 3, overexpression of HAP4 in a Δmig1 deletion background does not contribute to derepression of SUC2, but surprisingly restores the repressed state of the SUC2 promoter (compare lanes 2 and 4 in Fig. 3).

In an attempt to investigate the basis of this phenomenon, the effect of HAP4 overexpression was also analysed in other mutants that are defective in glucose repression. As shown in Fig. 3, the strong repressive effect of HAP4 overexpression is also present in a Δmig1Δmig2 double deletion and a Δhxk2 deletion strain (compare lanes 5 and 6, and 7 and 8, respectively). This shows that the repressive action of overexpression of HAP4 is not mediated by components of the glucose repression pathway that are known to bind to the SUC2 promoter (Herrero et al., 1998; Wu & Trumbly, 1998).

The Mig1p-regulated genes MAL61, encoding maltose permease, and HXT2, encoding a high-affinity glucose transporter, also exhibit HAP4↑-mediated repression. Furthermore, from whole-genome expression profiles of glucose-grown Δmig1Δmig2 and the Δhxk2 strains with and without a HAP4 overexpression cassette (J. M. Schuurmans & M. J. Teixeira de Mattos, unpublished data), only nine derepressed genes were found to show a twofold decrease due to overexpression of HAP4: SUC2, HXK1, HSP12, HXT16, MAL11, MAL32, YER067W and the

![Fig. 2. The expression of the respiratory gene CYT1 closely follows the expression of the HAP4 gene as determined by Northern analysis using RNA from cells grown in rich medium containing non-fermentable carbon sources (YPEG) or glucose (YPD). Transcripts from the HAP4 overexpression construct generate an additional band beneath the endogenous HAP4 mRNA. Note that the endogenous HAP4 gene is repressed by glucose and the HAP4 overexpression construct, which is regulated by the TDH3 promoter, is induced by glucose. Deletion of the MIG1 gene (Δmig1) leads to a very minor derepression of both the HAP4 and CYT1 transcripts and apparently does not contribute significantly to the effect of overexpression of HAP4. Note that the expression of CYT1 unaccountably increases in a Δmig1HAP4↑ strain during growth on non-fermentable carbon sources. PDA1 was used as loading control. Lanes: 1, wild-type; 2, HAP4↑; 3, 4, Δmig1; 5, 6, Δmig1HAP4↑.](http://mic.sgmjournals.org)

![Fig. 3. Overexpression of HAP4 causes repression of SUC2 in strains that have a defect in the glucose repression pathway. Northern analysis was performed to monitor the expression of the Mig1p/Mig2p-regulated SUC2 and JEN1 genes in glucose-repressed cells (YPD). PDA1 was used as loading control. SUC2 expression but not JEN1 expression is repressed by overexpression of HAP4. Lanes: 1, wild-type; 2, Δmig1; 3, HAP4↑; 4, Δmig1HAP4↑; 5, Δmig1Δmig2; 6, Δmig1Δmig2HAP4↑; 7, Δhxk2; 8, Δhxk2HAP4↑.](http://mic.sgmjournals.org)
HAP4 expression (Fig. 4b) in the wild-type (not shown) and HAP4 repressive effect of (Fig. 4a). This is of interest, since it demonstrates that the HAP4 shows the repressive action of mutant cells grown on raffinose, a sugar that Suc2p SUC2 Mig2p. As shown in Fig. 3, the JEN1 gene, which encodes lactate permease and which is regulated by Mig1p and the co-repressor Mig2p (Bojunga & Entian, 1999), was not repressed under conditions of HAP4 overexpression.

HAP4 †-mediated repression is also evident under SUC2-inducing conditions

We further studied whether HAP4 †-mediated repression also occurs under conditions where the SUC2 gene is required for growth and is fully induced. Wild-type and mutant cells grown on raffinose, a sugar that Suc2p (invertase) hydrolyses to fructose and melibiose, indeed show the repressive action of HAP4 overexpression (Fig. 4a). This is of interest, since it demonstrates that the repressive effect of HAP4 overexpression also occurs if the glucose repression system is not functional (compare HAP4 † and wild-type in Fig. 4a). Consistently, activating the glucose repression pathway by addition of 2-DOG to raffinose-growing cells results in strong repression of SUC2 expression (Fig. 4b) in the wild-type (not shown) and HAP4 overexpression strains, but not in the Δmig1HAP4 † strain. Glucose repressors other than Mig1p apparently play a minor role relative to the repressive effects of Mig1p and of overexpression of HAP4. Taken together, these findings show that the strong repression mediated by overexpression of HAP4 does not require glucose metabolism and is independent of glucose signalling.

**Fig. 4.** (a) HAP4 †-mediated repression of SUC2 under inducing conditions, viz. growth on 3% raffinose. Lanes: 1, wild-type; 2, HAP4 †; 3, Δmig1; 4, Δmig1HAP4 †. (b) Transcriptional response of SUC2 before and after addition of 2-DOG in HAP4-overexpressing strains with normal (HAP4 †; lanes 1 and 2) and defective (Δmig1HAP4 †; lanes 3 and 4) glucose repression signalling. Raffinose-grown overnight cultures were inoculated at OD600 = 0.3 into fresh medium containing 3% raffinose or 3% raffinose with 0.02% 2-DOG. After 3 h samples were taken for RNA isolation. ACT1 was used as loading control. Addition of 2-DOG to raffinose-grown cells results in strong glucose signalling, repressing the endogenous HAP4 gene (upper band of the HAP4 mRNA) and inducing the HAP4 overexpression construct which is regulated by the glucose-inducible TDH3 promoter (lower band of the HAP4 mRNA).

**HAP4 overexpression prevents release from a novel glucose-repressed state**

We further analysed whether overexpression of HAP4 in wild-type and Δmig1 cells affects the kinetics of glucose repression. For this reason glucose was added to wild-type and mutant cells that were grown on the non-fermentable carbon sources ethanol and glycerol and samples for RNA isolation were taken at 0, 5, 10, 20, 40, 60, 90 and 180 min after glucose addition. As shown in Fig. 5, in HAP4-overexpressing cells glucose repression of SUC2 is nearly instantaneous and persists in time, similar to the transcriptional response of wild-type cells (data not shown). This is expected since the repressive action of Mig1p is strong and fast (De Vit et al., 1997). However, when the Δmig1 strain is analysed for glucose repression kinetics, a peculiar dynamic expression pattern is observed that consists of three different phases. (i) The first phase is characterized by a strong induction of SUC2 expression, occurring immediately after glucose has been added (see 10 and 20 min time-points in Fig. 5). Apparently a glucose-dependent transcriptional activator is functional at the SUC2 promoter that is normally masked by the presence of the stronger repressor Mig1p. (ii) The second and most important phase occurs 40 min after glucose addition and is characterized by a near complete loss of SUC2 mRNA. This novel phase shows that a strong repression system is active that is not dependent on Mig1p. In fact, since this repressed state is also found in other mutants that have a defect in the glucose repression pathway, like Δmig1Δmig2

**Fig. 5.** Glucose repression kinetics in the HAP4 †, Δmig1 and Δmig1HAP4 † strains. Changes in expression of the SUC2 gene were monitored before and after glucose (3%) was added to cells grown on non-fermentable carbon sources (YP, 2% ethanol and 2% glycerol). When the Mig1p repressor is absent, a highly dynamic expression pattern appears that can be divided into three phases (see Results). This pattern was also observed using a glucose concentration of 6%. At the indicated time points (min) samples were taken for RNA isolation. ACT1 was used as loading control.

MALx2 homologues FSP2 and YGR287C. This indicates that a small, specific group of genes is affected by HAP4 †-mediated repression. HAP4 †-mediated repression, however, does not extend to all genes regulated by Mig1p and/or Mig2p. As shown in Fig. 3, the JEN1 gene, which encodes lactate permease and which is regulated by Mig1p and the co-repressor Mig2p (Bojunga & Entian, 1999), was not repressed under conditions of HAP4 overexpression.

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DISCUSSION

In this report we show that a combination of mutations that stimulate respiration and diminish glucose repression act additively to generate resistance against 2-DOG. The additive effect is likely to be due to the fact that Hap4p and Mig1p act largely independently in regulation of carbon source metabolism. This is further illustrated by the fact that deletion of MIG1 in the HAP4-overexpressing strain did not contribute to stronger derepression/activation of respiratory genes. From this perspective it was surprising to find that target genes of the Mig1p repressor were strongly repressed due to overexpression of HAP4. This unexpected repressive effect is not due to increased activity of the glucose repression pathway, since HAP4↑-mediated repression also occurred under conditions other than growth on glucose. Furthermore, deletion of genes that encode factors functionally related to Mig1p, viz. the MIG2 and HXK2 genes, did not abolish HAP4↑-mediated repression. Apparently, a repressive mechanism is acting that is distinct from the glucose repression pathway. The kinetics of glucose repression of SUC2 in time in the Δmig1 and Δmig1HAP4↑ strains revealed a novel repression state of SUC2 that is maintained by overexpression of HAP4 in the double mutant, but released in the Δmig1 mutant. The repressed state that is maintained by overexpression of HAP4 can be released by blocking the activity of the respiratory chain. These data suggest that carbon source regulation of SUC2 involves not only the glucose repression system but also control by the respiratory activity in the cell.

The question arises why a system has evolved in which the expression of SUC2 follows the respiro-fermentative state of the cell. To answer this question we have to bear in mind that Suc2p (invertase) is required and expression of the SUC2 gene is induced during respiro-fermentative growth on raffinose or sucrose. The respiratory capacity in these conditions is high and the fermentative capacity low. Nevertheless, part of these sugars is fermented to ethanol. Overexpression of HAP4 is likely to enhance the respiratory capacity further so that less sugar will be fermented. This can be expected to result in a decrease in the glycolytic flux and a subsequent increase in glucose concentration in the periplasmic space. It should be noted that relatively minor changes in the balance between respiration and fermentation would have severe effects due to large differences in energy yield between fermentation and respiration. Hence, it is hypothesized that respiratory feedback control may down-regulate invertase activity in order to decrease the periplasmic glucose concentration. Two unfavourable effects of increased periplasmic glucose may be prevented by this control mechanism. First, it may prevent the activation of several signal transduction pathways, i.e. the glucose repression pathway, cAMP signalling and the Snf3/Rtg2 system, stabilizing the balance between respiration and fermentation. Second, it may prevent glucose and also fructose molecules being lost by diffusion from the periplasmic space into the extracellular medium. Loss of fructose has been observed to occur when wild-type cells were grown until near glucose depletion in medium containing a mixture of sucrose and glucose (Raamsdonk, 2000). We suggest that respiratory feedback control is the regulatory mechanism that maintains periplasmic glucose concentrations at a low level and hence stabilizes the balance between respiration and fermentation.

In this system, low periplasmic glucose concentrations may be sensed by the glucose sensor Snf3p, an HXT homologue that acts at low glucose concentrations (Ozcan & Johnston, 1999). Interestingly, Snf3p interacts specifically with Mth1p, a factor involved in glucose repression (Gamo et al., 1994; Schmidt et al., 1999). The origin of the signal provided by overexpression of HAP4 – increased respiratory activity – may be, for instance, the ADP/ATP ratio, the redox state of the cell or oxidative stress formed by mitochondrial activity. Which pathway/signalling mechanism(s) actually is (are) involved is not known.

Glucose-induced mRNA turnover of SUC2 may also be important for the apparently fast degradation of all SUC2 mRNA (Cereghino & Scheffler, 1996) after glucose has been added to the culture. The repression that is mediated by HAP4↑ on the other hand is unlikely to be due to increased mRNA turnover, since the JEN1 gene does not show HAP4↑-mediated repression (see Fig. 3), whereas the mRNA of JEN1, similar to SUC2 mRNA, is degraded by a glucose-induced mechanism (Andrade & Casal, 2001).

Glucose repression kinetics of SUC2 in the Δmig1 strain consists of ‘normal’ derepression starting at least 1 h after glucose addition, which is unexpectedly slow. When the HAP4 gene is overexpressed, derepression does not occur. However, derepression in this strain can be triggered by blocking mitochondrial function with antimycin A (data not shown). Previously, we have shown that overexpression of HAP4 results in a marked increase in mitochondrial biogenesis (Lascaris et al., 2002). Since mitochondrial biogenesis in the Δmig1 deletion strain is glucose-repressed, similar to the wild-type strain, it may be that the changes in expression of the SUC2 gene correlate with slow repression by glucose of mitochondrial biogenesis and/or function.
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