Functional Analysis of the Zn$_2$Cys$_6$ Transcription Factors Oaf1p and Pip2p

DIFFERENT ROLES IN FATTY ACID INDUCTION OF $\beta$-OXIDATION IN SACCHAROMYCES CEREVISIAE*

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Fatty acid induction of the peroxisomal $\beta$-oxidation machinery in Saccharomyces cerevisiae involves transcriptional control of genes regulated by the oleate response element (ORE). Glucose as the preferred carbon source antagonizes this effect. Induction is dependent on the Zn$_2$Cys$_6$ family member Oaf1p and Pip2p, which bind to this element as a heterodimer. We show here by ectopically expressing both components and LexA fusion derivatives that this transcription factor complex is only active in the presence of oleate. In contrast to Pip2p, Oaf1p is responsive to oleate activation in the absence of the other component of the heterodimer. Therefore, it is the exclusive receptor of the oleate signal. Pip2p is active also under noninducing conditions but is effectively inhibited when complexed with Oaf1p in the absence of inducer. It contributes to the transactivation properties of the Oaf1p-Pip2p heterodimer and is required for efficient binding of Oaf1p to OREs in vivo. Repression of ORE-dependent transcription by glucose occurs via both Oaf1p and Pip2p. By dissecting functional domains of both proteins, we identified a region required for regulated activity of the C-terminal activation domain. These findings allow us to postulate a model for carbon source-regulated transcription of peroxisomal protein genes.

In the yeast Saccharomyces cerevisiae fatty acids such as oleate cause a massive proliferation of the peroxisomal compartment accompanied by the induction of $\beta$-oxidation (1). Glucose represses these effects apparently because it is utilized preferentially to alternative carbon sources such as fatty acids (2–4). Glucose repression predominantly affects gene expression via both Oaf1p and Pip2p. By dissecting functional domains of both proteins, we identified a region required for regulated activity of the C-terminal activation domain. These findings allow us to postulate a model for carbon source-regulated transcription of peroxisomal protein genes.

Due to the presence of an oleate response element (ORE) in their promoters (9, 10). This upstream activating sequence is minimally defined by an inverted repeat of CGG triplets separated by a 15–18-nucleotide spacer. It constitutes the binding target for the transcription factors Oaf1p and Pip2p (11, 12). These two proteins are quite similar with an overall identity of 40% and belong to the Zn$_2$Cys$_6$ protein family of fungal transcription factors. Usually, members of this family require homodimerization to be able to bind their cognate sites. Oaf1p and Pip2p, however, predominantly bind to OREs as a heterodimeric complex (13, 14). Transcription of Pip2 is itself up-regulated by oleate via an ORE in its promoter and thus requires Pip2p and Oaf1p, whereas expression of OAF1 is independent of these factors and comparable in glucose, ethanol, and oleate medium (14).

Functional domains other than the N-terminal Zn$_2$Cys$_6$ DNA-binding motifs have not yet been identified in Oaf1p or Pip2p. The domain structures of Oaf1p and Pip2p may deviate from the functional organization of a prototypic Zn$_2$Cys$_6$ protein homodimer (15) as they have to allow the formation of a heterodimer in order to function properly. Moreover, the mechanisms required to convert Zn$_2$Cys$_6$ proteins from an inactive to an active state are apparently not conserved. Activation of Gal4p, for instance, depends on its interaction with Gal80p- and Gal3p-binding galactose (16, 17), whereas phosphorylation via the Snf1p (Cat1p) protein kinase suffices to activate Cat8p and Sip4p (18–20). These recent findings challenge the idea of a common principle in Zn$_2$Cys$_6$ protein regulation and highlight the requirement for a detailed study on the inducer-mediated activation of the Oaf1p-Pip2p transcription factor.

This report is concerned with the individual roles of Pip2p and Oaf1p in activation of transcription by fatty acids and its repression by glucose and addresses the domain structure of Oaf1p and Pip2p. The results obtained are integrated into a model for carbon source-dependent transcription of ORE-regulated genes.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Yeast strains and plasmids used are listed in Tables I and II, respectively. All DNA manipulations and subcloning were done in Escherichia coli strain DH10B.

Yeast Media and Growth Conditions—For $\beta$-galactosidase assays overnight cultures propagated in YP medium (1% yeast extract, 2% bacteropone) containing 2% glucose were diluted to an A$_{600}$ of 0.3 either in YP medium supplemented with 0.5% glucose (for strains derived from BJ1991) or in synthetic medium (0.67% yeast nitrogen base, 0.5% glucose, amino acids as required) lacking tryptophan (for strains derived from L40) and grown for 8 h. The cultures were then

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1 The abbreviations used are: ORE, oleate response element; AD, trans-activation domain; DMS, dimethyl sulfate; PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair.
shifted to YP medium containing 2% glucose (YPD), 2% ethanol (YPE), or 0.2% oleic acid plus 0.02% Tween 80, adjusted to pH 7.0 with NaOH (YPO), and further grown until the cultures reached an 

The annealed oligonucleotides PIP2-321a (5'-GATCTTTAATAGTAGGTCGAGG-3') and PIP2-500b (5'-GATCTGTGCCCAT-3') containing a stop codon prior to a SalI site were ligated into the BamHI site of pBTMPIP2-996. The oligonucleotide-dependent SalI site was used to excise the region of PIP2 that was 3' to its BamHI site as described for pBTMPIP2-321C. To clone pBTMPIP2-823C (the proximal 2483 bp of PIP2), the annealed linker oligonucleotides PIP2-500a/PPIP2-500b were ligated into the PIP2 BoI site of pBTMPIP2-996, and the region of PIP2 that was 3' to its BoI site was excised as above. To clone pBTMPIP2-975C (the proximal 2925 bp of PIP2), the annealed linker oligonucleotides ∆Neo, SalI-1 (5'-CATGACATCGAGCTTACACCC-3'), and ∆Neo, SalI-2 (5'-TGACGGTAAAAGACTGAGG-3'), were ligated into the larger (8.0 kb) of the two fragments obtained after a digest of pBTMPIP2-996 with Ncol and SalI. To clone pBTMPIP2-94N (the distal 2709 bp of PIP2, i.e. the 2709 base pairs counted from the 3'-end of PIP2), a PCR was performed with the primer pair PIP2-94N (5'-TAAAGAATCTACAGTGGAAAGG-3') and HR12 (5'-GGTCTGTTATATACGGG-3') using plasmid pBTMPIP2-996 as a template. The PCR product was cut with EcoRI to obtain a 1.5-kb fragment that was ligated into the larger (6.3 kb) of the two fragments obtained after pBTMPIP2-996 was cut with EcoRI. To clone pBTMPIP2-180N (the distal 3448 bp of PIP2), a PCR was performed with the primer pair PIP2-180 (5'-TTGAAGATTTTATGAGCTGACGATT-3') and HR12 using pBTMPIP2-996 as a template. The PCR product was cut with EcoRI and the obtained 1164-bp fragment was ligated into the EcoRI-digested 6.3-kb pBTMPIP2-996 fragment as described for pBTMPIP2-94N. To clone pBTMPIP2-322N (the distal 2202 bp of PIP2), a PCR was performed with the primer pair PIP2-322 (5'-TTGAAGATTTTATGAGCTGACGATT-3') and HR12 using pBTMPIP2-996 as a template. 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with *Bcl*I followed by a complete *Cla*I digestion. Plasmid pBTMPiP2-500-832I (a 996-bp *Bam*HI/*Bcl*I internal deletion of *PIP2*) was constructed by digesting pBTMPiP2-996 with *Bam*HI followed by a partial digestion with *Bcl*I and a self-ligation of the obtained 7-kb fragment. To clone pBTMPiP2-180NHI (the distal 723 bp of *OAF1*), a PCR was made using genomic DNA from BJ1991 wild type as a template. The amplified 3.2-kb *OAF1* fragment was cut with *Sal*I and *Sst*I and ligated into the appropriately cut pBTM116.

To clone pBTMOAF1-1047 (representing the entire proximal 3063 bp of *OAF1*), a PCR was made using genomic DNA from BJ1991 wild type as a template. The amplified 2.8-kb *OAF1* fragment was cut with *Sst*I and *Sal*I and ligated into the appropriately cut pBTM116.

To clone pYCOAF1-1047 using plasmid pYC-PIP2 as a template. The fragment (1.5 kb) was cut with *Bam*HI and *Sal*I, and the largest of the obtained fragments (6.3 kb) was isolated and self-ligated. To clone pBTMOAF1-776N (the distal 816 bp of *OAF1*), plasmid pBTMOAF1-1047 was digested with *Nde*I and *Sal*I and subsequently treated with Klenow enzyme to generate blunt ends. The fragment was isolated and self-ligated. To clone pBTMOAF1-807NHI (the distal 723 bp of *OAF1*), a PCR was made using genomic DNA from BJ1991 wild type as a template. The PCR product was cast with EcoRI and *Sal*I and ligated into the appropriately cut pBTM116.
Functional Analysis of Zn\textsubscript{2}Cys\textsubscript{6} Proteins Oaf1p and Pip2p

**FIG. 1.** Overexpression of Pip2p is not sufficient to induce FOX3 ORE-dependent transcription in the absence of oleate. A. Western blot showing myc-tagged Pip2p. The fusion protein was expressed from the ADH2 promoter in the yeast strain yHPR107 (wild type + ADH2-PIP2-myc9) in glucose (G), ethanol (E), and oleate (O) media and detected using a monoclonal anti-myc antibody. Strain yHPR106 (wild type + ADH2-PIP2) acted as a negative control. Equal protein loading was ensured by determining the constitutively expressed Kar2p using a polyclonal anti-Kar2p antibody. B, FOX3 ORE-CYC1-lacZ reporter gene activities in ethanol- (E) and oleate-grown (O) cells. β-Galactosidase activity of the yeast strain yHPR115 (wild type + ADH2-PIP2) was compared with that of wild type strain yHPR111 (wild type) in ethanol and oleate media. Strains yHPR114 (pip2Δ + ADH2-PIP2) and yHPR112 (pip2Δ) were used to ensure the functionality of the ADH2-PIP2 construct.

**FIG. 2.** Suppression of a pip2Δ deletion by overexpressed Oaf1p is an ORE-dependent and oleate-specific event. A, detection of myc-tagged Oaf1p and Pip2p using Western blot analysis. The tagged proteins were expressed from the ADH2 promoter in strains yHPR93 (pip2Δ + ADH2-OAF1-myc9) and yHPR109 (oaf1Δ + ADH2-PIP2-myc9) in ethanol (E) and oleate (O) media and detected using a monoclonal anti-myc antibody. Strains yHPR92 (pip2Δ + ADH2-OAF1) and yHPR108 (oaf1Δ + ADH2-PIP2) expressing the appropriate untagged versions served as negative controls. Kar2p was monitored as an internal loading control. B, carbon source-dependent activities of the FOX3 ORE-CYC1-lacZ reporter gene. Expression of β-galactosidase was tested in the yeast strain yHPR118 (pip2Δ + ADH2-OAF1) in ethanol (E) and oleate (O) media and compared with that of wild type strain yHPR111 (wild type), yHPR112 (pip2Δ), and yHPR117 (oaf1Δ + ADH2-OAF1). In parallel, strain yHPR116 (oaf1Δ + ADH2-PIP2) was compared with yHPR111 (wild type), yHPR113 (oaf1Δ), and yHPR114 (pip2Δ + ADH2-PIP2).

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**RESULTS**

Activation of the Oaf1p-Pip2p Heterodimer Strictly Depends on Oleate—Strains deleted in Pip2 or OAF1 are unable to induce ORE-regulated genes in the presence of oleate (11, 12). Expression of one partner of the heterodimer, Pip2p, is, however, itself induced by oleate via an ORE in its promoter (14). It was therefore tested whether the requirement for oleate by the induction machinery could be bypassed by overexpressing Pip2 in the absence of oleate. For that purpose a copy of Pip2 or a myc9-tagged variant thereof was placed under the control of the ADH2 promoter and introduced into a wild type strain. The ADH2 promoter was chosen because of its high activity in the absence of glucose. As expected, expression of Pip2p-myc9 was completely repressed in glucose medium but was high in ethanol and oleate media (Fig. 1A). In the absence of oleate,
however, increased FOX3 ORE-dependent expression of a CYC1-lacZ reporter construct (Fig. 1B) was not detected.

Induction of Transcription by Oleate Occurs via Oaf1p—Since oleate was necessary to activate the Oaf1p-Pip2p heterodimer even when Pip2p was abundantly present, one of the two proteins or both of them should receive the oleate-derived signal. It was therefore tested whether a single component of the heterodimer when overexpressed from the ADH2 promoter suffices to activate a FOX3 ORE-driven reporter gene in an oleate-specific manner. Amounts of myc9-Oaf1p in a pip2Δ strain were comparable in ethanol and oleate media (Fig. 2A) indicating that the higher reporter gene activity observed for a corresponding strain (pip2Δ + ADH2-OAF1) in the presence of oleate was due to oleate-triggered transcription (Fig. 2B). Overexpression of Pip2p-myc9 in oaf1Δ cells also yielded similar quantities in the presence of ethanol and oleate (Fig. 2A). However, excess Pip2p only weakly stimulated expression of the reporter gene, although some oleate-dependent increase was obtained (Fig. 2B; oaf1Δ + ADH2-PIP2). This finding may suggest that the trans-activation potential of Pip2p also increased in the presence of oleate. The effect obtained may have been limited by Pip2p being virtually unable to bind to the FOX3 ORE in the absence of Oaf1p (Ref. 14 and data not shown). An assay that did not depend on binding to OREs was therefore required to allow a better comparison of the intrinsic transcriptional activities of Oaf1p and Pip2p.

For this purpose Pip2p and Oaf1p were fused to LexA and tested for their ability to activate a lexA<sup>prog</sup>-lacZ reporter construct. Both proteins remained functional in their fusion to LexA as they partially complemented the oleate phenotype in the pip2 and oaf1 deletions, respectively. As a result of being under the control of the constitutive ADH1 promoter, the fusion constructs were expressed to comparable levels in glucose, ethanol, and oleate media (not shown). In wild type cells, LexA-Oaf1p was able to mediate significant transcriptional activation only in the presence of oleate (Fig. 3A). To demonstrate that this oleate-specific expression was not dependent on dimers eventually formed with wild type Pip2p or Oaf1p, the fusion protein was also tested in strains deleted in PIPE, OAF1, or both genes. Importantly, the activation profiles detected in such strains did not significantly deviate from that in the wild type strain. LexA-Pip2p was a strong transcriptional activator in the presence of ethanol or oleate, but oleate-specific induction was not observed in the wild type or any of the mutant strains tested (Fig. 3B). This is in contrast to a preliminary result published earlier by our group (12). However, the present, much more systematic investigation (see also Figs. 5 and 6) unambiguously showed that LexA-Pip2p fusions are not acti-
activated by oleate. Although both proteins possess trans-activating properties, only Oaf1p is able to mediate the oleate signal.

Glucose Directly Inhibits Oaf1p and Pip2p Function—Oleate induction is repressed by the presence of glucose, partly because PIP2 expression is repressed (14). However, it seemed possible that the activity of the Oaf1p-Pip2p complex is additionally directly inactivated by glucose. It was therefore tested whether the activities of the constitutively expressed LexA fusions of Oaf1p and Pip2p can be repressed by glucose in the presence of oleate. As shown in Table III, glucose effectively abolished the transcriptional activity of Pip2p observed in the presence of oleate both in wild type and in oaf1Δpip2Δ cells. Oleate-induced activation of Oaf1p was also significantly impaired when glucose was present. Since similar results were obtained when the Oaf1p fusion was expressed in an oaf1Δpip2Δ strain, it was concluded that Oaf1p is only able to respond efficiently to oleate in the absence of glucose and that both factors are direct targets of glucose repression.

Both Oaf1p and Pip2p Are Required for Formation of an ORE Footprint in Vivo—Since overexpressed Oaf1p can circumvent the requirement for Pip2p in ORE activation, the apparent inability of the Oaf1p homodimer to contribute markedly to oleate induction in wild type cells was reinvestigated. One likely explanation was a low ORE binding activity of an Oaf1p homodimer. Evidence for this has previously been obtained in vitro (14). The binding site occupancy of the CTA1 ORE was therefore tested in vivo by genomic footprinting using DMS-treated genomic DNA from oleate-grown cells. DMS acts as a chemical probe for protein-DNA interactions since it only methylates guanine residues that are not protected by proteins (22). Fig. 4 shows a segment of the CTA1 promoter containing the ORE. Bands correlating to guanines within the ORE were quantified and intensities obtained from DNA of oaf1Δ, pip2Δ, or oaf1Δpip2Δ mutant strains were compared with those of wild type DNA. Differences were largely restricted to guanines of the conserved CGG triplets of the ORE (arrowheads numbered 1–4) indicating that these residues were particularly involved in DNA-protein complex formation. Importantly, these guanines were only weakly protected from methylation in the absence of Pip2p, Oaf1p, or both factors demonstrating that a low in vivo binding affinity of an Oaf1p homodimer is indeed one reason for the drastic phenotype observed in a pip2Δ strain.

Oaf1p and Pip2p Have a Similar Domain Structure—The results described above demonstrate that despite their homology, Oaf1p and Pip2p do not possess identical functions in the regulation of fatty acid catabolism. It was therefore attempted to assign salient features of both transcription factors to discrete regions of the two proteins. LexA fusions with distinct regions of Pip2p and Oaf1p were tested for their transcriptional activities on different carbon sources. In all cases, comparable expression of fusion proteins was ensured by Western blotting using polyclonal anti-LexA antibody (not shown). As shown in Fig. 5A, all versions of Pip2p that were truncated at the C terminus (Pip2p-179C to Pip2p-975C) had completely lost reporter gene activity suggesting that the C-terminal region of

TABLE IV

| Strain                  | Glucose | Ethanol | Oleate | Glucose + Oleate |
|-------------------------|---------|---------|--------|-----------------|
| Wild type + lexAPIP2Δ   | 767 ± 84| 2160 ± 8| 2004 ± 152| 666 ± 74       |
| Wild type + lexAOAF1Δ   | 1194 ± 310| 2810 ± 46| 2767 ± 443| 1050 ± 329     |
| Wild type + VP16-LexA   | 1794 ± 517| 3326 ± 310| 1303 ± 264| 1107 ± 108     |

of the conserved CGG triplets of the ORE (arrowheads numbered 1–4) indicating that these residues were particularly involved in DNA-protein complex formation. Importantly, these guanines were only weakly protected from methylation in the absence of Pip2p, Oaf1p, or both factors demonstrating that a low in vivo binding affinity of an Oaf1p homodimer is indeed one reason for the drastic phenotype observed in a pip2Δ strain.

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Pip2p has essential trans-activation potential. None of these constructs complemented the oleate induction phenotype in strain MF24-6x pip2Δ. Even with plasmids pYCPIP2-975C and pYCPIP2-832C, which expressed the respective truncations without the LexA moiety from the native PIP2 promoter, no such complementation could be achieved (not shown). The sufficiency of the C-terminal region for trans-activation was demonstrated by Pip2p-833N (the C-terminal 163 amino acids of Pip2p) and Pip2p-971N (the C-terminal 26 amino acids of Pip2p), which highly induced expression of the reporter gene on ethanol and oleate (Fig. 5A).

After having defined an essential trans-activation domain (AD) of Pip2p to its last 26 amino acid residues, it was tested whether Oaf1p possessed a similarly located AD. As shown in Fig. 5B, Oaf1p-901C and Oaf1p-1021C were also deficient in activating transcription of the lexA-op-lacZ reporter gene. Consistently, these two constructs as well as pYCOAF1-901C expressing the LexA-less Oaf1p-901C from the native OAF1 promoter could not complement an oaf1Δ deletion on oleate (not shown). Oaf1p-902N and Oaf1p-1021N possessed strong trans-activation properties demonstrating that Oaf1p also contained an essential AD within its C-terminal 27 amino acid residues.

The ADs of both Oaf1p and Pip2p still responded to glucose repression, as is demonstrated in Table IV, particularly by the activities of Pip2p-971N and Oaf1p-1021N in the simultaneous presence of oleate and glucose. Since only an approximately 3-fold repression by glucose was observed, it was tested in a control experiment whether LexA fused to the heterologous VP16 AD exhibited a similar effect. Although a minimal derepression on ethanol compared with glucose (1.8-fold) was obtained, no significant glucose repression in the presence of oleate was observed in the case of the VP16 construct. Although, a minor glucose repression effect seems indeed exerted on Pip2p and Oaf1p via their ADs, further constructs were generated to search for other sites involved in glucose repression.

Deletion of the N-terminal 93 amino acids of Pip2p (Pip2p-94N) eliminating the DNA binding domain resulted in non-complementation of the oleate induction phenotype of a pip2Δ mutation but was still repressible by glucose comparable to the full-length LexA-Pip2p-996 construct (Fig. 6A). Deleting the N-terminal 179 residues (Pip2p-180N) generally increased the activity approximately 10-fold compared with full-length Pip2p, but a 16-fold repression of activity in the presence of glucose was still observed. This suggested that the region between amino acids 93 and 179 was involved in negative regulation but not in glucose repression. By deleting a further 143 amino acids from the N terminus (Pip2p-322N), an inactive fusion protein was generated. Since neither the complementary C-terminal deletion (Pip2p-321C; Fig. 5A) nor a fusion of LexA with amino acid residues 179–321 of Pip2p (Pip2p-180N, 321C) exhibited trans-activation, it was concluded that this latter region did not represent an independent AD. It appeared to aid in the function of the C-terminal AD in the context of the full-length protein and was therefore termed auxiliary domain.

A further internal deletion that lacked amino acids 325–832 (Pip2p-324–833I) showed an approximately 6-fold increase in activity when compared with full-length Pip2p but was still susceptible to glucose repression (16-fold). Thus Pip2p contains a second inhibitory domain within amino acid residues 324–832. Since a smaller internal deletion (Pip2p-500–833I) retained activity levels comparable to full-length Pip2p, this inhibitory domain (ID II) is localized between amino acids 324 and 500. These data suggest that the auxiliary domain may represent the main target for glucose repression in Pip2p.
oleate phenotype of an binding domain of Oaf1p and was not able to complement the was generated. This fusion protein lacked the putative DNA Therefore, Oaf1p-181N roughly corresponding to Pip2p-180N rapid termination of ORE-dependent transcription. activities of both Pip2p and Oaf1p are directly repressed leading to a Oaf1p-Pip2p transcription factor. Under noninducing conditions, Oaf1p is transcriptionally silent and inhibits Pip2p activity. Upon addition of oleate Oaf1p is converted into a transcriptional activator that enables the heterodimer to induce the expression of Pip2 and consequently of peroxisomal protein genes. In the presence of glucose, the activities of both Pip2p and Oaf1p are directly repressed leading to a rapid termination of ORE-dependent transcription. inhibitory domains have to be located N-terminal to amino acid 776. The lack of reporter gene activity using Oaf1p-626N or Oaf1p-568N suggested that an inhibitory domain is located between amino acid residues 568 and 776 (ID II) that irreversibly masked the AD similarly to the case of Pip2p-322N. In Pip2p, retaining a more N-terminal auxiliary domain restored activity (compare Pip2p-322N and Pip2p-180N in Fig. 6A). Therefore, Oaf1p-181N roughly corresponding to Pip2p-180N was generated. This fusion protein lacked the putative DNA binding domain of Oaf1p and was not able to complement the oleate phenotype of an oaf1Δ deletion (not shown). However, Oaf1p-181N was able to activate a lexAop-lacZ reporter gene. Importantly, its activity was well regulated by oleate. Thus, an auxiliary domain could be assigned to the region between position 181 and 568, and a region necessary for responsiveness to oleate was located between amino acid residues 181 and 776. Since activity mediated by Oaf1p-181N was increased 3-fold compared with full-length Oaf1p under all conditions tested, a second inhibitory region (ID I) was likely to be partially eliminated by deleting the N-terminal 180 amino acid residues of Oaf1p.

**DISCUSSION**

A heterodimer of two fungal zinc cluster transcription factors, Oaf1p and Pip2p, has previously been shown to be necessary for ORE-dependent oleate induction of transcription in the yeast S. cerevisiae (13, 14). Results reported here led to the conclusion that Oaf1p is the sole receptor for the oleate signal. This is supported by the following observations: 1) Oaf1p overproduced in the absence of Pip2p (in a pip2Δ mutant) allows oleate induction of a reporter gene. 2) A LexA-Oaf1p fusion protein clearly mediated induction of a lexA operator-driven reporter gene in an oleate-depndent manner in a pip2☐oaf1Δ double mutant. 3) Neither LexA-Pip2p nor any of the tested LexA-Pip2p deletion constructs exhibited any significant oleate induction. It is therefore at least unlikely that oleate activation of Pip2p was masked by an interaction with the LexA moiety in the respective fusion with full-length Pip2p.

Our results show that oleate induction occurs via the Oaf1p component of the heterodimer. On the other hand, oaf1Δ and pip2Δ mutations have very similar phenotypes on oleate (13, 14). This obviously leads to questions concerning the role of Pip2p. An in vivo footprinting experiment carried out in this study demonstrates that ORE-protein binding is only efficient when an Oaf1p-Pip2p heterodimer can be formed. However, it is clear that Pip2p is more than a simple adapter protein since as a LexA fusion protein it mediates transcriptional activity comparable to that of the respective oleate-activated Oaf1p fusion protein. This strongly suggests that at least after the activation of the heterodimer by oleate, Pip2p contributes significantly to its trans-activation function.

Overexpression of native Pip2p on ethanol did not lead to significant ORE-dependent activation of transcription although its trans-activator potential was comparable on ethanol and oleate in a LexA-Pip2p fusion. It appears that in a Pip2p-Oaf1p heterodimeric complex, Pip2p activity is regulated by oleate in an indirect way. We therefore postulate that Pip2p activity is effectively inhibited by its interaction with Oaf1p unless oleate is present. Convincing evidence exists for Pip2p being complexed to Oaf1p also in the absence of oleate (13, 29). Pip2p present in excess to Oaf1p because of overproduction would not bind to OREs and consequently also not could activate transcription. In contrast, the LexA-Pip2p fusion protein does not require ORE binding and therefore could exhibit activity also in the absence of Oaf1p. Since LexA-Pip2p expressed from the ADH1 promoter would be present in excess to wild type Oaf1p, it is plausible that Oaf1p was not able to inhibit effectively lexAop-lacZ-dependent transcription under these conditions even if it could form dimers with LexA-Pip2p.

The detailed molecular mechanisms of carbon source regulation of activity of Pip2p-Oaf1p heterodimer are not yet clear. Studies carried out here have demonstrated that Pip2p and Oaf1p are remarkably similar not only in their amino acid sequence but also in their domain structure (Fig. 7). ADs were identified in both proteins immediately adjacent to the C terminus, similar to previously identified ADs in other members of the Zn2Cys6 protein family (15). Two inhibitory domains have been assigned to both proteins (ID I and ID II). They appear not involved in receiving any carbon source signal. Similar properties have been assigned to the middle region of other Zn2Cys6 protein family members (30–36). Whereas this led to the proposal of a weakly conserved central homology domain or middle homology region (15, 30, 37), the role of this region in negatively regulating transcriptional activity remains poorly understood. It may be required to mask the AD
via inter- or intramolecular interactions in the absence of inducer, thereby keeping the transcription factor in a locked conformation and ensuring minimal activity.

An auxiliary domain was required for regulated activity of both Pip2p and Oaf1p. In Pip2p, the main glucose-responsive element appears to be part of this domain. In Oaf1p, this region may also possess regulatory functions, since activation by oleate has been shown here to depend on a region between amino acid residues 181 and 776. Given the presence of the auxiliary domain in all active fusion proteins larger than 60 kDa, it may well be that it also contains the nuclear localization signal of the two proteins. It is worth noting that gain of function point mutations in the \( \text{Zn}_{2}\text{Cys}_{6} \) proteins Pdr1p and Pdr3p were reported to be clustered in a short, similarly located region (34, 35). Future work will have to clarify whether the auxiliary domain is directly involved in mediating carbon source regulation or serves as a hinge for interactions of other domains in Oaf1p or Pip2p.

In summary, the data obtained in this study allow us to draw a model of the role of Oaf1p and Pip2p in fatty acid-induced transcription of genes encoding peroxisomal proteins (Fig. 8). On nonfermentable but non-inducing carbon sources such as ethanol, Oaf1p is inactive. The intrinsically derepressed activity of Pip2p is suppressed in the heterodimeric complex with Oaf1p, and OREs therefore remain virtually silent. Since some genes encoding peroxisomal proteins (e.g. \( \text{CTA1} \)) are expressed to a fairly high level under such conditions, it is possible that derepression of peroxisomal functions is accomplished solely by promoter elements other than the ORE including the Adr1p-binding element (2). During specific induction of peroxisome proliferation by fatty acids, Oaf1p becomes activated, which is most likely also linked to an exposure of the Pip2p AD. This allows autoregulatory induction of Pip2p synthesis and the formation of maximal amounts of the heterodimeric transcription factor, which efficiently binds to OREs. The inducing effect of oleate can be overcome by glucose. Several mechanisms clearly contribute to glucose repression of genes encoding peroxisomal functions (8, 38, 39). One further mechanism identified in the course of this study involves a direct inhibition of the activities of Pip2p and Oaf1p. Inactivation of the ORE-binding transcription factor ensures a rapid termination of ORE-dependent transcription and a shift toward glucose utilization whenever this carbon source is available.

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