Mutations at different sites in members of the Gpr1/Fun34/YaaH protein family cause hypersensitivity to acetic acid in *Saccharomyces cerevisiae* as well as in *Yarrowia lipolytica*

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**Keywords**

GPR1; YCR010c; YNR002c; acetic acid stress; *Yarrowia lipolytica*; *Saccharomyces cerevisiae*.

**Abstract**

The Gpr1 protein of the ascomycetous yeast *Yarrowia lipolytica* belongs to the poorly characterized Gpr1/Fun34/YaaH protein family, members of which have thus far only been found in prokaryotes and lower eukaryotes. *Trans*-dominant mutations in the *GPR1* gene result in acetic acid sensitivity of cells at low pH. Moreover, Gpr1p is subjected to phosphorylation at serine-37 in a carbon source-dependent manner. Here we show that several mutations within the ORFs of the GPR1 orthologues of *Saccharomyces cerevisiae*, YCR010c (ATO1) and YNR002c (ATO2), also *trans*-dominantly induce acetic acid hypersensitivity in this yeast. We demonstrate that the C-termini of mutated Gpr1p, Ycr010cp and Ynr002cp are necessary for the triggering of acetic acid sensitivity. Phosphorylation of *Y. lipolytica* Gpr1p was also affected by several mutations. Data further suggest that Gpr1p exists in an oligomeric state.

**Introduction**

The Gpr1 protein of the ascomycetous yeast *Yarrowia lipolytica* is an integral protein of the plasma membrane with six transmembrane domains and hydrophilic N- and C-termini (Augstein et al., 2003). It belongs to a growing group of highly conserved proteins that are mainly present in prokaryotes and lower eukaryotes (mainly fungi) but not in higher multicellular eukaryotes such as vertebrates or plants. Many of these organisms carry several homologues of these proteins. *Yarrowia lipolytica* harbours six *GPR1* homologous genes. So far only one of the *Y. lipolytica* homologues (*GPR1*) and its three orthologues in *Saccharomyces cerevisiae* (YCR010c, YNR002c and YDR384c) are under investigation. The members of the Gpr1/Fun34/YaaH protein family have the motif N-P-[AV]-P-[LF]-G-L-x-[GSA]-F in common. Besides this family signature the hydrophobic transmembrane regions are conserved, whereas major parts of the hydrophilic N- and C-termini are quite heterologous. Prokaryotic orthologues of Gpr1p completely lack the hydrophilic N-terminal part. In many Gpr1p orthologues of several fungi, including two Gpr1p orthologues of *S. cerevisiae* (Ycr010cp and Ynr002cp), the N-terminal motif AFGGTLNPG is highly conserved as well. Gpr1 proteins that have amino acid substitutions within this motif (L65Q [Gpr1-2p], G62S [Gpr1-3p], G63D [Gpr1-4p]) *trans*-dominantly induce hypersensitivity of *Y. lipolytica* cells to protonated acetic acid (Augstein et al., 2003). In addition, one amino acid exchange caused by very low amounts of mutant Gpr1p even when an excess of wild-type Gpr1p is present (Augstein et al., 2003). Acetic acid hypersensitivity is already known to be caused by very low amounts of mutant Gpr1p even when an excess of wild-type Gpr1p is present (Augstein et al., 2003). The deletion of *GPR1*, however, has no effect on acetic acid sensitivity. To explain these data Augstein et al. (2003) suggested that Gpr1p functions as a sensor for acetic acid and that it is involved in the regulation of acetic acid adaptation processes. In the absence of acetic acid an active state of the protein is thought to inhibit acetic acid adaptation processes, whereas the presence of acetic acid would trigger the inactivation of Gpr1p and thereby allow the activation of acetic acid adaptation processes. Furthermore, it has been proposed that the acetic acid inducing mutations of Gpr1p prevent an inactivation of the protein, thus resulting in the permanent inhibition of acetic acid adaptation, even in the presence of acetic acid (Augstein et al., 2003).
We have also shown that Gpr1p as well as Gpr1-1p and Gpr1-2p are subjected to phosphorylation and dephosphorylation at serine-37 in a carbon source-dependent manner (Gentsch & Barth, 2005). Although acetate induces the fast phosphorylation of almost all Gpr1 proteins, no correlation between phosphorylation of Gpr1p and acetic acid sensitivity caused by mutant Gpr1p was found. It is therefore suggested that Gpr1p is also involved in the metabolic adaptation to new carbon sources (Gentsch & Barth, 2005).

The present data on the function of the Gpr1 orthologues in S. cerevisiae, YCR010c (ATO1, ADY2), YNR002c (FUN34, ATO2) and YDR384c (ATO3), are similarly puzzling. Data indicate that Ycr010cp (Ady2p) is essential for the mediated acetate uptake in S. cerevisiae (Paiva et al., 2004). Palková et al. (2002) suggested that the Gpr1p orthologues in S. cerevisiae are ammonia exporters that are expressed in a War1p-dependent manner (Schüller et al., 2003). Furthermore, all three genes are highly induced under carbon-limiting conditions (Boer et al., 2003) and during the stationary phase after growth on YPD medium (Gasch et al., 2000). YNR002c is induced by sorbic acid in a War1p-dependent manner (Schüller et al., 2004). Reduced levels of YDR384c transcripts were found in Δgcn4 cells of S. cerevisiae, while induction of the YDR384c promoter occurs in respiratory deficient ρ0 petite cells (Guaranella & Butow, 2003). Induction of the S. cerevisiae GPR1 orthologues was also altered during diauxic shift, when induction of glyoxylate cycle enzymes occurs (Haurie et al., 2001; Lorenz & Fink, 2001; Tachibana et al., 2005). However, YCR010c, YNR002c and YDR384c were not found among genes that become activated by Haa1p (Keller et al., 2001), a transcription factor that was shown to activate genes that are responsible for adaptation to weak hydrophilic acids such as acetic acid and propionic acid (Fernandes et al., 2005).

In this study we show that mutated GPR1 (GPR1-1 and GPR1-2) but also several mutations within the ORFs of the S. cerevisiae genes YCR010c and YNR002c trans-dominantly induce acetic acid sensitivity of respective S. cerevisiae transformants. The C-terminal parts of Gpr1p, Ycr010cp and Ynr002cp were found to play a crucial role in triggering acetic acid sensitivity. Moreover, the data suggest that Gpr1p may exist in an oligomeric state.

### Materials and methods

#### Strains, media and cultivation

Yarrowia lipolytica and S. cerevisiae strains were grown in complete YPD medium and minimal medium with the following composition: 1.7 g yeast nitrogen base (YNB; Difco) without amino acids, 10 g L⁻¹ glucose, 30 mM acetate or a mixture of both as carbon sources, and if required 20 mg L⁻¹ uracil, 60 mg L⁻¹ leucine, 20 mg L⁻¹ histidine and/or 20 mg mL⁻¹ tryptophane. If acetate was added to glucose-grown cultures the final acetate concentration was also 30 mM. The medium was adjusted to pH 5.5 or in the case of acetic-acid-containing media to pH 4 by HCl. Solid media contained 2% agar. Yarrowia lipolytica PO1d-GR1-2 was obtained after homologous and integrative transformation of PO1dGR1 with the BamHI/HindIII fragment of the plasmid pYLG2 (encodes Gpr1-2p). After selection on plates containing 1.25 mg mL⁻¹ 5-fluoroorotic acid, transformants were tested phenotypically for acetic acid sensitivity and genotypically by Southern blotting.

Strains used are listed in Table 1. Yarrowia lipolytica cells were cultivated at 28°C, S. cerevisiae at 30°C. Batch cultures were shaken at 220 r.p.m. Escherichia coli DH5αC [F80lacZAM15, recA1, endA1, gyrA96, thi-1, hsdR17 (rK- mG+) supE44, relA1, deoRA (lacZYA-arg F') U169] was used as a host strain for all plasmid manipulations. Escherichia coli cells were grown in Luria–Bertani (LB) medium (1% peptone, 0.5% yeast extract and 1% NaCl).

#### DNA manipulations

All basic DNA manipulation procedures were performed according to Sambrook et al. (1989) and Ausubel et al. (1997). Plasmid preparations from E. coli were performed according to standard protocols (Ausubel et al., 1997). PCR reactions were performed with Combizyme DNA polymerase (InViTek) or Pwo polymerase (Roche). Except PacI (New England Biolabs) all restriction endonucleases were purchased from MBI Fermentas. T4-DNA-Ligase was from Promega. DNA sequencing was carried out according to Sanger et al. (1977) using the CEQ™ DTCS-Quick Start Kid

### Table 1. Yeast strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. lipolytica PO1d</td>
<td>MatA leu2-270 ura3-302 xpr2-322</td>
<td>Le Dall et al. (1994)</td>
</tr>
<tr>
<td>Y. lipolytica PO1dΔgr1</td>
<td>MatA leu2-270 ura3-302 xpr2-322Agr1::URA3)</td>
<td>Augstein et al. (2003)</td>
</tr>
<tr>
<td>Y. lipolytica PO1-Gpr1-2</td>
<td>MatA leu2-270 ura3-302 xpr2-322Agr1::GPR1-2</td>
<td>this study</td>
</tr>
<tr>
<td>S. cerevisiae DBY747</td>
<td>MatA, his3-1, leu2-3, ura2-112, trp1-289, ura3-352</td>
<td>ATCC 44774</td>
</tr>
<tr>
<td>S. cerevisiae YN99-72</td>
<td>MATa, his3Δ200, trp1Δ63, ura3-52, YCR010c::KanMX4loxP, YDR384c::KanMX4</td>
<td>EUROFAN II</td>
</tr>
<tr>
<td>S. cerevisiae YN99-89</td>
<td>MATa, his3Δ200, trp1Δ63, ura3-52, YNR002c::KanMX4</td>
<td>EUROFAN II</td>
</tr>
</tbody>
</table>

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from Beckman Coulter and the CEQ 2000XL Sequencer. Transformation of autonomously replicating plasmids into 
E. coli and Y. lipolytica was performed by electroporation (Dower et al., 1988; Barth & Gaillardin, 1996) and according to Schiestl & Gietz (1989) in the case of S. cerevisiae. Yeast transformants were selected on glucose minimal medium lacking leucine (Y. lipolytica) or uracil (S. cerevisiae). For selection of E. coli transformants LB medium with ampicillin (100 mg L\(^{-1}\)) was used. Plasmid re-isolation from S. cerevisiae transformants was performed according to Hoffman & Winston (1987).

**Construction of plasmids**

Basic vectors used for the construction of the plasmids given below are listed in Table 2.

**Plasmids for Y. lipolytica**

Plasmids coding for C-terminally HA-tagged Gpr1p variants were constructed by ligation of PCR amplified 2x HA sequences into the BamHI/SalI sites of the plasmids pTSC1, pTSC2 and pTSC3. The plasmid encoding the Gpr1-green fluorescent protein (GFP) fusion was described previously (Augstein et al., 1993). In the case of S. cerevisiae, Y. lipolytica, S. pombe and Y. lipolytica, the fusion was described previously (Augstein et al., 2003).

The introduction of the Flag tag at the N-terminus of Gpr1p and Gpr1-2p (plasmids pG1-FLAG and pG2-FLAG) was achieved by overlap extension PCR and ligation of the final PCR product into NheI/Eco91I opened pYLG3.

For construction of plasmids coding for C-terminal truncated Gpr1p, part of the GPR1 gene was PCR amplified using reverse primers that inserted a stop codon and a Sal

**Table 2. Plasmids used**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pINA237</td>
<td>Y. lipolytica/E. coli shuttle vector with LEU2 and ARS18/CEN of Y. lipolytica</td>
<td>Fournier et al. (1993)</td>
</tr>
<tr>
<td>pYLG2</td>
<td>GPR1 ORF from strain B204-12C-156 (GPR1-2) under control of GPR1 promoter from Y.</td>
<td>A. Augstein unpublished</td>
</tr>
<tr>
<td></td>
<td>lipolytica PO1d</td>
<td></td>
</tr>
<tr>
<td>pYLG1</td>
<td>GPR1 ORF from strain B204-12C-112 (GPR1-1) under control of GPR1 promoter from Y.</td>
<td>A. Augstein unpublished</td>
</tr>
<tr>
<td></td>
<td>lipolytica PO1d</td>
<td></td>
</tr>
<tr>
<td>YEp351-3HA</td>
<td>Plasmid containing 3x the haemagglutinin epitope</td>
<td>Tzschoppe et al. (2000)</td>
</tr>
<tr>
<td>pYLG3-GFP</td>
<td>pINA237-derived vector containing GPR1 fused to GFP(S65T) under control of GPR1 promoter</td>
<td>Augstein et al. (2003)</td>
</tr>
<tr>
<td>pTSC1</td>
<td>GPR1 gene from strain PO1d fused to lacZ gene of E. coli in pINA237</td>
<td>Augstein et al. (2003)</td>
</tr>
<tr>
<td>pTSC2</td>
<td>ORF from GPR1-2 gene with GPR1 promoter from Y. lipolytica PO1d fused to lacZ gene of E. coli in pINA237 (same backbone as plasmid as pTSC1)</td>
<td>A. Augstein unpublished</td>
</tr>
<tr>
<td>pTSC3</td>
<td>ORF from GPR1-1 gene with GPR1 promoter from Y. lipolytica PO1d fused to lacZ gene of E. coli in pINA237 (same backbone as plasmid as pTSC1)</td>
<td>A. Augstein unpublished</td>
</tr>
<tr>
<td>p426MET25</td>
<td>S. cerevisiae/E. coli shuttle vector with URA3 marker gene that allows protein expression under the MET25 promoter (2 μ plasmid)</td>
<td>Mumberg et al. (1994)</td>
</tr>
</tbody>
</table>
obtained encode Ycr010c-L75Q and Ynr002c-L74Q that contain mutations analogous to that of Gpr1-2p as well as Ycr010c-L259D and Ynr002c-L258D with Gpr1-1p analogous mutations under control of the MET25 promoter. Fragments from these plasmids containing the relevant mutations were also ligated into the plasmids p416YCR and p416YNR to achieve the expression of mutated Ycr010cp and Ynr002cp under control of the authentic YCR010c and YNR002c promoters. Plasmids encoding C-terminally truncated Ycr010c and Ycr010c-L75Q proteins were constructed using a reverse primer that introduced a stop codon and a ClaI site after the codons of tyrosine-267 and alanine-258. The PCR products obtained were ligated into HindIII/ClaI opened p416YCR and p416YCR-L75Q. Plasmids encoding truncated Ynr002cp and Ynr002c-L74Qp with tyrosine-266 or alanine-257, respectively, as C-terminal amino acid were constructed analogously. Overlap extension PCR was applied to construct plasmids that express YNAYA-deleted variants of Ycr010cp and Ynr002cp.

Random mutagenesis of YRC010c, YNR002c and YDR384c

To identify further amino acids within the sequence of Ycr010cp, Ynr002cp and Ydr384cp whose substitution resulted in acetic acid sensitivity, we performed mutagenic PCR (Shafikhani et al., 1997) using Taq-Polymerase (MBI Fermentas) and YCR010c, YNR002c and YDR384c containing plasmids as PCR templates. Amplification of the respective genes was performed in the presence of 0.05, 0.1 and 0.15 mM MnCl2. The PCR products obtained were ligated into the SpeI/ClaI site of the vector p426MET25 and then transformed into E. coli. From each transformation plate, corresponding to different MnCl2 concentrations, 12 E. coli colonies were selected, their plasmids isolated and the insert sequenced. Plasmids were prepared from pooled E. coli cells from that plate where the 12 isolated plasmids contained the most single base pair mutations in their inserts. These plasmids were used for transformation of S. cerevisiae DBY747, YN99-89 and YN99-72. The yeast transformants were cultivated on glucose minimal medium and replica plated onto plates containing acetic acid. Plasmids from transformants showing no or reduced growth on acetic acid minimal medium were isolated and amplified in E. coli. To confirm that the acetic acid sensitivity had been caused indeed by the identified mutations, the respective plasmids were retransformed into S. cerevisiae YN99-72, YN99-89 and DBY747 and the growth of the respective transformants on acetic acid minimal medium was monitored again. To identify mutations within the ORFs of YCR010c and YNR002c, the inserts of plasmids isolated from three E. coli clones were sequenced.

SDS-PAGE and Western blotting

Preparation of whole protein extracts from Y. lipolytica cells and transformants was performed according to Volland et al. (1994) as described previously (Gentsch & Barth, 2005). When further processing of raw cell extracts was required, cells were disrupted with chilled glass beads (0.45 mm) in TSP buffer (100 mM Tris/HCl, pH 7.4, 400 mM sorbitol, one tablet of Roche protease inhibitor cocktail complete per 50 mL). The resulting homogenate was centrifuged at 3000 g for 10 min at 4 °C to remove cell debris and intact cells.

Dephosphorylation of proteins was performed with λ protein phosphatase (λ-PPase, New England Biolabs) as described previously (Gentsch & Barth, 2005). Protein concentrations were determined using the BCA assay (Pierce) following the manufacturer’s instructions, with bovine serum albumin as standard.

Samples were separated in 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels (to separate the phosphorylated from the dephosphorylated form of Gpr1p, 20-cm-long SDS gels were used) and semi-dry-blotted onto PVDF membranes (Millipore) according to standard protocols. Detection of Gpr1p constructs was performed with 1:2000 diluted anti-Gpr1p antiserum (Gentsch & Barth, 2005) and secondary anti-rabbit antibodies (1:10 000 dilution, GE Healthcare) using the ECL-Plus Western blotting detection system (GE Healthcare).

Results

Several mutations in the ORFs of YCR010c and YNR002c as well as Y. lipolytica GPR1-1 and GPR1-2 trans-dominantly induce acetic acid sensitivity of S. cerevisiae transformants

Amino acid exchanges in the C-terminus (Gpr1-1p) and N-terminus (Gpr1-2p) of Gpr1p induce acetic acid sensitivity in Y. lipolytica (Augstein et al., 2003). To investigate whether the mutant forms of Gpr1p can also induce acetic acid sensitivity in S. cerevisiae or may have other effects, plasmids (2 μ) carrying GPR1-1 and GPR1-2 under control of the MET25 promoter were transformed into the S. cerevisiae strains YN99-89 and YN99-72 (a strain with the same genetic background as YN99-89 but deleted for all three GPR1 orthologues). The two strains were also transformed with similar plasmids expressing Ycr010cp and Ynr002cp, the S. cerevisiae orthologues of Gpr1p, containing mutations analogous to those of Gpr1-1p and Gpr1-2p, i. e. substitution of glycine—259 (Ycr010cp) and —258 (Ynr002cp) by aspartate and substitution of leucine —75 (Ycr010cp) and —74 (Ynr002cp) by glutamine. Ydr384cp, the third Gpr1p orthologue in S. cerevisiae, shows no homology to Gpr1p within the regions that are mutated in Gpr1-1p and Gpr1-2p. Growth of transformants was tested on minimal
medium containing acetic acid at pH 4. Interestingly, S. cerevisiae transformants of both strains that expressed mutated Y. lipolytica Gpr1p or mutated S. cerevisiae Ycr010cp and Ynr002cp were unable to grow in the presence of acetic acid whereas transformants expressing the respective wild-type proteins grew normally (Fig. 1). This effect was not due to the overexpression of the mutated constructs triggered by a high plasmid copy number and the strong MET25 promoter as transformants that expressed mutant Ycr010c and Ynr002c proteins under the control of their authentic promoters (on centromeric plasmids) also could not grow on acetic-acid-containing minimal medium (data not shown).

In order to find further sites within the protein sequences of the Gpr1p orthologues in S. cerevisiae whose mutation results in acetic acid sensitivity, the ORFs of all three genes were subjected to random mutagenesis by mutagenic PCR and the respective plasmids were transformed into the S. cerevisiae strains DBY747, YN99-89 and YN99-72. Altogether, 11 transformants expressing mutated Ycr010cp and four transformants expressing mutated Ynr002cp could be found. Nine YCR010c plasmids encoded Ycr010cp with one amino acid substitution (plasmid 1: F212S, plasmid 2: N145D, plasmid 3: E144G, plasmid 4: A88V, plasmid 5: N75D, plasmid 13: G147D, plasmid 14: S265L) whereas mutated Ynr002cp encoded by the other plasmid harboured three amino acid exchanges (plasmid 15: L74P, A87T and M268I). Amino acids whose substitutions resulted in acetic acid sensitivity are highlighted in Fig. 2. All mutations were still present (data not shown). These observations indicate that the identified mutations are acting dominantly. We did not find any acetic acid sensitivity-inducing mutation within the ORF of YDR384c, suggesting that this protein is probably not directly involved in acetic acid adaptation.

The C-termini of mutated Gpr1p, Ycr010cp and Ynr002cp are essential for triggering acetic acid sensitivity

Previous studies revealed that major parts of the hydrophilic N-terminus of Y. lipolytica Gpr1p can be deleted without any effect on acetic acid sensitivity (Augstein et al., 2003). It was of interest to investigate whether the hydrophilic C-terminal part of Gpr1p harbours any functionally important regions. For this purpose the Y. lipolytica strains PO1d and PO1dAgpr1 were transformed with plasmids encoding C-terminally truncated Gpr1p (wild-type), Gpr1p-1p and Gpr1p-2p (the resulting C-terminal amino acid sequences are shown in Fig. 3a).

As shown in Fig. 3, C-terminal truncation of Gpr1p-2p abolished the acetic acid sensitivity-causing effect of full-length Gpr1p-2p when expressed in strain PO1dAgpr1. This indicates that the C-terminal part of the mutated Gpr1p is essential for triggering acetic acid sensitivity. By contrast, PO1d transformants, which additionally harbour a chromosomal copy of wild-type GPR1, remained sensitive to acetic acid when they expressed Gpr1p-1p truncated to tyrosine-256 and alanine-247. These data suggest that the C-terminally untruncated Gpr1p molecules that are also present in strain PO1d are necessary to induce acetic acid sensitivity. We therefore propose that C-terminally truncated Gpr1p-2p
constructs interact with the chromosomally encoded wild-type Gpr1p and that the acetic acid sensitivity-causing effect of Gpr1-2p is transmitted to the intact C-terminus of chromosomally encoded Gpr1p. Truncation of plasmid encoded Gpr1-2p proteins beyond the YNAYA motif (plasmid pG2-F242) or the deletion of this motif itself (plasmid pG2-dYNAYA) restored growth of the respective PO1d transformants on acetic-acid-containing media. This suggests that the YNAYA motif is essential for the proposed interaction. The presence of YNAYA-deleted wild-type Gpr1p does not abolish acetic acid sensitivity when full-length Gpr1-2p is present, because a Y. lipolytica strain expressing chromosomally encoded Gpr1-2p (PO1d-GPR1-2) transformed with pG2-dYNAYA remained sensitive to acetic acid. In addition, PO1d transformants expressing Gpr1-1p truncated to tyrosine-256 and aspartate-248

Fig. 2. Alignment of the protein sequences of Yarrowia lipolytica Gpr1p and the Saccharomyces cerevisiae proteins Ycr010cp and Ynr002cp. Amino acids that are identical in all three proteins are in bold. Amino acids whose substitution resulted in acetic acid sensitivity are white on black background. The family motif NPAPL Gel and the YNAYA motif are highlighted on a grey background. Sequences that form putative transmembrane domains according to the HMMTOP program (Tusnady Simon, 1998, 2001) are underlined.

Fig. 3. (a) C-terminal sequences of truncated Gpr1p encoded by the corresponding plasmids. ‘pG1’ plasmids code for truncated wild-type Gpr1p, ‘pG2’ plasmids for truncated Gpr1-2p and ‘pG3’ plasmids for truncated Gpr1-1p. (b) Growth of PO1d and PO1dΔgpr1 transformants expressing C-terminally truncated wild-type Gpr1p and Gpr1-2p, respectively. In total, 3 × 10⁴ cells of PO1d and PO1dΔgpr1 transformants harbouring the stated plasmids were dropped onto plates with minimal medium and glucose or acetic acid as carbon sources. Cells were grown for 48 h at 28 °C.

Gpr1p -----MNTEI PDLKKQIDH NESD-----SD DPQIHDSDMA PVSRISSGP NEYIHIADQ 50
Ycr010cp MSDKQETSON TDELAPANPY YS8DHNDYVG VAEDERPSHD SLGKTYGEGD NNEYIYGRQ 60
Ynr002cp MSDKQETSON TDELAPANPY YS8DHNDYVG VAEDERPSHD SLGKTYGEGD NNEYIYGRQ 59

Gpr1p KFHRDFFYRA FGGTLPNGGA FPSRKKFNP APLGLSFAAL TTVLFSLCYT QARGVNP 110
Ycr010cp KFLKSDLYQA FGGTLPNGGA FPSRKKFNP APLGLSFAAL TTVLFSLCYT QARGVNP 120
Ynr002cp KFLRDDLFFEA FGGTLPNGGA FPSRKKFNP APLGLSFAAL TTVLFSLCYT QARGVNP 119

Gpr1p AVGALAPFGG VCGPAAGME WFGQNTFGIA ALTSGGFWLM SPFAAAYIPF VIGLLEAYEDE 180
Ycr010cp VGGCAMFGG LVQLIAGISE ILATENTFGTG ALCSYGGFWLM SPFAAAYIPF VIGLLEAYEDE 179
Ynr002cp VGGCAMFGG LVQLIAGISE ILATENTFGTG ALCSYGGFWLM SPFAAAYIPF VIGLLEAYEDE 178

Gpr1p IEVQNANPAE LFGLFIIITLM LTLCTLKKPA AFFGLFFMLM MLTFLVACAN VTNQHTGTAIG 229
Ycr010cp SDLNASLGYF LLGWAIFTPFL LTVCJOINSTV RYFLLSSFLA LTFLLSLSHG FANLQVTRA 240
Ynr002cp SDLNASALGFY LGWAIFTPFL LSVCTNIGHT MFFALFFFLA VTFLLSLSFIAN FTGVEGVTRA 239

Gpr1p GGNLGIITAP FGYNAYAGL ANPAGNSYVPA VPALMPFVKK D-- 270
Ycr010cp GGVGLVYAVF IAYNAYAGLY ATKNSYVLA RFPFLPSTER VIF 283
Ynr002cp GGVGLVYAVF IAYNAYAGLY ATKNSYVLA RFPFLPSTREAM HPFALPNDK VPF 282
(mutation of \textit{GPR1-1} results in the exchange of glycine-248 to aspartate) were sensitive to acetic acid whereas the respective PO1d\textregistered gpr1 transformants were not (data not shown).

The fusion of additional amino acids to the C-terminus of Gpr1p had similar effects on the growth of \textit{Y. lipolytica} transformants as C-terminal truncations. While C-terminally 2 × HA- or GFP-fused Gpr1-1p and Gpr1-2p still triggered acetic acid sensitivity of the respective PO1d transformants, the respective transformants of the \textit{GPR1} deletion strain PO1d\textregistered agpr1 were able grow on acetic-acid-containing media (Table 3).

We later investigated whether the C-terminal truncation of mutated Ycr010cp and Ynr002cp also abolished acetic acid sensitivity of the respective \textit{S. cerevisiae} transformants. For this purpose \textit{S. cerevisiae} strains YN99-89 and YN99-72 were transformed with plasmids encoding wild-type and mutated (L75Q) Ycr010c proteins that were C-terminally truncated to tyrosine-267 or alanine-258 as well as wild-type and mutated (L74Q) Ynr002c proteins that were C-terminally truncated to tyrosine-266 and alanine-257, respectively. In addition, plasmids encoding YNAYA-deleted wild-type and mutated Ycr010c and Ynr002c proteins were used for transformation. Growth tests on acetic-acid-containing media revealed that all of these transformants except those expressing undeleted Ycr010c-L75Qp and Ynr002c-L74Qp grew normally on acetic acid at low pH (data not shown). Thus, C-terminal truncations or the sole deletion of the YNAYA motif abolished the acetic acid sensitivity-causing effect of mutant Ycr010c and Ynr002c proteins. In contrast to chromosomally encoded Gpr1p (band shift), but did not

Western blot analysis also revealed that all C-terminally truncated proteins were not subjected to phosphorylation at serine-37. In contrast to chromosomally encoded Gpr1p no shift of the corresponding bands was observed after addition of acetate (Fig. 4a). Furthermore, λ protein phosphatase (λ-PPase) treatment resulted in the dephosphorylation of chromosomally encoded Gpr1p (band shift), except for pG2-A247-encoded proteins that induced acetic acid sensitivity in PO1d transformants (Fig. 4a).

C-terminal deletions affect Gpr1p phosphorylation at serine-37

Western blot analysis showed that acetic acid tolerance of all PO1d\textregistered agpr1 transformants expressing C-terminally truncated Gpr1-2p and of PO1d transformants expressing YNAYA-deleted Gpr1-2 proteins was not due to a failed expression or fast degradation of these proteins (Fig. 4a). Instead, pG2-F242- and pG2-dYNAYA-encoded proteins that did not trigger acetic acid sensitivity gave even stronger bands in Western blots than pG2-A247-encoded proteins that induced acetic acid sensitivity in PO1d transformants (Fig. 4a).

Western blot analysis also revealed that all C-terminally truncated proteins were not subjected to phosphorylation at serine-37. In contrast to chromosomally encoded Gpr1p no shift of the corresponding bands was observed after addition of acetate (Fig. 4a). Furthermore, λ protein phosphatase (λ-PPase) treatment resulted in the dephosphorylation of chromosomally encoded Gpr1p (band shift), but did not cause a band shift in the case of C-terminally truncated Gpr1p constructs (Fig. 4b, and data not shown).

Acetate-triggered phosphorylation of Gpr1p at serine-37 was also impaired by a C-terminal HA tag. Only a part of the

Table 3. Growth of \textit{Yarrowia lipolytica} PO1d and PO1d\textregistered agpr1 transformants expressing tagged Gpr1p constructs on acetic-acid-containing media

<table>
<thead>
<tr>
<th>Transformed plasmid</th>
<th>Encoded protein</th>
<th>Growth on acetic-acid-containing media</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYLG3</td>
<td>Gpr1p</td>
<td>+</td>
</tr>
<tr>
<td>pYLG2</td>
<td>Gpr1-2p</td>
<td>–</td>
</tr>
<tr>
<td>pTSC1-HA</td>
<td>Gpr1p C-terminally fused with 2xHA</td>
<td>+</td>
</tr>
<tr>
<td>pTSC2-HA</td>
<td>Gpr1-2p C-terminally fused with 2xHA</td>
<td>–</td>
</tr>
<tr>
<td>pTSC3-HA</td>
<td>Gpr1-1p C-terminally fused with 2xHA</td>
<td>–</td>
</tr>
<tr>
<td>pYLG3-GFP</td>
<td>Gpr1p C-terminally fused with GFP</td>
<td>+</td>
</tr>
<tr>
<td>pYLG2-GFP</td>
<td>Gpr1-2p C-terminally fused with GFP</td>
<td>–</td>
</tr>
<tr>
<td>pG1-FLAG</td>
<td>Gpr1p N-terminally fused with the Flag tag</td>
<td>+</td>
</tr>
<tr>
<td>pG2-FLAG</td>
<td>Gpr1-2p N-terminally fused with the Flag tag</td>
<td>–</td>
</tr>
</tbody>
</table>

Transformants were cultivated for 24h on minimal medium plates with glucose before they were replica plated onto plates with acetic acid, glucose/ acetic acid and glucose. Growth on acetic-acid-containing media is marked by ‘+’ for normal growth, ‘(+)’ for reduced growth, and ‘–’ for no growth.
HA fusion proteins became phosphorylated after acetate addition and not all molecules, as observed for untagged Gpr1p (data not shown).

Other mutations within Gpr1p and their effect on acetic acid sensitivity and phosphorylation of Gpr1p

We also looked for other regions of Gpr1p that may affect acetic acid sensitivity of respective transformants or phosphorylation of the Gpr1 protein. Deletion of the NPAPLGL motif, which is present in all proteins of the Gpr1/Fun34/YaaH protein family, abolished the acetic acid sensitivity-causing effect of the mutations of Gpr1-1p and Gpr1-2p. There was no effect on acetic acid sensitivity when wild-type Gpr1p was deleted for NPAPLGL. NPAPLGL-deleted Gpr1 proteins were detected at lower amounts compared with wild-type Gpr1p and they were only present in their dephosphorylated form (Fig. 4c). Neither addition of acetate (Fig. 4c) nor λ-PPase treatment (data not shown) had an effect on the electrophoretic mobility of the protein. However, we propose that the lower amount of these constructs alone is not responsible for the abolishment of acetic acid sensitivity as very low amounts of mutant Gpr1p were shown to induce acetic acid sensitivity (Augstein et al., 2003). Additionally, pG2-A247-encoded proteins that were hardly detectable by Western blotting (Fig 4a) induced acetic acid sensitivity. However, NPAPLGL-deleted proteins could become inactivated or dislocated.

Furthermore, we looked for effects triggered by mutations of several putative phosphorylation sites. The mutation of the highly conserved threonine-194, serine-197 and threonine-198 residues. Growth of PO1d and PO1dΔgpr1 transformants expressing respective proteins on acetic-acid-containing media is also marked in (c) and (d) by ‘+’ for regular growth, ‘+/−’ for reduced growth by and ‘−’ for no growth.

Fig. 4. Western blot analysis of Yarrowia lipolytica transformants expressing mutated Gpr1p constructs. Each lane was loaded with equal amounts of total cellular protein prepared from cells harvested after growth in glucose minimal medium (lanes G) and 30 min after addition of acetate to these cultures (lanes A). Protein extracts from PO1dΔgpr1 with pYLG3 (encodes Gpr1p) and pINA237 (empty control vector) served as positive (+) and negative (−) controls. (a, b) Analysis of protein extracts from PO1d (P) and PO1dΔgpr1 (Δ) transformants that have been transformed with stated plasmids encoding C-terminally truncated Gpr1-2p constructs. λ-PPase treatment of protein extract from pG2-Y256 containing transformants that were harvested 30 min after acetate addition (lanes A + P) resulted in dephosphorylation of chromosomally encoded phosphorylated Gpr1p while the electrophoretic mobility of pG2-Y256-encoded Gpr1p constructs remained unchanged (b). Specific bands are marked by arrows as follows: arrows 1 and 2: phosphorylated and dephosphorylated chromosomally encoded Gpr1p; arrows 3, 4, 5 and 6: pG2-Y256-, pG2-A247-, pG2-F242- and pG2-dYNAYA-encoded Gpr1p constructs, respectively. (c) Western blot analysis of cell extracts from PO1dΔgpr1 transformants with plasmids encoding Gpr1p with substituted threonine-194, serine-197 and threonine-198 residues. Growth of PO1d and PO1dΔgpr1 transformants expressing respective proteins on acetic-acid-containing media is also marked in (c) and (d) by ‘+’ for regular growth, ‘+/−’ for reduced growth by and ‘−’ for no growth.

HA fusion proteins became phosphorylated after acetate addition and not all molecules, as observed for untagged Gpr1p (data not shown).
as threonine-194, induced acetic acid sensitivity of the respective Y. lipolytica transformants. In contrast to Gpr1p-T194D, these proteins were detectable by Western blotting (Fig. 4d). We assume that the whole motif containing threonine-194, serine-197 and threonine-198 is important for the function of Gpr1p and might not tolerate structural changes. This motif forms a hydrophilic loop between the fourth and fifth transmembrane domain and could therefore be involved in the interaction with other proteins.

In contrast to C-terminally tagged Gpr1p-2p the N-terminal tagging of Gpr1p-2p with the Flag epitope did not influence acetic acid sensitivity in PO1d and PO1d Δgpr1 transformants (Table 3). In addition, N-terminal fusions had no impact on phosphorylation of Gpr1p at serine-37 after acetate addition (Fig. 5).

The fact that N-terminally fused constructs migrated more slowly than wild-type Gpr1p in SDS gels also implies that N-terminal processing does not take place in the case of the Gpr1 protein.

**Discussion**

In this study we show that several mutations within the ORFs of the GPR1 orthologues of S. cerevisiae YCR010c and YNR002c as well as Y. lipolytica GPR1-1 and GPR1-2 trans-dominantly induce acetic acid sensitivity in respective S. cerevisiae transformants. The C-termini of the mutated proteins seem to be responsible for conferring acetic acid sensitivity.

Our data showing that mutated Gpr1p as well as mutated Ycr010cp and Ynr002cp induce acetic acid sensitivity in S. cerevisiae imply that these proteins have similar functions in Y. lipolytica and S. cerevisiae. Like mutations in Y. lipolytica GPR1 (Tzschoppe et al., 1999), all mutations found within YCR010c and YNR002c are acting trans-dominantly. Deletion of all three GPR1 orthologues did not lead to acetic acid sensitivity of the resulting S. cerevisiae strain YN99-72. YCR010c, YNR002c and YDR384c were not found among genes activated by Haa1p, a transcription factor that was shown to activate genes involved in adaptation to less hydrophilic acids such as acetic acid (Keller et al., 2001).

Based on these earlier observations and our own data we propose that Gpr1p as well as Ycr010cp and Ynr002cp are not directly involved in the protection of cells against acetic acid but instead suppress acetic acid adaptation in the absence of acetic acid. As already hypothesized by Augstein et al. (2003), the observed mutations are thought to prevent a deactivation of these proteins, which is thought to occur in the presence of acetic acid, resulting in a permanent inhibition of acetic acid adaptation processes. However, the molecular mechanisms underlying these processes are not yet understood.

Several residues of Ycr010cp and Ynr002cp whose substitution resulted in acetic acid sensitivity were found within the N-terminal motif AFGGTLNPG. In addition, three out of four originally analysed Y. lipolytica GPR1 mutant strains contain mutations within this motif (Augstein et al., 2003), indicating that this region is important for the function of Gpr1p and its two S. cerevisiae orthologues. Other amino acid residues whose substitution resulted in acetic acid sensitivity are located between the second and third and between the fourth and fifth transmembrane domains and within the C-terminus of Ycr010cp and Ynr002cp (Fig. 3). Assuming that Gpr1p, Ycr010cp and Ynr002cp have six transmembrane domains, all those residues are located at the same site of the plasma membrane. It might be hypothesized that these residues form a three-dimensional structure whose integrity is essential for an interaction with a ligand or other proteins and that certain mutations within these sites disturb this interaction. It is, however, not known whether they are located at the extra- or at the intracellular site of the plasma membrane. Only for Ynr002cp an extra-cellular localization of the C-terminus has been suggested by experimental data (Kim et al., 2003). But because that study relied on experiments with C-terminally modified Ynr002cp it is questionable whether it reflects the correct topology of Ynr002cp as C-terminal modifications (truncations and fusions) of Gpr1p and its two S. cerevisiae orthologues seem to impair the function of these proteins. Thus, the topology might also be affected.

A particular role for the function of Gpr1p, Ycr010cp and Ynr002cp seems to be dedicated to their hydrophilic C-termini, as C-terminal deletions abolished the acetic acid sensitivity-causing effect of mutated Ycr010cp and Ynr002cp in S. cerevisiae and mutated Gpr1p in Y. lipolytica PO1d Δgpr1 transformants. C-terminal tagging of mutant Gpr1p had similar effects as C-terminal truncations. These data suggest that the C-termini of these proteins are...
Acetic acid hypersensitivity in \textit{S. cerevisiae} and \textit{Y. lipolytica}

According to the previously proposed model for the function of Gpr1p (Augstein et al., 2003) it could thus be assumed that the hydrophilic C-terminal parts of Ycr010cp, Ynr002cp and Gpr1p mediate the inactivation of acetic acid adaptation processes. The deletion of C-terminal parts or C-terminal fusion might affect interactions of Gpr1p and its orthologues with other proteins that are part of a signalling cascade that inhibits acetic acid adaptation processes. However, transformants that coexpressed wild-type Gpr1p and mutated Gpr1p C-terminally truncated up to alanine-247 acetic acid were sensitive to acetic acid, suggesting an interaction between wild-type and C-terminally truncated Gpr1p. We therefore conclude that Gpr1p might generally interact with itself and forms di- or oligomers. As mutant Gpr1p truncated beyond the YNAYA motif or deleted for this motif was unable to induce acetic acid sensitivity even in the presence of wild-type Gpr1p we further assume that the YNAYA motif might be essential for this di- or oligomerization.

Furthermore, data also suggest that Gpr1p cannot be functionally substituted by one of the five other Gpr1p homologues that exist in \textit{Y. lipolytica}. If this was the case, respective POI1\Delta gpr1p transformants should also be sensitive to acetic acid because the acetic acid sensitivity-causing effect of C-terminally truncated or tagged Gpr1p-2p could be mediated by one of these Gpr1p homologues.

As recently reported, acetate induced a fast phosphorylation of almost all Gpr1p molecules at serine-37 (Gentsch & Barth, 2005). However, C-terminally truncated Gpr1 proteins were not subjected to this phosphorylation after acetate addition to the culture medium and the phosphorylation of C-terminally tagged Gpr1p was also impaired. In addition, the mutation of Gpr1-1p that is characterized by an amino acid exchange in the C-terminal part of the protein hampers phosphorylation (Gentsch & Barth, 2005). A possible explanation for this effect could be that the kinase that phosphorylates Gpr1p recognizes the C-terminus although phosphorylation takes place at the N-terminal part, and that alterations within the C-terminus prevent proper binding of this kinase. It is not yet clear how phosphorylation of chromosomally encoded wild-type Gpr1p was hindered in POI1 transformants expressing pG1/2-Y256-encoded proteins.

N-terminally fused Gpr1p constructs migrated at a lower rate in SDS gels than nonfused Gpr1p. This implies that Gpr1p does not undergo an N-terminal processing, because if this was the case, after the cleavage of a signal sequence the final size of N-terminally tagged Gpr1p should be equal to the size of untagged Gpr1p. Furthermore, an N-terminal signal sequence is not suggested for Gpr1p by the SignalP and TargetP program (Emanuelsson et al., 2000; Bendtsen et al., 2004a). We therefore assume that Gpr1p enters the nonclassical secretory pathway for localization to the cytoplasmic membrane, as also suggested by the SecretomeP program (Bendtsen et al., 2004b).

In contrast to C-terminal tags and truncations, N-terminal fusions seem to have no impact on the function of Gpr1p.

All amino acids whose substitution or deletion altered acetic acid sensitivity are located within conserved regions of Gpr1p and its \textit{S. cerevisiae} orthologues, suggesting that other members of the Gpr1/Fun35/\textit{yaaH} protein family might be involved in acetic acid adaptation in the respective organisms.

\textbf{References}


