Yeast genes YOL002C and SUL1 are involved in neomycin resistance

Laboratório de Bioquímica e Fisiologia de Microrganismos, Núcleo de Pesquisa em Ciências Biológicas, Universidade Federal de Ouro Preto, CEP 35.400.000, Ouro Preto, MG, Brazil
*Author for correspondence: Tel./Fax: +31 35591680, E-mail: imcastro@cpd.ufop.br

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Summary

In previous studies we suggested the importance of the control of plasma membrane H⁺-ATPase by a phosphatidylinositol-like pathway for cellular proton extrusion in Saccharomyces cerevisiae (Brandão et al. 1994; Cocetti et al. 1998). The observations that provided the model above include the inhibition of the glucose-induced activation of the plasma membrane H⁺-ATPase as well as the inhibition of the glucose-induced external acidification by neomycin, a known inhibitor of the phosphatidylinositol turnover in eukaryotic cells. In this work, using two libraries, we isolated two yeast clones that were able to prevent the inhibition of glucose-induced activation of the H⁺-ATPase by neomycin. We show that the YOL002C gene, which encodes a protein of unknown function, and the SUL1 gene, which is a sulphate transporter belonging to the major facilitator superfamily, suppress growth inhibition by neomycin. However, they are not required for glucose-induced activation of the plasma membrane H⁺-ATPase. The resistance of the clones to neomycin is probably related to the level and/or activity of proteins functioning as drug extrusion pumps.

Introduction

The plasma membrane H⁺-ATPase of yeast is a proton pump that plays an essential role in the physiology of this organism by maintaining the transmembrane electrochemical proton gradient necessary for nutrient uptake and by regulating the intracellular pH (Serrano et al. 1986). Addition of glucose or related fermentable sugars to yeast grown on nonfermentable carbon sources is known to trigger an extensive series of regulatory effects (Thevelein 1991). Short term effects include rapid activation of plasma membrane H⁺-ATPase (Sychorová & Kotyk 1985) and cellular proton extrusion (Sigler & Hofer 1991; Laphatits & Kotyk 1998). The identity of the signalling pathway involved in glucose-induced H⁺-ATPase activation remains unclear. A suggestion that cAMP synthesis would play a specific role in glucose-induced activation of H⁺-ATPase activity (Portillo & Mazón 1986; Ulaszewski et al. 1989) has been contradicted (Becher dos Passos et al. 1992; Brandão et al. 1994). Previous studies suggested that yeast cells might contain a similar phosphatidylinositol (PI) pathway as is present in mammalian cells and that glucose activation of plasma membrane H⁺-ATPase is a consequence of glucose stimulation of PI turnover (Brandão et al. 1994; Cocetti et al. 1998; Tanaka et al. 2000). Neomycin, a known inhibitor of this pathway, prevents glucose activation of the H⁺-ATPase. The predicted amino acid sequence of PMA1 gene product does not show a consensus sequence for cAMP-dependent protein phosphorylation (Serrano 1989). However, there are sites for other protein kinases (Kolarov et al. 1988).

In this report, we have transformed the SP1 strain with two different yeast genomic DNA libraries and we selected neomycin-resistant transformants. We have studied the glucose-induced activation of the H⁺-ATPase and cellular proton extrusion in these transformants and we compared the effects to those in the wild-type strain. We have found that the YOL002C or SUL1 genes, which when overexpressed in a wild-type strain reduce the growth inhibition by neomycin, are not necessary for glucose-induced proton extrusion, suggesting that the gene products are primarily acting as on drug extrusion pumps and not as components of the hypothetical glucose-induced PI pathway controlling the H⁺-ATPase.

Selection of transformants

The Saccharomyces cerevisiae strain SP1 (MATa leu2 his3 trp1 ade8 can1 ura3) was transformed with two different S. cerevisiae genomic DNA libraries in the multicopy
tively, showed glucose-induced H⁺-ATPase activation (Figure 1) and more pronounced levels of glucose-induced proton extrusion even in the presence of 20 mM of neomycin when compared to the wild-type strain SP1 (Table 1).

Cloning and deletion of the YOL002C and SULI genes

Plasmid DNAs were rescued from yeast transformants (Del Sal et al. 1988) and amplified in Escherichia coli TOP10F: F⁺, merA, (mvr-bsdRMS-merBC), 800MlacA-M15, lacX74, deoR, recA1, araD139, A(araM,leu)1, galU, galK, ñ-3, rsiP, endA1, napG. Restriction analysis and Southern blotting analysis (DIG DNA Labeling and Detection Kit – Boehringer Mannheim) of these clones, using internal probes, revealed that two pairs of clones contained the same insert.

Suitable restriction fragments of the clone 162C08, responsible for suppression of growth inhibition by neomycin, were subcloned into M13mp19 and sequenced by the chain termination method, using T7 DNA polymerase. The clone 162C08 contains an open reading frame which is 2775 bp in length and homologous to SULI and also part (500 bp) of the gene YBR 293W. The SULI gene has been characterized from Neurospora crassa (Ketter et al. 1991), rat hepatocyte (Bissig et al. 1994) and S. cerevisiae (Smith et al. 1995). In S. cerevisiae, SULI encodes a high affinity sulphate transporter. The 3.3 kb fragment XhoI–XbaI, containing the SULI gene, was cloned into a vector pYEPlac195, cut with HindIII–XbaI and ligated with HindIII–Xba I of pSK⁺ (Stratagene). This construction was cut with EcoRV and one fragment of 2.0 kb was replaced by a SspI fragment of URA3. The deleted gene was cloned into a pYPlac128 vector. Integration of the SULI::URA3 construct (Ito et al. 1983) was confirmed by Southern blot analysis (Sambrook et al. 1989), using a radioactively labelled probe with α²³P dCTP and high prime of Boehringer Mannheim Company.

Another clone, 152B17, which suppresses the phenotype of neomycin inhibition contained a fragment of 984 bp, which codes for YOL002C, and another fragment that codes for the PHO80 gene. After subcloning, we concluded that YOOL002C gene, but not PHO80, was involved in neomycin resistance. The gene

Table 1. Estimated $V_{max}$ (mmol h⁻¹ g⁻¹ cell dry wt) of proton extrusion in wild-type, neomycin-resistant transformants and the respective deletion mutants, after 100 mM glucose addition.

<table>
<thead>
<tr>
<th>Strains →</th>
<th>Wild-type SP1 (n = 3)</th>
<th>SP1 + clone 162C08 (SULI) (n = 3)</th>
<th>SP1 + clone 152B17 (YOL002C) (n = 3)</th>
<th>SULI A (n = 3)</th>
<th>YOL002C A (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM glucose</td>
<td>0.98 ± 0.06</td>
<td>1.67 ± 0.07</td>
<td>1.21 ± 0.10</td>
<td>1.38 ± 0.11</td>
<td>1.33 ± 0.08</td>
</tr>
<tr>
<td>100 mM glucose</td>
<td>0.76 ± 0.05</td>
<td>1.32 ± 0.11</td>
<td>0.85 ± 0.03</td>
<td>0.88 ± 0.06</td>
<td>0.80 ± 0.02</td>
</tr>
<tr>
<td>+ 10 mM neomycin</td>
<td>0.36 ± 0.10</td>
<td>0.84 ± 0.05</td>
<td>0.69 ± 0.02</td>
<td>0.74 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>100 mM glucose</td>
<td>0.36 ± 0.10</td>
<td>1.37 ± 0.06</td>
<td>0.84 ± 0.05</td>
<td>0.69 ± 0.02</td>
<td>0.74 ± 0.04</td>
</tr>
</tbody>
</table>

Figure 1. Glucose-induced activation of plasma membrane 11⁺-ATPase in the presence of 20 mM of neomycin: (çı) wild-type strain SP1, ( ● ) SP1 with YEP13 vector, ( □ ) SP1 with YRP 7 vector, ( ▲ ) SP1 with clone 162C08, ( ■ ) SP1 with clone 152B17.
YOL002C was disrupted after linearization and integrative transformation of the fragment cloned in pYIPPlac128, into the SP1 strain. The YOL002C transcript is highly expressed in presence of glucose and is expressed at lower levels in cells grown in glycerol or glycerol plus oleate (Karpichev & Small 1998).

Phenotypic tests

To study the phenotype of the strains with the disrupted SUL1 and YOL002C genes, the mutants were grown in YP-glycerol medium. Table 1 shows the glucose-induced extracellular acidification for the wild-type, transformants and deletion mutants. The levels of glucose-induced proton extrusion were higher in transformant strains in the absence of neomycin. The proton extrusion was inhibited by neomycin in wild-type as well in deletion mutants. Since the plasma membrane H⁺-ATPase is the major system responsible for cellular proton extrusion in yeast and fungi in general (Sigler & Hofer 1991), our data suggest that YOL002C and SUL1 are not related to H⁺-ATPase activation.

Figure 2 shows that overexpression of these genes increases neomycin resistance, as demonstrated in the 10⁻¹ serial dilutions for clone 162C08, which expresses several copies of the SUL1 gene, and clone 152B17, which expresses only one extra copy of the YOL002C gene. Deletion of YOL002C gene resulted in a phenotype similar to the wild-type strain. Curiously, however, deletion of SUL1 gene generated a strain that presented a phenotype characterized by an intermediate resistance to the drug as compared to the wild-type and the overexpressing clone. It thus seems that the mechanisms involved in growth inhibition by neomycin and H⁺-ATPase activation, and consequently proton extrusion are completely independent, and that apparently SUL1 and YOL002C are only important for neomycin resistance.

In conclusion, this study provides new information about the function of YOL002C: it is involved in drug resistance. Even though we cannot offer, at this time, an explanation for the resistance to neomycin presented by SUL1 deleted strain, our results clearly show that overexpression of SUL1 or YOL002C in a wild-type strain of S. cerevisiae resulted in better growth in medium containing neomycin. Furthermore, our results with the mutants in which the YOL002C or SUL1 genes were disrupted eliminate the possibility that these genes are involved in glucose-induced H⁺-ATPase activation. The proton extrusion did not change compared with the results presented by the wild-type strain. Thus, the mechanism of resistance can be explained by amplification of the levels or enhancement of the activity of membrane proteins which function as drug extrusion pumps. For both genes, the hydropathy profile clearly shows multiple hydrophobic regions which are characteristic of membrane proteins. Topology predictions indicate several putative transmembrane regions for these genes: 12 in SUL1 and 7 in YOL002C. In microorganisms, one of the strategies used to resist toxic compounds is the overproduction of broad specificity transporters, which extrude drugs out of cells. In S. cerevisiae, a network of more than a dozen genes is involved in the multidrug resistance phenotype (Kolaczkowski et al. 1998). These pumps belong either to the ATP-binding cassette (ABC), MDR transporters or to the major facilitator superfamilies (MFS). The ABC-MDR transporters mediate resistance to most classes of clinically and agriculturally important fung-

![Figure 2](image-url)
cides and also to many antibiotics, herbicides and other

toxic compounds.

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