Biogenesis of Candida albicans Can1 permease expressed in Saccharomyces cerevisiae

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Abstract The Candida albicans CAN1 gene, encoding a high-affinity permease for arginine, lysine and histidine, was tagged at its C-terminus with a c-myc epitope and introduced into strains of Saccharomyces cerevisiae lacking basic amino acid permeases. The expression levels of Ca-Can1p were influenced by the available nitrogen source, being almost negligible when cells were grown in the presence of ammonia. Ca-Can1p was shown to follow the secretory pathway in S. cerevisiae. Ca-Can1p activity was not detected in a secretion-defective sec1 mutant grown at a non-permissive temperature. Shr3p, an ER protein that participates in the biogenesis of amino acid permeases was also required for the functional expression of Ca-Can1p. The shr3 mutation does not affect the affinity for substrate but does decrease the number of Can1p molecules reaching the plasma membrane.

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Key words: Basic amino acid permease; Secretory pathway; Heterologous expression; (Candida albicans); (Saccharomyces cerevisiae)

1. Introduction

The amino acid permease Can1 from Candida albicans (Ca-Can1p) can be functionally expressed in Saccharomyces cerevisiae [1]. Ca-Can1p actively transports lysine, arginine and histidine across the plasma membrane [1,2]. Its protein sequence (571 amino acids) is strongly homologous to two permeases specific for basic amino acids of S. cerevisiae, Sc-Can1p (57.3% identity, 75.5% similarity) and Sc-Lyplp (49.7% identity, 73.3% similarity) and to the lysine-specific permease LysP of Escherichia coli [3]. The Ca-Can1p differs from Sc-Can1p in its substrate specificity and affinity to substrates: Ca-Can1p transports all three basic amino acids with high affinity \( K_{T,Arg} = 18 \mu M, K_{T,Lys} = 12 \mu M, K_{T,His} = 37 \mu M \) [4] whereas Sc-Can1p is specific for arginine and lysine [4] and prefers arginine to lysine \( K_{T,Arg} = 10 \mu M, K_{T,Lys} = 180 \mu M \) [5].

In S. cerevisiae, secreted and plasma membrane proteins reach their destination via the secretory pathway, i.e. they move in transport vesicles from the endoplasmic reticulum (ER) where they are synthesized, to the Golgi and finally to the plasma membrane. Many genes encoding components participating in the sorting, post-translational modification and intracellular transport through the secretory pathway have been characterized [6]. Mutations in these secretory (sec) genes exhibit phenotypes of a general nature; they are non-specific and affect a great number of different exported proteins. Recently, the existence of a specific transport molecular chaperone in the secretory pathway has been discovered. This non-essential integral ER membrane protein, Shr3p, is necessary for the in vivo localization of amino acid permeases to the plasma membrane of S. cerevisiae [7]. Shr3p is required for the packaging of amino acid permeases into ER derived COP11 transport vesicles [8].

Using S. cerevisiae strains defective in the uptake of basic amino acids, we show that the functional heterologous expression of C. albicans Can1 permease depends upon an operational secretory pathway and requires the Shr3 chaperone.

2. Materials and methods

2.1. Strains, plasmids, media and cultivation

S. cerevisiae strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Two types of minimal media were used: YNB-NH4 consisted of the yeast nitrogen base w/o amino acids (6.7 g/l) and 2% glucose; YNB-Pro was prepared from yeast nitrogen base w/o amino acids and ammonium sulphate (1.7 g/l) supplemented with proline (1 g/l) as a source of nitrogen, and 2% glucose. Cells were cultivated aerobically at 30°C unless otherwise specified.

2.2. Disruption of the SHR3 gene

The strain HS 100-3C was transformed with a linear EcoRI-SauI fragment of DNA derived from pPL288 containing shr3A::hisG-URA3-hisG [9,8] to create AM 8. AM 8 was propagated on media containing 5-fluoro-orotic acid [10] to attain the unmarked deletion of shr3A resulting in AM 8-1. Correct disruption and deletion were checked by Southern blots. The 650 bp fragment PstI-ClaI containing a part of the SHR3 gene (410 bp) was excised from pPL210 [7], labelled with digoxigenin and used as a probe for Southern blots.

2.3. Transport measurements

The uptake of amino acids was measured as described previously using \(^{14}C\)-labelled amino acids [11]. In all experiments, cells from early exponential phase of growth were used (OD\textsubscript{600} = 0.5). If the transport was measured in strains with functional Gap1 permease, its transport activity was saturated by addition of 1 mM citrulline to the cell suspension 1 min before addition of the radioactively labelled substrate.

2.4. Epitope tagging

The C. albicans amino acid permease Can1 was tagged at its C-terminus with the c-myc epitope using yeast multi-copy vector YEpmyc181 and yeast centromeric vector YCPmyc111 [12]. Plasmid pCA2-4 containing the C. albicans CAN1 gene was first digested with SstI, the ends were made blunt using the Klenow fragment of DNA polymerase I, and finally digested BglII. In this way we generated a 2.9 kb fragment with BglII site in the promoter region and a blunt end at 3'-region of CAN1 gene. This fragment with the last 30 nucleotides and stop-codon of CAN1 gene truncated was inserted into vectors YEpmyc181 and YCPmyc111 that had previously been linearized with XmaI (filled in to form blunt ends by treatment with the Klenow fragment of DNA polymerase I) and subsequently digested with BamHI (compatible with BglII), to create pCAmyc181 and pCAmyc111, respectively. These constructs encode a Ca-Can1p with the last 10 amino acid residues of the native C. albicans Can1p (AWDKFWANVA) replaced by one arginine (R) and 11 amino acids (EQKLISEEDLN) of the c-myc epitope.

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2.6. Plasma membrane preparation

Partial purified plasma membranes were prepared according to Zinner et al. [14] with some modifications. Yeast cells in the exponential phase of growth were harvested by centrifugation, washed twice by distilled water and suspended in breaking buffer (50 mM Tris-HCl, pH 7.5; 250 mM sucrose; 5 mM EDTA; 0.5 mM t-glutathione). All subsequent steps were carried out at 4°C. Chilled glass beads (0.45 mm diameter) were added to the meniscus and the cells were broken by vigorous vortex mixing (30 s pulses repeated 6 times). A mixture of protease inhibitors (final concentration 1 mM phenylmethylsulfonfluoride and 1 μM each of leupeptin, antipain, pepstatin and chymostatin) was added before and between each two pulses. The resulting homogenate was diluted 3-fold with breaking buffer (2 x sample buffer), vortex mixed again, unbroken cells and large debris were removed by centrifugation at 3500 rpm for 5 min and then at 4000 rpm for 5 min (Sorvall RC-5B, rotor SS34). The plasma membrane-enriched fraction was collected by centrifugation for 40 min at 14,000 rpm. This membrane pellet was resuspended in glycerol medium (50 mM phosphate buffer, pH 6.3; 20% glycerol; 1 mM EDTA) containing the mixture of proteinase inhibitors and applied to a discontinuous gradient made using 1 volume 53% (w/w) and 2 volumes 43% (w/w) sucrose. The plasma membranes were recovered at the 43/53% sucrose interface after centrifugation in a swinging bucket rotor (SW28) at 100,000 × g for 4 h. The recovery was 40% in the first and 70% in the second centrifugation step. The purified plasma membranes withdrawn from the 43/53% sucrose interface were sedimented after dilution as described above, and re-suspended in glycerol medium containing the mixture of proteinase inhibitors. Protein concentrations were determined by the method of Bradford [15]. The plasma membrane ATPase activity was estimated according to Sychrova and Kotyk [16] after each purification step.

3. Results and discussion

3.1. Epitope tagging

To study the properties of heterogeneously expressed C. albi-
cans CAN1 permease in Saccharomyces cerevisiae we tagged the CAN1 with -myc epitope. The tagging was accomplished using two S. cerevisiae-E. coli shuttle vectors YEpmycl81 and YCpmycl11 that allowed the in-frame fusion of the C. albi-
cans CAN1 gene with a sequence encoding the epitope of the human c-myc protein [12]. The c-myc epitope tagged Ca-
CAN1 gene (Ca-CAN1myc) was introduced into the S. cerevisiae strain HS 60-2B (can1 lyp1 ura3Δ leu2Δ) on either multi-
cyto- or centromeric vector (pCAMycl181 or pCAMycl111, re-
spectively). First, the uptake of 100 μM lysine into the transformed cells was measured to check the activity of the tagged Ca-CAN1myc constructs. The initial rates were 7.5 nmol (mg dry mass)⁻¹ min⁻¹ and 1.4 nmol (mg dry mass)⁻¹ min⁻¹ in strains harbouring multi-copy or centromeric plasmids, respectively (Fig. 1A). The amount of Ca-CaN1mpowc permease in the cells was estimated using Western blots. Immunoreac-
tive signals were detected in total protein extracts of cells transformed with the epitope tagged Ca-CAN1myc constructs but not in cells containing either the untagged Ca-CAN1 or YEpmycl81. The relative amounts of Ca-CaN1mpowc observed on Western blots corresponded to initial rates of lysine uptake (Fig. 1). In the case of cells transformed with the centromeric vector, the weak signal observed on Western blots was accom-
panied by low transport activity. As usual for highly hydro-
phobic proteins, the immunodetected Can1pmyc had an appar-
et mass smaller (~58 kDa; Fig. 1b) than that one calculated from the amino acid sequence (~63 kDa). An unidentified polypeptide with an apparent molecular mass of about 110 kDa was detected (also observed by Hein et al. [17]). The presence of this crossreacting protein seems to be strain and media dependent, as it appears only in strains of S. cerevisiae derived from Σ1278b and only in cells grown on minimal media [18].

To verify that the replacement of last 10 amino acid resi-
dues of the Ca-CaN1 by c-myc epitope did not change the permease transport activity and/or substrate specificity, we compared transport activities of Ca-CaN1mpowc permease with untagged Ca-CaN1. Both Ca-CAN1myc construct and un-
tagged Ca-CAN1 gene were cloned into multi-copy vector YEplp352 with URA3 marker gene and expressed in the strain HS 100-3C which lacks Gap1 transport activity in contrast to strain HS 60-2B that was originally used. Both tagged and untagged permeases transported all three substrates, but in case of Ca-CAN1mpowc the initial rates of uptake were a bit lower, e.g., the initial uptake rate measured using 100 μM lysine into the HS 100-3C [pCAMycl352] was 10.2 nmol (mg dry mass)⁻¹ min⁻¹ whereas in HS 100-3C [pCA2-4ΔBE] it was 19.7 nmol (mg dry mass)⁻¹ min⁻¹. However, the decrease of the initial rate of lysine uptake is probably not caused by the change of tagged permease affinity to its substrates as the detailed ki-
netic analysis showed that the $K_T$ for lysine uptake did not differ for both Can1pmyc and Can1p ($K_T$,Can1p-myc = $K_T$,Can1p = 13 μM).

3.2. Ca-CAN1mpowc transport activity in the presence of ammonia

It is known that the expression levels and activities of sev-
Fig. 1. Uptake of 100 μM lysine in the presence of 1 mM citrulline (a) and Western blotting protein analysis (b) in *S. cerevisiae* strain HS 60-2B (*canl* *lyspl* ura3Δ len2) transformed with *C. albicans* CAN1p*roc* construct in multi-copy vector (pCAmyc111; 1) or centromeric vector (pCAmyc111; 2).  

### Table 2

**List of plasmids**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Composition</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YEp352</td>
<td>5.2 kb, <em>URA3</em>, 2μ, <em>AmpR</em>, <em>ORI</em></td>
<td>[22]</td>
</tr>
<tr>
<td>pCA2-4</td>
<td>5.6 kb fragment containing <em>C. albicans</em> <em>CANI</em> in YEp352</td>
<td>[1]</td>
</tr>
<tr>
<td>pCA2-4ABE</td>
<td>3.4 kb fragment containing <em>C. albicans</em> <em>CANI</em> in YEp352</td>
<td>[1]</td>
</tr>
<tr>
<td>pLY7-9</td>
<td>5.2 kb fragment containing <em>LYP1</em> in pFL61</td>
<td>[11]</td>
</tr>
<tr>
<td>YEpmyc181</td>
<td>5.8 kb, LEU2, 2μ, <em>AmpR</em>, <em>ORI</em></td>
<td>[12]</td>
</tr>
<tr>
<td>YCPmyc111</td>
<td>6.1 kb, LEU2, <em>CEN4</em>, <em>ARS1</em>, <em>AmpR</em>, <em>ORI</em></td>
<td>[12]</td>
</tr>
<tr>
<td>pCAmyc181</td>
<td>2.9 kb BglII- XbaI fragment containing c-myc-tagged <em>C. albicans</em> <em>CANI</em> in YEpmyc181</td>
<td>this work</td>
</tr>
<tr>
<td>pCAmyc111</td>
<td>2.9 kb BglII- XbaI fragment containing c-myc-tagged <em>C. albicans</em> <em>CANI</em> in YCPmyc111</td>
<td>this work</td>
</tr>
<tr>
<td>pCAmyc352</td>
<td>3.9 kb BglII-XbaI fragment containing c-myc-tagged <em>C. albicans</em> <em>CANI</em> in YEp352</td>
<td>this work</td>
</tr>
<tr>
<td>pPL210</td>
<td>1.4 kb Accl fragment containing <em>SHP3</em> in pRS316</td>
<td>[7]</td>
</tr>
<tr>
<td>pPL288</td>
<td>5.7 kb EcoRI-XbaI fragment containing sh3A5::hisG <em>URA3</em> hisG in pBSII SK(+)</td>
<td>[8]</td>
</tr>
</tbody>
</table>

Fig. 2. Immunodetected amounts of Canlp**roc** in cells of strain HS 100-3C [pCAmyc352] grown in minimal media containing either proline (YNB-Pro) or ammonia (YNB-NH₄) and corresponding initial rates (J₀ in nmol (mg dry mass)⁻¹ min⁻¹) of 100 μM lysine uptake.

**3.3. Biogenesis of the heterologous permease**

The biogenesis of heterologously expressed Ca-Canlp in *S. cerevisiae* was studied and compared with the biogenesis of the endogenous *S. cerevisiae* *Lyp1* system [11] which shares high sequence similarity with Ca-Canlp. First, the transport activities of both of these permeases were measured in temperature sensitive sec1-1 mutants (Fig. 3a,b). At non-permissive temperature the secretory vesicles of sec1-1 cells cannot fuse with the plasma membrane [20]. HS 35-4A (*canl* *lypl* sec1-1) was determined in cells grown in minimal media containing either ammonium ions or proline as sole nitrogen sources available in the growth medium [19]. To estimate if heterologously expressed Ca-Canlp, under control of its own promoter is influenced by the presence of ammonia in the growth medium, the transport activity and the amount of Ca-Canlp*roc* was determined in cells grown in minimal media containing either ammonium ions or proline as sole nitrogen sources. Fig. 2 shows that initial rates of lysine uptake and also the amount of permease molecules in cells grown in YNB-NH₄ is lower compared to cells grown in YNB-Pro. Apparently, Ca-Canlp expression is inhibited by the presence of ammonia. In addition, we observed that after the cells were transferred from YNB-NH₄ to 1% glucose (i.e., without any source of nitrogen), the initial rate of lysine uptake increased about 5-fold within 3 h, while the high lysine uptake activity in cells from YNB-Pro slowly decreased after cells were transferred to 1% glucose (data not shown).

In a similar experiment the amount of Ca-Canlp*roc* was determined using Western blots of proteins isolated from cells of HS 35-4A [pCAmyc352] pregrown in YNB-NH₄ and incubated at permissive or restrictive temperatures in YNB-Pro. Surprisingly, the amount of immunodetected Canlp**roc** in cells incubated at the permissive temperature was lower than that in cells incubated at the restrictive temperature (Fig. 3c) in spite of the fact that the initial rate of transport was almost negligible at the restrictive temperature. This phenomenon could be explained if the Ca-Canlp*roc* accumulated in secretory vesicles incubated at 37°C turnover at rates appreciably slower than the Ca-Canlp*roc* in the plasma membrane of cells incubated at 25°C. If purified plasma membranes were ana-
Fig. 3. Uptake of 100 mM lysine in HS 35-4A (can1 lyp1 secl-1 ura3) transformed with pCA2-4ABE (a), or pLY7-9 (b), and amounts of Can1p<sup>rec</sup> in HS 35-4A [pCAMyc352] determined by immunoblotting (c). Cells were harvested from YNB-NH<sub>4</sub> and resuspended in YNB-Pro. After 2-h incubation at permissive (25°C, dashed lines) or restrictive (37°C, full lines) temperatures; (i) in (a) and (b), the protein-synthesis inhibitor cycloheximide (0.4 mM) was added and a half of the cells was transferred from restrictive to permissive temperature; (ii) in (c), total protein extracts were prepared from HS 35-4A [pCAMyc352].<br>Fig. 4. Uptake of 100 μM histidine in the presence of 1 mM citrulline into strains PLAS 1-6A (SHR3; squares) and PLAS 23-4B (shr3; circles) both transformed either by pCA2-4ABE (full lines) or by YEp352 without any insert (dashed lines). s<sub>i</sub>, internal concentration of histidine in nmol (mg dry mass)<sup>-1</sup>.

Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lysine</th>
<th>Arginine</th>
<th>Histidine</th>
<th>K&lt;sub&gt;T&lt;/sub&gt;</th>
<th>J&lt;sub&gt;max&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS 100-3C [pCAMyc352]</td>
<td>(SHR3)</td>
<td>10.2</td>
<td>19.4</td>
<td>11.9</td>
<td>13</td>
</tr>
<tr>
<td>AM 8-1 [pCAMyc352]</td>
<td>(shr3)</td>
<td>2.1</td>
<td>3.6</td>
<td>2.5</td>
<td>13</td>
</tr>
<tr>
<td>HS 100-3C [pLY 7-9]</td>
<td>(SHR3)</td>
<td>14.7</td>
<td>1</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>AM 8-1 [pLY 7-9]</td>
<td>(shr3)</td>
<td>2.6</td>
<td>0.9</td>
<td>0.9</td>
<td>1</td>
</tr>
</tbody>
</table>

J<sub>0</sub>, initial rate of transport in nmol (mg dry mass)<sup>-1</sup> min<sup>-1</sup>,
K<sub>T</sub>, half-saturation constant in μM.
J<sub>max</sub>, maximum rate of transport in nmol (mg dry mass)<sup>-1</sup> min<sup>-1</sup>.
crease in the uptake activity into shr3 mutants (≈20% of the SHR3 strain; Fig. 4) suggests that the heterologous Ca-Canlp is recognized by Shr3p. To verify this observation a shr3 null mutant strain also carrying mutations in CAN1, GAP1 and LYP1 (AM 8-1) was constructed. This strain was transformed with multi-copy vectors containing either the Sc-LYPL gene or the Ca-CANlpmyc construct. Transport activities of both permeases in shr3 mutant were measured. The initial rates of the amino acid transport in these strains with disrupted shr3 gene were about 5 times lower compared to the uptake in similarly transformed SHR3 wild-type strains (Table 3). A detailed kinetic analysis showed that the $K_T$ of lysine uptake mediated by C. albicans Can1 system did not change, whereas the maximum velocity decreased about 5 times compared to the SHR3 cells (Table 3). This data suggests that the absence of Shr3p influences the amount of permease molecules in the plasma membrane but not the permease substrate affinity. These results were confirmed using Western blots (Fig. 5) of total cell extracts and isolated plasma membranes (the ATPase activity in purified plasma membranes was about 4 μmol Pι min⁻¹ (mg protein)⁻¹ for the shr3 mutant and 3 μmol Pι min⁻¹ (mg protein)⁻¹ for the SHR3 strain). The decreased amount of Can1pmyc in total cell extracts of the shr3 mutants indicates that Can1p accumulated in the ER is probably degraded.

In conclusion, heterologous amino acid permease Can1p follows in S. cerevisiae the secretory pathway and its amount in the plasma membrane is influenced by the amino acid permease specific transport chaperone Shr3p analogously as S. cerevisiae own amino acid transport system Lyp1.

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