Utilization of green fluorescent protein as a marker for studying the expression and turnover of the monocarboxylate permease Jen1p of Saccharomyces cerevisiae

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Green Fluorescent protein (GFP) from Aequorea victoria was used as an in vivo reporter protein when fused to the C-terminus of the Jen1p lactate permease of Saccharomyces cerevisiae. The Jen1 protein tagged with GFP is a functional lactate transporter with a cellular abundance of 1670 molecules/cell, and a catalytic centre activity of 123 s$^{-1}$. It is expressed and tagged to the plasma membrane under induction conditions. The factors involved in proper localization and turnover of Jen1p were revealed by expression of the Jen1p-GFP fusion protein in a set of strains bearing mutations in specific steps of the secretory and endocytic pathways. The chimaeric protein Jen1p-GFP is targeted to the plasma membrane via a Sec6-dependent process; upon treatment with glucose, it is endocytosed via END3 and targeted for degradation in the vacuole. Experiments performed in a Dola64 mutant strain showed that ubiquitination is associated with the turnover of the permease.

Key words: yeast, transport, secretion, ubiquitination, endocytosis

INTRODUCTION

It has been shown that the product of the gene JEN1 is required for the uptake of lactate and other monocarboxylates in the yeast Saccharomyces cerevisiae [1]. To date, this is the first and only gene known to be involved in monocarboxylate transport in fungi. An exhaustive study carried out recently revealed that the S. cerevisiae homologues of mammalian monocarboxylate permeases do not transport monocarboxylic acids across the plasma membrane [2], in contrast with what had been suggested from computer analyses [3,4]. Jen1p is therefore the only model of a membrane monocarboxylate transporter (MCTs; for a review, see [5]), and of permeases in general. In the case of plasma membrane proteins, an effort is being orchestrated by several groups towards the understanding of their trafficking, localization and degradation. Previous studies performed in our laboratory have shown that JEN1 expression is mediated under glucose catabolite repression at distinct levels: transcription, mRNA turnover and carrier inactivation [6]. Jen1p is rapidly inactivated on addition of glucose to induced cells, undergoing an irreversible catabolite-inactivation process [6]. In the present study, green fluorescent protein (GFP) (for a recent review, see [7]) from Aequorea victoria was used as in vivo reporter protein, fused to the C-terminus of the Jen1p monocarboxylate permease of S. cerevisiae. Our studies focused on the expression, trafficking and turnover of Jen1p.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The S. cerevisiae strains used in this study are listed in Table 1. Plasmid pFA6a-GFPS65T-KanMX6, kindly provided by A. Wach (Institut fur Angewandte Mikrobiologie, Biozentrum, Universitat Basel, Basel, Switzerland), was used for the construction of the JEN1-GFP chimera. The plasmid YEp96, which contains a synthetic yeast Ubiquitin (Ub) gene under the control of the copper-inducible CUP1 promoter, was used [8] for the overexpression of Ub. The cultures were maintained on slants of 1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar. To promote growth, either a complex medium containing 1% (w/v) yeast extract and 1% (w/v) peptone (YP medium) or a synthetic minimal medium comprising 0.67% (w/v) Difco yeast nitrogen base, supplemented with adequate quantities of auxotropic requirements (YNB medium), were used. Carbon sources were either glucose (2%, w/v) or D,L-lactic acid (0.5%, v/v; at pH 5.0). Strains with temperature-sensitive alleles were grown at 24 °C (the permissive temperature) or 37 °C (the restrictive temperature); other strains were grown at 30 °C. Cultures were always harvested.

Table 1 Yeast strains used in this paper

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tr>
<td>CEN/PK113-ED</td>
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<td>[8]</td>
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<tr>
<td>CEN/PK 113-13D-Djen1</td>
<td>MATa ura3-52 Djen1</td>
<td>[2]</td>
</tr>
<tr>
<td>BLC 491-U2</td>
<td>MATa ura3-52 JEN1::GFP Kar1</td>
<td>The present work</td>
</tr>
<tr>
<td>NY17</td>
<td>MATa sec4-4 ura3-52</td>
<td>[9]</td>
</tr>
<tr>
<td>RH1623</td>
<td>MATa his4 leu2 ura3 bar1-1 end3-1</td>
<td>[10]</td>
</tr>
<tr>
<td>BLC 492</td>
<td>MATa ura3-52 sec4-4 JEN1::GFP Kar1</td>
<td>The present work</td>
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<tr>
<td>BLC 493</td>
<td>MATa his4 ura3 bar1-1 end3-1</td>
<td>The present work</td>
</tr>
<tr>
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<td>MATa his3-d200 leu2-3,112 ura3-52 lys2-801 trp1 doa4::LEU2</td>
<td>[11]</td>
</tr>
<tr>
<td>MHY623</td>
<td>MATa his3-d200 ura3-52 lys2-801 trp1</td>
<td>[11]</td>
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Abbreviations used: CMAC-Arg, 7-amino-4-chloromethylcoumaryl-arginine amide dihydrochloride; GFP, green fluorescent protein; ORF, open reading frame; Ub, ubiquitin.

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Table 2: Oligonucleotides used to construct and check the JEN1–GFP fusion

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<td>S1</td>
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</tr>
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<td>K2</td>
<td>5′-GTGTGATCTGCTCTCAGATGCGTTGAC-3′</td>
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<td>5′-CTGCTGTGACATGCAGCTGCTGACGACGAC-3′</td>
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during the exponential phase of growth. Glucose-containing media, i.e. YP (YPD) or YNB (YNBD) were used for the growth of yeast cells under conditions of repression. Conditions for the induction of yeast were obtained by incubating cells previously grown under repression conditions in YNB medium with d,L-lactic acid for 4 h. Media contained 0.1 mM CuSO4 for experiments involving overexpression of Ub. Cell growth was monitored by measuring the attenuation (D) of appropriately diluted cell suspensions at 600 nm.

Genetic methods

Crossoes, isolations of diploids, sporulation and tetrad analysis were achieved using standard methods [13].

JEN1-GFP chimera DNA fragment and transformation

A genetic chimera formed between JEN1 and GFP was made to yield a fusion gene at the chromosomal JEN1 locus. The flanking homology PCR cassette technique [14,15] was used. Primer S1 (Table 2) has 46 bp of DNA that are homologous with the 3′-end of the JEN1 open reading frame (ORF), not including the stop codon, followed by 21 bp of sequence derived from the 3′-end of the GFP reporter gene in the plasmid pFA6a-GFPS65T-KanMX6. Primer S2 (Table 2) has 45 bp DNA homologous with first 45 nucleotides downstream of the JEN1 ORF, followed by 19 bp of sequence derived from the 3′-end of the ADH1 terminator in the plasmid pFA6a-GFPS65T-KanMX6A. The resulting PCR product of 2.4 kb was purified, and used to transform cells of the strain CEN.PK 113-5D by the improved lithium- 

Transport assays

Cells incubated under de-repressed conditions were harvested by centrifugation, and washed twice in ice-cold deionized water to a final concentration of about 25±40 mg dry weight/ml. Conical centrifuge tubes containing 30 μl of 0.1 M KH2PO4 buffer at pH 5.0 and 10 μl of the yeast suspension were incubated for 2 min at 25 °C. The reaction was started by the addition of 10 μl of an aqueous solution of 4000 d.p.m./nmol of radiolabelled [1-14C]lactic acid (sodium salt; Amersham Biosciences, Piscataway, NJ, U.S.A.) at pH 5.0. The reaction was stopped by dilution with 5 ml of ice-cold water. The reaction mixtures were filtered immediately through GF/C membranes (Whatman Biosystems Ltd., Maidstone, Kent, U.K.) and the filters were washed with 10 ml of ice-cold water and transferred to scintillation fluid (Opti-Phase HiSafe II; LKB, Gaithersburg, MD, U.S.A.). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer equipped with a d.p.m. correction factor. For non-specific adsorption of °C, labelled lactic acid was added at time zero after the cold water. To determine the best fitting transport kinetics to the experimental initial-uptake rate values, and to estimate the kinetic parameters, a computer-assisted non-linear regression analysis (GraphPAD software; San Diego, CA, U.S.A.) was used. All the experiments were repeated at least three times, and the data reported represent the average values.

Microscopy

Living cells were examined with a Leitz Aristoplan epifluorescence microscope with filter cube 1001 HQ-FITC (Chroma Technology, Brattleboro, VT, U.S.A.) for GFP excitation, and filter A for 7-amin-4-chloromethylcoumaryl-l-arginine amide dihydrochloride (CMAC-Arg; Molecular Probes, Eugene, OR, U.S.A.). For staining of vacuolar lumen, cells were incubated with CMAC-Arg according to the manufacturer’s instructions. For the capture of images, an Apogee charge-coupled-device camera was used, and the micrographs were processed for display using Image Pro Plus software.

Cell protein measurement

Total cell protein was measured by the Lowry assay, after the digestion of cells overnight in 1 M NaOH.

Fluorescence spectroscopy

A Hitachi RF-5001PC fluorimeter was used to scan the excitation and emission spectra of whole-cell suspensions. Spectra of cells expressing JEN1±GFP were normalized for cell density, and were corrected for background by subtraction of spectra that were recorded for cells expressing JEN1 cultured under identical conditions. Emission spectra were collected between 500 and 550 nm, with excitation set at 489 nm, and excitation and emission slit widths of 3 nm.

Catalytic-centre activity and cellular abundance estimation

The catalytic-centre activity and cellular abundance of JEN1±GFP protein were estimated by the method of Kruckeberg et al. [17].

Immunoblotting

Immunoblot analyses were performed following the method of Kruckeberg et al. [17]. Anti-GFP antibody was purchased from Molecular Probes, and diluted 1:500.

RESULTS

Construction of a Jen1–GFP fusion protein

A genetic chimera formed between JEN1 and GFP was made to yield a fusion gene at the chromosomal JEN1 locus. The flanking homology PCR cassette technique [14,15] was used. Primer S1 (Table 2) has 46 bp of DNA that are homologous with the 3′-end of the JEN1 ORF, not including the stop codon, followed by 21 bp of sequence derived from the 3′-end of the GFP reporter gene in the plasmid pFA6a-GFPS65T-KanMX6. The resulting PCR product of 2.4 kb was purified, and used to transform cells of the strain CEN.PK 113-5D by the improved lithium- 

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Figure 1 Strategies followed for the construction of the JEN1–GFP fusion (A) and for the verification of gene fusion by analytical PCR (B)

(A) Primers S1 and S2 were used to amplify a GFP-kanMX6 cassette. Primer S1 was homologous with the last 46 nt of JEN1, and primer S2 was homologous with the first 45 nt downstream of JEN1. After transformation of the CEN.PK113-5D strain with the PCR product, a strain named BLC491-U2 was obtained, bearing the Jen1±GFP fusion protein. (B) Cells from single colonies of BLC491-U2 (JEN1–GFP) and of CEN.PK113-5D (WT) were lysed as described in the Materials and methods section. Of the cell suspension of each strain, 1 l l was used directly for PCR analysis according to the previously described strategy. The PCR products were loaded on to a 0.7% (w/v) agarose gel, and the size of the fragments obtained are indicated in the Figure.

cerevisiae strain CEN.PK113-5D was transformed with this PCR product to yield the chimaeric JEN1±GFP gene (Figure 1A). The correct fusion of the targeted gene at the genomic locus was verified by analytical PCR performed on whole cells. The primers used to confirm the correct fusion are summarized in Table 2, and shown in Figure 1(B). Six independent PCRs were performed, with three of them using the strain CEN.PK113-5D as a control and primer pairs A1 and A2, A1 and K2, and A2 and K3. Using the same primers, three PCRs were performed using the strain BLC491-U2, bearing the JEN1±GFP fusion. The analytical PCR results clearly confirmed that the PCR-generated DNA fragment was correctly integrated into the CEN.PK113-5D genomic JEN1 locus.

Jen1 fused with GFP is a functional lactate transporter

De-repressed cells of the strains CEN.PK113-5D and BLC491-U2 were obtained under the conditions described in the Materials and methods section, and after 4 h in YNB/lactic acid medium the cells were analysed for their capacity to transport labelled lactic acid. The results obtained at pH 5.0 are presented in Figure 2, showing that both strains display activity for the lactate permease. The kinetic parameters observed were of the same order of magnitude: for the CEN.PK113-5D strain, \( V_{\text{max}} \) was estimated to be 0.28 nmol/s per mg of dry mass, and \( K_{m} \) was estimated to be 0.44 mM; for the BLC491-U2 strain, \( V_{\text{max}} \) was estimated to be 0.30 nmol/s per mg of dry mass, and \( K_{m} \) was estimated to be 0.49 mM. Under the same experimental conditions, transport of labelled lactic acid was evaluated in cells not expressing JEN1 (strain CEN.PK113-13D-jen1). In this strain, the initial uptake rates were significantly lower than those observed in cells expressing JEN1. Furthermore, first-order kinetics were found, indicative of the absence of a mediated transport mechanism for the acid across the plasma membrane (Figure 2). Cells of the strain W303-1a, obtained under identical experimental conditions [1], displayed similar kinetic parameters:

\[ V_{\text{max}} = 0.40 \text{ nmol/s per mg of dry mass and } K_{m} = 0.69 \text{ mM.} \]

Cultures of strains CEN.PK113-5D and BLC491-U2 were grown in YNB-glucose and YNB-lactic acid liquid and solid media. After 48 h, no differences were found between them in terms of the final biomass or the growth rates.

Jen1–GFP is localized in the plasma membrane

The expression and subcellular localization of Jen1±GFP fusion protein was monitored over time in living cells by fluorescence microscopy. BLC491-U2 cells were grown overnight in YNB-glucose medium until they reached a \( D_{\text{max}} \) of approx. 0.5, and

![Figure 2](image-url)
Cells growing exponentially in YNB-glucose were harvested by centrifugation, washed twice with deionized water, and incubated in YNB-lactate. Equal volumes of cells were resuspended in low-melt agarose and observed by epifluorescence microscopy. Photos were then taken at the times indicated.

Figure 3 Photographs of a time-course study showing the localization of fluorescent JEN1–GFP in living cells

Figure 4 Time course of JEN1–GFP inactivation

(A) Induced cells of the BLC491-U2 strain were treated with glucose or with sorbitol (as a control for osmotic shock), to a final concentration of 110 mM, and examined after continued incubation. (B) Induced cells of the BLC491-U2 strain were examined after 1 h of glucose treatment (final concentration 110 mM) and stained with CMAC-arginine, either detecting GFP fluorescence or CMAC-arginine fluorescence [17]. CMAC-arginine is a vacuole-specific stain. PC, phase contrast.

were subsequently transferred to YNB medium containing lactic acid [0.5 % (w/v) at pH 5.0] as the sole carbon and energy source. After relocating the cells to medium with lactic acid, the expression and localization of Jen1±GFP, the lactic acid concentration and the attenuance of the culture were measured simultaneously over a 24 h period. In Figure 3, representative photographs of Jen1±GFP fluorescence in cells at various time points are shown. The fluorescence was almost undetectable within the first 2 h. After that, it started to become clearly localized to the plasma membrane, and increased gradually. The maximum fluorescence intensity in the plasma membrane was achieved 4±6 h after induction of the medium with lactic acid.
After a prolonged incubation, the signal was lost from the plasma membrane, and the Jen1±GFP fusion protein began to be internalized by endocytosis, and was subsequently delivered to the vacuole. The metabolite content present in the medium culture was examined by HPLC, and it was found that consumption of lactic acid occurred during the whole experiment (results not shown).

**Catalytic-centre activity of the Jen1–GFP lactate transporter** Light absorption and fluorescence emission spectra were recorded from cell suspensions expressing JEN1 or JEN1±GFP. The specific fluorescence of purified GFP is indistinguishable from membrane protein±GFP fusions in Yqs (A. Kruckeberg, unpublished results). The fluorescent signal from Jen1±GFP was used to determine the cellular concentration and catalytic-centre activity of the fusion protein, using purified GFP as a fluorescent standard. The cellular abundance of the Jen1±GFP chimera proved to be in 1670 molecules/cell, and the estimated value for the catalytic-centre activity was 123 s⁻¹. All the fluorescence signal appeared to reside at the plasma membrane in the cells expressing JEN1±GFP, when examined by fluorescence microscopy (results not shown).

**Inactivation of Jen1–GFP by glucose**

Cells of the strain BLC491-U2 induced with lactate were treated with glucose or sorbitol (final concentration 110 mM), and examined after a continued period of incubation (Figure 4A). In glucose-treated cells, after 5 min the fluorescent signal was almost completely lost from the plasma membrane, and appeared in punctuate structures. After 30 min of incubation, it accumulated in a single, large globular structure. In sorbitol-treated cells, the fluorescence remained in the plasma membrane. To identify the globular structure observed in cells after 30 min of glucose addition, the cells were incubated with CMAC-Arg, which stains the vacuole [17]. The GFP fluorescence clearly co-localized with vacuoles stained by CMAC-Arg (Figure 4B).

**Jen1–GFP is targeted to the plasma membrane via a Sec6-dependent process**

The role of the secretory pathway in the trafficking of Jen1±GFP to the plasma membrane was assessed in a strain with a temperature-sensitive allele of the SEC6 gene [19,20]. The growth of 10 complete tetrads originating from the parental cross between BLC491-U2 and Dsec6-4 strains was evaluated on YPD medium, both at the permissive temperature of 23 °C and at the restrictive temperature of 37 °C, and on YPD medium supplemented with Geneticin. One haploid strain, BLC492, was selected, which exhibited normal growth on YPD—geneticin, and which was temperature-sensitive at 37 °C. When the expression of the Jen1±GFP fusion protein was induced in this strain grown at the permissive temperature, the plasma membrane was clearly labelled by GFP (Figure 5). When the fusion protein was expressed in cells maintained at the restrictive temperature of 37 °C, the fluorescence developed to a similar level, but it was completely excluded from the plasma membrane. Instead, it accumulated in globular bodies within the cell (Figure 5). The sec6-4 phenotype has been reported to be reversible, when cells are restored to the permissive temperature [19]. After sec6-4 cells expressing Jen1±GFP were shifted from the restrictive to the permissive temperature for 2 h, their plasma membranes were distinctly labelled with fluorescence (Figure 5). This assay was performed in the presence of cycloheximide in order to prevent the de novo synthesis of protein.

**Endocytosis is involved in the removal of Jen1–GFP from the plasma membrane**

Strains with the end3-1 or end4-1 alleles display temperature-sensitive defects in endocytosis [10]. The growth of 10 complete tetrads originating from the parental cross between BLC491-U2 and an end3-1 strain was evaluated on YPD medium, both at the permissive temperature of 23 °C and at the restrictive temperature of 37 °C and on YPD supplemented with Geneticin. One haploid strain, BLC493, was selected, which exhibited normal growth on YPD—Geneticin medium, and which was temperature-sensitive at 37 °C. The expression of JEN1±GFP was induced for 4 h, both in the BLC493 (end3-1) strain and in the BLC491-U2 strain at 23 °C. After 4 h, there was a clear localization of the fluorescence to the plasma membrane in both strains (Figure 6). Each culture was then divided into four aliquots. Two were maintained at the permissive temperature, and two were transferred to the restrictive temperature. Glucose was added to one of the aliquots to a final concentration of 2% (w/v) at each temperature. All the cultures were visualized by fluorescence microscopy after 30 min (Figure 6). In the absence of glucose, there was a strong fluorescent staining of the plasma membrane in all cultures, both at 23 °C and at 37 °C. The addition of glucose led to an accumulation of the fluorescence in the vacuole both in BLC491-U2 and in BLC493 cells at the permissive temperature, but only in BLC491-U2 cells at the restrictive temperature. In BLC493 cells at 37 °C, there was a retention of JEN1±GFP in the plasma membrane, which persisted with prolonged incubation. Taken together, these results indicate that, upon glucose treatment,
Figure 6 Jen1–GFP is retained in the plasma membrane in cells blocked in endocytosis

Jen1±GFP expression was induced for 4 h, in both the BLC493 and BLC491-U2 strains at 23 °C, under the conditions described in the Materials and methods section. After 4 h of induction, each culture was divided into four aliquots. Two were maintained at the permissive temperature, and two were transferred to the restrictive temperature. Glucose was added to one of the aliquots at each temperature to a final concentration of 2% (w/v). All the cultures were visualized by fluorescence microscopy after 1 h.

Jen1±GFP is endocytosed via END3, and subsequently targeted to the vacuole.

Western blot analysis of the effect of glucose addition in cells blocked in endocytosis

A Western blot analysis was performed using an anti-GFP antibody. The strains BLC 491-U2 and BLC-493 were induced in lactate for 4 h. After this period of induction, a sample of each culture was collected and glucose was added to the culture medium to a final concentration of 2% (w/v). Of each culture, two more samples were collected after 30 and 60 min of glucose addition. Lysates containing 15 l g of protein were resolved on an SDS/10% polyacrylamide gel. After blotting, the degradation products were only observed in the BLC491-U2 strain (Figure 7). No degradation products could be observed in the strain with a defect in the endocytic pathway, supporting the results previously obtained by fluorescence microscopy (Figure 7).

Figure 7 Western-blot analysis of Jen1–GFP protein level

The strains used were BLC491-U2 (END3 JEN1–GFP) (lanes labelled 'A') and BLC 493 (end3 JEN1–GFP) (lanes labelled 'B'). Detection of Jen1±GFP was performed with anti-GFP antibody. Time after glucose addition is shown.

Figure 8 Activity of the lactate transporter in induced cells upon the addition of glucose, using 1 mM of labelled lactic acid, pH 5.0

(A) Activities are shown for wild-type cells (●) and Ddoa4 cells (●●●). (B) Ddoa4 cells transformed with the plasmid YEp96, carrying the wild-type Ub gene, were induced in the presence (●●●) or absence (●●●) of Cu2+. The final glucose concentration was 2% (w/v).
Overexpression of Ub partially restored endocytosis of the lactate transporter in a Doa4Δ strain

We then set out to determine whether the Ub pathway was involved in the endocytosis of the lactate permease upon glucose treatment. The Doa4Δ Ub-isopeptidase has been shown to have a key role in Ub-dependent degradation in vivo [21], and a doa4Δ mutant has reduced levels of free Ub [21]. We used the yeast strain MHY623 lacking the Ub protein hydrolase Doa4ΔNpi2. Cultures of induced cells of MHY501 and MHY623 strains were supplemented with glucose to a final concentration of 2 % (w/v), and samples were collected over time to estimate the activity of the carrier. Upon glucose treatment, the inactivation of the carrier is substantially reduced in a Doa4Δ strain when compared with the wild type (Figure 8A). These results suggest that the internalization step of endocytosis of the Jen1 permease is dependent on the Doa4 protein. The Doa4Δ phenotype can be complemented with an overproduction of Ub [22]. This can be achieved by transforming the mutant strain with the plasmid YEp96. This [24]. All of these solute permeases are high-affinity transporters [25]. In all cases tested, the fusion proteins retain solute transport function, with kinetics similar to those of the wild-type protein. The genetic chimera formed between Jen1 and GFP is also a functional lactate transporter. The expression pattern and intracellular trafficking of the fusion protein, monitored by fluorometry and epifluorescence microscopy, has been determined. We observed that the protein is strongly localized at the plasma membrane in induced cells. The maximum expression at the plasma membrane was obtained between 4±6 h after induction, and this result was in accordance with the results obtained previously by determining the activity of the carrier [26].

The quantification of the emission from GFP in the Jen1±GFP protein allowed us to estimate a value of 123 s⁻¹ for the catalytic-centre activity of the lactate transporter in vivo. This is the first empirical estimate of a catalytic-centre activity for a yeast monocarboxylate transport protein. The value obtained is of the same order of magnitude as the one obtained for the GLUT1 human glucose transporter at 30 °C [27], and for the Hxt7±GFP transporter [28]. All of these solute permeases are high-affinity transporters. The value obtained seems to reflect a high activity of this transporter molecule in cells under induction conditions.

The factors involved in proper localization and turnover of the Jen1 protein were also explored by expression of the Jen1±GFP fusion in a set of strains with mutations affecting specific steps in the secretory and endocytic pathways. We have shown that SEC6 is involved in delivering Jen1±GFP to the membrane. The results obtained in a strain defective in the ENO3 gene indicate that endocytosis is the mechanism involved in the process of cataloylative inactivation of the carrier. Glucose triggers a rapid degradation of the lactate permease in the vacuole. Upon treatment with glucose, Jen1p was removed from the membrane, internalized by endocytosis and accumulated in the vacuole for degradation. The data resulting from the inactivation of Jen1±GFP by glucose, together with the results observed in the time-course experiments (Figure 3), indicate that the permease constitutively undergoes a moderate rate of turnover, in addition to a rapid, stress-stimulated turnover.

We used a strain defective in the Ub-protein hydrolase Doa4ΔNpi2 to determine whether the binding of Ub was a signal required for the internalization of the carrier. Jen1p undergoes internalization for vacuolar degradation in a manner dependent on Doa4Δ. This pattern of behaviour is also reported in the literature for other plasma membrane proteins whose internalization is substantially reduced in doa4Δ mutant cells [28±32].

The results obtained with cells featuring impairments in the Doa4 protein showed that ubiquitination of the lactate permease signals its endocytosis. These observations taken together re-inforce previous evidence that indicated that there is a general pattern in the mechanism of endocytosis followed by all the plasma membrane proteins studied to date in yeast, as has been suggested by Hicke [33]. The results obtained in the present study suggest that glucose-induced proteolytic degradation (catalolybte inactivation) of Jen1p seems to occur independently of the proteasome, i.e. it occurs in the vacuole after internalization by glucose. Future experiments will be designed to determine this type of ubiquitination that the Jen1p permease undergoes in S. cerevisiae, and the mechanisms that regulate the endocytosis of this transporter.

We thank Professor Karel van Dam and his laboratory for their generous assistance. We also thank Professor Cecilia Leao for a critical reading of the manuscript and for valuable discussions. We are grateful to Professor Rosario Lagunas and Dr Pillar Lucaro for their helpful information and suggestions. Sandra Paiva received a Ph.D. fellowship from the Portuguese Government (IPRAV/XXI-BD/1839/98) and a FEBS short-term fellowship for the work performed in The Netherlands. This work was supported by the Portuguese grant POCI/1999/BME/36 625.

REFERENCES


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