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 šictoria* was used as an *in vivo* reporter protein when fused to the C-terminus of the Jen1 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 D*doa4* 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 monocarboxylate transporter in yeast, and its study can provide important insights into the function of the mammalian monocarboxylate transporters (`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 catabolic-inactivation process [6]. In the present study, green fluorescent protein (GFP) (for a recent review, see [7]) from *Aequorea šictoria* was used as *in vivo* reporter protein, fused to the C-terminus of the Jen1 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* chimaera. 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 auxotrophic 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 permis- sive 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

Strain	Genotype	Source or reference
CEN.PK113-5D	*MATa ura3-52*	[8]
CEN.PK 113-13D-D*jen1*	*MATa ura3-52* D*jen1*	[2]
BLC 491-U2	*MATa ura3-52 JEN1::GFP Kan^r*	The present work
NY17	*MATa sec6-4 ura3-52*	[9]
RH1623	*MATa his4 leu2 ura3 bar1-1 end3-1*	[10]
BLC 492	*MATa ura3-52 sec6-4 JEN1::GFP Kan^r*	The present work
BLC 493	*MATa his4 ura3 bar1-1 end3-1 JEN1:GFP Kan^r*	The present work
MHY501	*MATa his3-D200 leu2-3,112 ura3-52 lys2-801 trp1*	[11]
MHY623	*MATa his3-D200 ura3-52 lys2-801 trp1 doa4::LEU2*	[11]

Abbreviations used: CMAC-Arg, 7-amino-4-chloromethylcoumaryl-L-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

The 21 underlined nt of primer S1 anneal to the 5«-end of the *GFP* ORF in plasmid pFA6a-GFPS65T-KanMX6A. The underlined 19 nt of primer S2 anneal to the 3«-end of the *ADH1* terminator in plasmid pFA6a-GFPS65T-KanMX6A.

Oligonucleotide	Sequence
S1	5«-GATTCGAACGTCTCAAAGACATATGAGGAGCATATTGAGACCGTTAGTAAAGGAGAAGAACTTTTC-3«
S2	5«-GTTACATAGAGAAGCGAACACGCCCTAGAGAGCAATGAAAAGTGAGGATGGCGGCGTTAGTATC-3«
K2	5«-CGATAGATTGTCGCACCTG-3«
K3	5«-CCATCCTATGGAACTGCCTC-3«
A1	5«-GGCCTATCCAAGGATGCTGTC-3«
A2	5«-GGCCCATTCAGTGCAAGAACC-3«

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 $CuSO_4$ for experiments involving overexpression of Ub. Cell growth was monitored by measuring the attenuance (*D*) of appropriately diluted cell suspensions at 600 nm.

Genetic methods

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

JEN1-GFP chimaeric DNA fragment and transformation

A genetic chimaera 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 5«-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-acetate method [16]. Transformed cells were grown at 30 °C in YPD media for 4 h and then spread on to YPD plates containing 200 mg/l Geneticin (G418 from Life Technologies; Gaithersburg, MD, U.S.A.). To purify transformants from the background, each large colony was re-streaked on to fresh YPD/Geneticin plates. Only those clones that grew after both means of selection were analysed further as potentially the sought-after transformants by analytical PCR, as described by Kruckeberg et al. [17]. Molecular biology techniques were performed using standard procedures published previously [18].

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 KH_2PO_4 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 L-[1-^{14}C]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 facility. For non-specific adsorption of ^{14}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-amino-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].

Immunobloting

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 DNA fragment consisting of the *GFP±kanMX6* cassette with short-flanking regions that were homologous with the *JEN1* locus at the 3«-end of the ORF was produced by PCR. *S.*

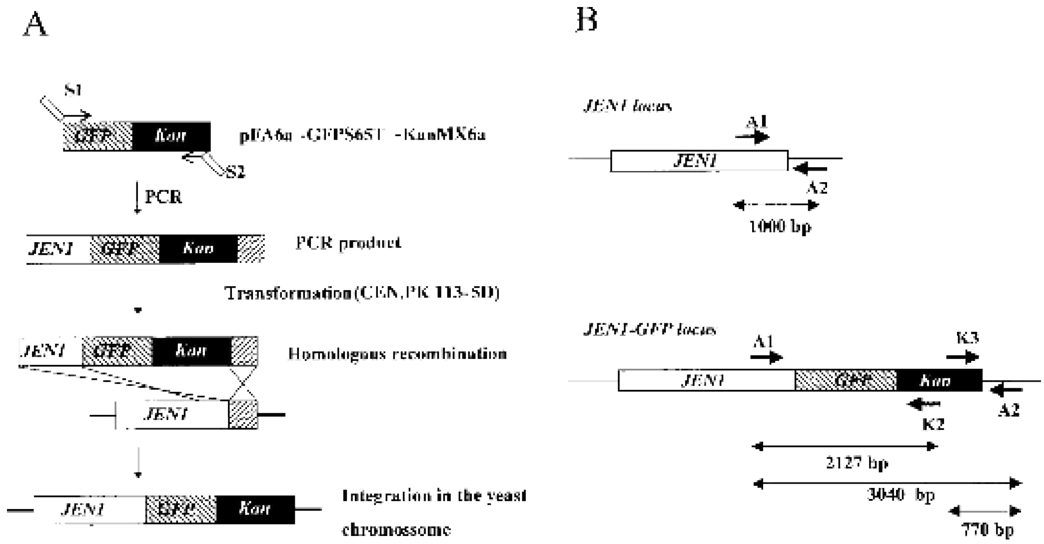

Figure 1 Strategies followed for the construction of the JEN1–GFP fusion (A) and for the verification of gene fusion by analytical PCR (B)

(*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.PK 113-5D 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.PK 113-5D (WT) were lysed as described in the Materials and methods section. Of the cell suspension of each strain, 1 Il 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_{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_{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.PK 113-13D-D*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:

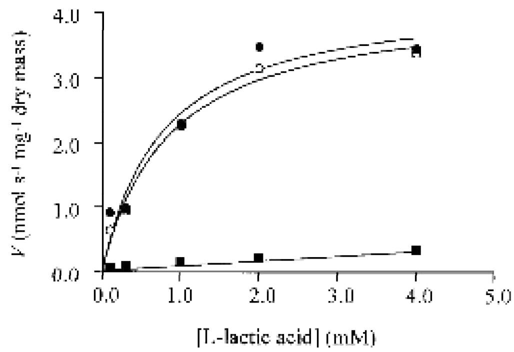

Figure 2 Initial uptake rates of labelled lactic acid at pH 5.0 by YNB/lactic acid-derepressed cells of various yeast strains

Shown are the results of experiments performed with strains CEN.PK 113-5D (E), BLC491-U2 (D) and CEN.PK 113-13D-D*jen1* (+). Uptake studies were performed as described in the Materials and methods section. Cells that were growing exponentially in YNB-glucose were harvested by centrifugation, washed twice with deionized water, and incubated in YNB-lactate for 4 h before the transport assay.

a V_{max} of 0.40 nmol/s per mg of dry mass and a K_m of 0.69 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_{600} of approx. 0.5, and

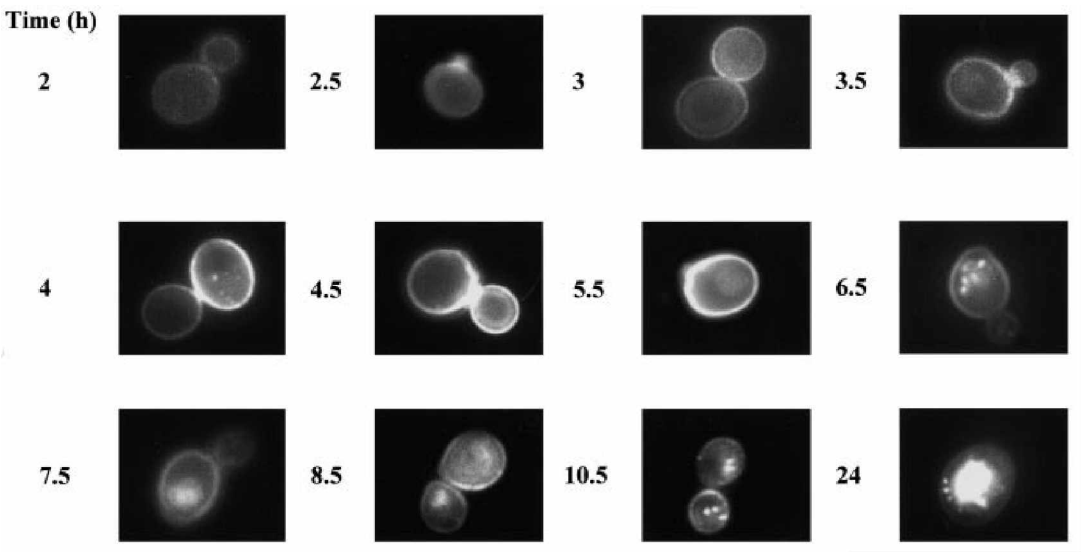

Figure 3 Photographs of a time-course study showing the localization of fluorescent JEN1–GFP in living cells

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.

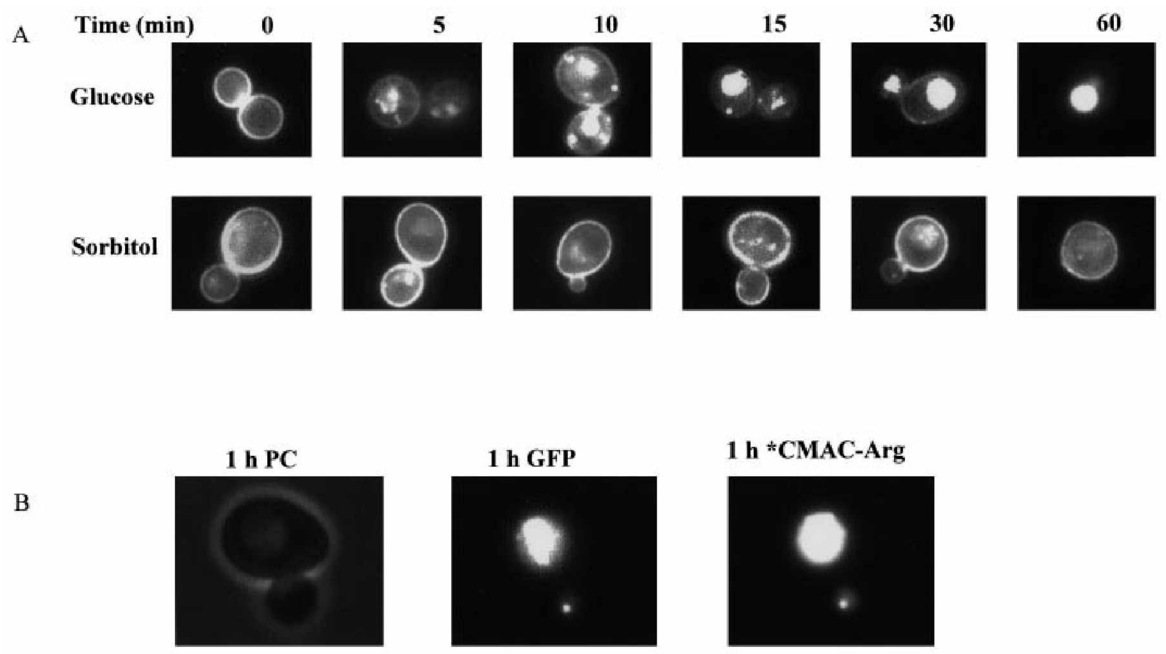

Figure 4 Time course of JEN1–GFP inactivation

(*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.

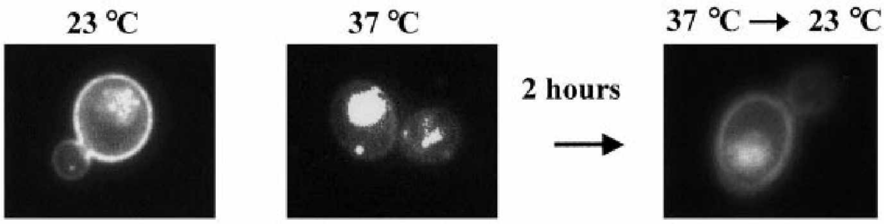

Figure 5 Jen1–GFP localization in a secretory mutant

Jen1±GFP expression was induced in the BLC492 strain. BLC492 strain was derived from the parental cross between BLC491-U2 strain and a *sec6-1* strain with a temperature-sensitive defect in fusion of the secretory vesicles with the plasma membrane. Cells were induced in YNB-containing lactic acid (0.5 %, v/v, pH 5.0) for 4 h at the permissive temperature (23 °C) or at the restrictive temperature (37 °C). After this assay, the cells induced at the restrictive temperature were treated with 10 lg/ml cycloheximide, and incubated at 23 °C for a further 2 h.

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 Vivo* (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 chimaera proved to be in 1670 molecules/cell, and the estimated value for the catalytic-centre activity was 123 s ". 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 the BLC491-U2 and D*sec6-4* 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 noSo* 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,

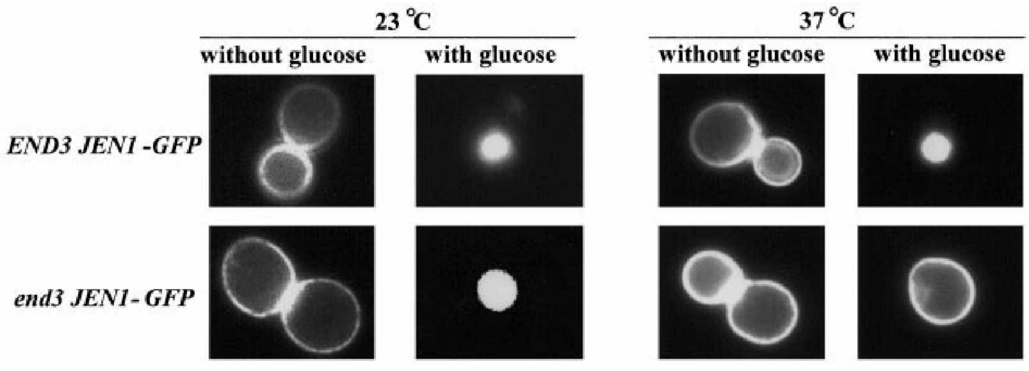

Figure 6 Jen1–GFP is retained in the plasma membrane in cells blocked in endocytosis

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.

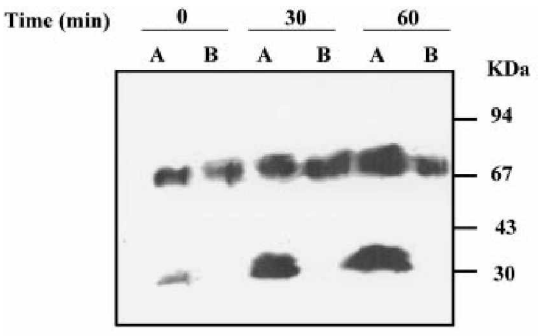

Figure 7 Western-blot analysis of Jen1–GFP protein level

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.

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 Ig 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).

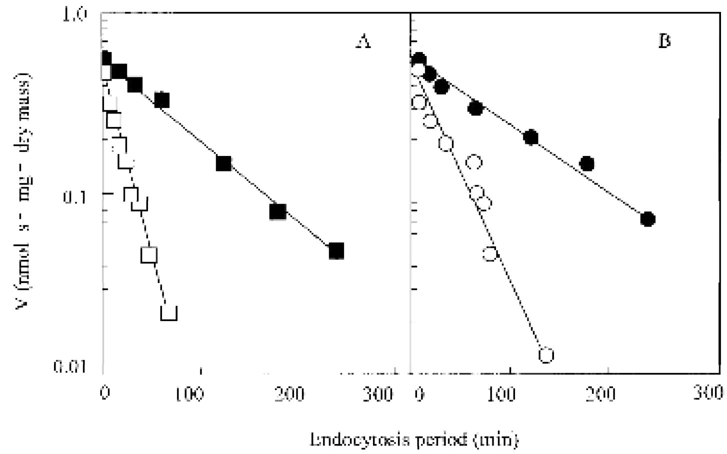

Figure 8 Activity of the lactate transporter in induced cells upon the addition of glucose, using 1 mM of labelled lactic acid, pH 5.0

(*A*) Activities are shown for wild-type cells (*) and D*doa4* cells (+). (*B*) D*doa4* cells transformed with the plasmid YEp96, carrying the wild-type *Ub* gene, were induced in the presence (D) or absence (E) of Cu^{2+}. The final glucose concentration was 2% (w/v).

Overexpression of Ub partially restored endocytosis of the lactate transporter in a Ddoa4 strain

We then set out to determine whether the Ub pathway was involved in the endocytosis of the lactate permease upon glucose treatment. The Doa4p Ub-isopeptidase has been shown to have a key role in Ub-dependent degradation *in Vivo* [21], and a *doa4* mutant has reduced levels of free Ub [21]. We used the yeast strain MHY623 lacking the Ub protein hydrolase Doa4/Npi2. 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 D*doa4* 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 D*doa4* phenotype can be complemented with an overproduction of Ub [22]. This can be achieved by transforming the mutant strain with the plasmid YEp96. This multi-copy plasmid encodes a synthetic *Ub* gene under the control of the *CUP1*-inducible promoter. The overexpression of Ub `rescued' the internalization of the permease in D*doa4* cells. However, in the presence of copper, no effect was found, in comparison with that which was observed for the strain D*doa4* not transformed with YEp96 (Figure 8B).

DISCUSSION

The properties and regulation of solute transporter proteins *in Vivo* have been investigated further by tagging them with the GFP of *A. Šictoria*. The analysis of a number of *S. cerevisiae* solute transporter proteins as fusion proteins with GFP has been reported previously: some of the tagged proteins are members of sugar-transporter family [3,23]. Furthermore, the hexose transporters Hxt2 [17] and Hxt7 [24], and the Pho84 phosphate transporter [25] have been tagged with GFP. In all cases tested, the fusion proteins retain solute transport function, with kinetics similar to those of the wild-type protein. The genetic chimaera formed between Jen1 and GFP is also a functional lactate transporter. The expression pattern and intracellular trafficking of the fusion protein, monitored by fluorimetry 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 " 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 37 °C [27], and for the Hxt7±GFP transporter [24]. 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 *END3* gene indicate that endocytosis is the mechanism involved in the process of catabolite 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 Doa4/Npi 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 Doa4p. This pattern of behaviour is also reported in the literature for other plasma membrane proteins whose internalization is substantially reduced in *doa4* 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 reinforce 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 (catabolite 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 the type of ubiquitination that the Jen1p permease undergoes in *S. cerevisiae*, and the mechanisms that regulate the endocytosis of this transporter.

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