Subcellular localization and functional expression of the glycerol uptake protein 1 (GUP1) of *Saccharomyces cerevisiae* tagged with green fluorescent protein

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GFP (green fluorescent protein) from *Aequorea victoria* was used as an *in vivo* reporter protein when fused to the N- and C-termini of the glycerol uptake protein 1 (Gup1p) of *Saccharomyces cerevisiae*. The subcellular localization and functional expression of biologically active Gup1–GFP chimaeras was monitored by confocal laser scanning and electron microscopy, thus supplying the first study of *GUP1* dynamics in live yeast cells. The Gup1p tagged with GFP is a functional glycerol transporter localized at the plasma membrane and endoplasmic reticulum levels of induced cells. The factors involved in proper localization and turnover of Gup1p were revealed by expression of the Gup1p–GFP fusion protein in a set of strains bearing mutations in specific steps of the secretory and endocytic pathways. The chimaerical protein was targeted to the plasma membrane through a Sec6-dependent process; on treatment with glucose, it was endocytosed through

END3 and targeted for degradation in the vacuole. Gup1p belongs to the list of yeast proteins rapidly down-regulated by changing the carbon source in the culture medium, in agreement with the concept that post-translational modifications triggered by glucose affect proteins of peripheral functions. The immunoelectron microscopy assays of cells expressing either Gup1–GFP or GFP–Gup1 fusions suggested the Gup1p membrane topology: the N-terminus lies in the periplasmic space, whereas its C-terminal tail has an intracellular location. An extra cytosolic location of the N-terminal tail is not generally predicted or determined in yeast membrane transporters.

Key words: endocytosis, green fluorescent protein (GFP), GUP1, localization, protein topology, yeast.

INTRODUCTION

Yeasts are exposed to highly variable changes in their environment, which depend on the availability and quality of nutrients, the temperature, pH, radiation and oxygen and water activities. The survival of yeast cells depends on their ability to recognize these changes and adapt to them appropriately and rapidly. Yeasts have developed response mechanisms based on adjustments to their metabolism and other cellular processes, to sustain growth and proliferation and to protect cells against the potentially detrimental effects of stress challenges [1]. To react to the osmotic changes, for example, yeasts have developed strategies, involving a number of cellular reactions, which allow them to survive in the environment [2]. The mechanisms responsible for this adaptation to altered osmolarity have been studied in *Saccharomyces cerevisiae* and many other yeast species [3]. A strategy in osmoadaptation is the increase in the cellular cytoplasm of specific osmolytes. The most important molecule, which has been evolutionarily selected for this function in *S. cerevisiae*, is glycerol. The accumulation in the cytoplasm of this chemically inert osmolyte could counterbalance hyperosmotic stress [4].

In *S. cerevisiae*, the pathways for glycerol metabolism not only address the production and the utilization of this compound, but they also have an important and proven role in controlling and balancing cellular metabolism [2]. Glycerol synthesis starts with an intermediate of glycolysis, i.e. dihydroxyacetone phosphate, and occurs in two steps: catalysis by two isoforms of NAD-dependent glycerol-3-phosphate dehydrogenase (*GPD1* and *GPD2*) and then by two isoforms of the glycerol-3-phosphatase (*GPP1* and *GPP2*). Glycerol can also be employed by *S. cerevisiae*

as a carbon and energy source, after being phosphorylated by the glycerol kinase (*GUT1*) and then oxidized to dihydroxyacetone phosphate by a mitochondrial glycerol-3-phosphate dehydrogenase (*GUT2*). An active mechanism for putative glycerol uptake has also been described in *S. cerevisiae*; physiological analyses suggest that, in ethanol-, glycerol- and acetate-growing *S. cerevisiae* cells, import of glycerol is guaranteed by a proton-symport system [5,6]. The putative genes responsible for glycerol uptake, *GUP1* and *GUP2*, have been identified by a transposon-mediated screening that allowed the characterization of mutants not capable of importing glycerol [7]. It is noteworthy that Gup1p is essential for glycerol-mediated recovery from salt stress in mutant strains unable to produce glycerol when they are grown in the presence of a small amount of glycerol, thus suggesting that Gup1p might be responsible for the glycerol symporter activity in *S. cerevisiae* [7]. However, recent reports do not exclude the possibility that the Gup1p has a more complex function in yeast cellular metabolism. A deletion of the *GUP1* gene resulted in an increase of triacylglycerols (triglycerides) and a concomitant decrease in phospholipids synthesis [8]. As reported by Bonangelino et al. [9], *GUP1* seems to be implicated in vacuolar protein sorting and it also appears to be involved in bipolar bud site selection [10]. Gup1p seemed to belong to the major facilitator superfamily [11], but recent insights based on sequence and function homology study of this protein indicate that it could be included in the Superfamily of membrane-bound *O*-acyltransferases [12,13]. Moreover, a recent report by Neves et al. [14] has shown that, in mutant yeast cells (deleted in *GUP1*, *GUP2* and *GUT1*), grown under salt stress conditions and collected during diauxic shift, a glycerol/H⁺ symport was

Abbreviations used: CLSM, confocal laser scanning microscopy; DAPI, 4',6'-diamidino-2-phenylindole; ER, endoplasmic reticulum; GFP, green fluorescent protein; IEM, immunoelectron microscopy; LFH, long flanking homology; ORF, open reading frame; SFH, short flanking homology.

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Table 1 Yeast strains used

Strain	Genotype	Reference
W303-1A	<i>MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100</i>	[50]
BHY54	Isogenic to W303-1A but <i>gup1::His51</i>	[7]
BHY66	Isogenic to W303-1A but <i>MATa gpd1::LEU2 gut1::KanMX gup1::His51</i>	[7]
RS80P2	Isogenic to W303-1A but <i>GUP1::GFP Kan^r</i>	The present study
NY17	<i>MATa sec6-4 ura3-52</i>	[51]
RH1800	<i>MATa his4 leu2 ura3 bar1-1</i>	[30]
RH1623	<i>MATa his4 leu2 ura3 bar1-1 end3-1</i>	[30]

still detectable. However, the same mutant strain did not show any glycerol-active transport when cultured in a non-fermentable carbon source, i.e. ethanol [7]. The authors interpreted the above findings as indicating that *GUP1*, *GUP2* and *GUT1* are associated with glycerol transport, but none of them could be considered as responsible for the glycerol-active transport in *S. cerevisiae* [14]. To understand the mechanisms behind Gup1p, we constructed *GUP1* gene fusions with the GFP (green fluorescent protein) gene, which has proved to be a useful tool for monitoring biochemical and cellular phenomena [15,16]. In the present study, the subcellular localization and functional expression of biologically active Gup1-GFP chimaeras have been monitored by confocal laser scanning and electron microscopy. To our knowledge, this is the first study of *GUP1* dynamics in live yeast cells and its membrane topology.

MATERIALS AND METHODS

Strains and growth conditions

The *Saccharomyces cerevisiae* strains used in the present study are listed in Table 1. The *Escherichia coli* strains employed in this study were DH5 α (F⁻, Φ 80d*lacZ*Δ*M15*, Δ*lacZYA-argF*) and BL21 [*E. coli* B F⁻, ompT, hsdS (r_B⁻, m_B⁻), gal, dcm]. Standard procedures were used for manipulating bacterial cells and recombinant DNA [17,18]. Yeast cultures were grown using either a rich [YP medium = 1 % (w/v) yeast extract and 2 % (w/v) peptone] or a minimal medium [YNB medium = 0.67 % (w/v) yeast nitrogen base, supplemented with adequate quantities of auxotrophic requirements]. Carbon sources for yeast cell growth were glucose (2 %, w/v), galactose (2 %, w/v) or glycerol (2 %, v/v). Yeast strain maintenance was achieved by plating on to YP-glucose or YNB-glucose medium supplemented with 2 % (w/v) agar. All yeast strains were grown at 30 °C except for strains with temperature-sensitive alleles, which were grown at permissive (23 °C) or restrictive (37 °C) temperatures. Cultures were always harvested during the exponential phase of growth. To study *GUP1* gene regulation, glucose-containing media were used for the growth of yeast cells under repression conditions, whereas yeast induction was obtained by incubating cells, previously grown under repression conditions, for 4 h in YP or YNB medium supplemented with glycerol or galactose.

Plasmid construction

To clone the *GUP1* gene in C-terminal fusion with the yeast-enhanced GFP gene (yEGFP) [19], the two genes were PCR-amplified separately using the *GUP1*-specific GUPforH (forward)/GUPrevB (reverse) and the EGFP-specific GFPforB (forward)/GFPprevE (reverse) primer pairs respectively. This yielded two amplicons, named GUP1_N and EGFP_C. N-terminal fusion of the *GUP1* gene with the EGFP cistron was performed by amplifying both sequences, by PCR, with the EGFP-specific GFPforH (forward)/GFPprevB (reverse) and the *GUP1*-speci-

fic GUPforB (forward)/GUPrevE (reverse) primer pairs, thereby producing two other amplicons, named EGFP_N and GUP1_C (see primer sequences in Table 2). After digestion with BamHI, the GUP1_N/EGFP_C and EGFP_N/GUP1_C couples were separately ligated with T4 DNA ligase and further digested with HindIII and EcoRI. After purification by agarose gel electrophoresis, the *GUP1*-EGFP and EGFP-*GUP1* fusion genes were separately cloned into the HindIII and EcoRI sites of pYES2 vector (Invitrogen, Carlsbad, CA, U.S.A.), downstream of the *GAL1* promoter. The two recombinant clones obtained, designated pY-GUPGFP and pY-GFPGUP, were used to transform yeast cells by the improved lithium acetate method [20].

GUP1-GFP chimaera construction and its homologous recombination

A genetic fusion between *GUP1* and GFP was performed to produce a chimaerical gene at the genomic *GUP1* locus of the W303-1A strain, by using the flanking homology PCR cassette method [21]. As a first approach, the SFH (short flanking homology) PCR (SFH-PCR), using the 5SFH/3SFH primer pair, was attempted (Table 2). Primer 5SFH has the first 40 bp at its 5'-end homologous with the 3'-end of the *GUP1* ORF (open reading frame) (excluding the stop codon) followed by 20 bp of sequence homologous with the 5'-end of the GFP reporter gene inserted into the plasmid pFA6a-GFP(S65T)-kanMX6. Primer 3SFH has its first 40 bp complementary to the 40 nt downstream of the *GUP1* ORF, followed by 20 bp complementary to the 3'-end of the *ADHI* terminator present in the plasmid pFA6a-GFP(S65T)-kanMX6. The resulting amplicon of 2504 bp was purified and then used to transform yeast cells [20]. Because of our inability to obtain a transformant yeast line by the above SFH-PCR strategy, we subsequently used the LFH (long flanking homology) technique (LFH-PCR) [22,23]. This method utilizes dual-step PCR to produce a sufficient amount of the ORF targeting module with much longer flanking homology than those obtained with SFH-PCR. In fact, by using 5LFH/pB and pC/3LFH primer pairs separately (Table 2), a 224 bp fragment (with its terminus localized 13 bp upstream of the *GUP1* stop codon) and a 161 bp fragment (with an initial sequence of 9 bp downstream of the *GUP1* stop codon) were obtained. In a first fusion-PCR step, the 224 bp fragment was fused to the previously produced 2504 bp sequence and the amplicon obtained was gel-purified and fused to the 161 bp fragment in a second fusion PCR [22]. The final PCR product was used as the template for a further PCR performed using 5LFH/3LFH primer pair (Table 2).

The final amplification product (2831 bp) was first sequenced to evaluate the absence of any mutation event along the entire construct sequence, and then directly transformed into yeast cells [20]. Transformed cells were plated on to YPD plates and grown at 30 °C for 24 h and then replica-plated on to YPD plates supplemented with 200 mg/l Geneticin (G418; Invitrogen, Carlsbad, CA, U.S.A.). Yeast colonies positive to the first screening were

Table 2 Oligonucleotides used

The restriction sites introduced are shown in boldface. The 20 underlined nucleotides of primer 5SFH anneal to the 5'-end of the GFP ORF in the plasmid pFA6a-GFP(S65T)-kanMX6. The underlined 20 nt of primers 3SFH anneal to the 3'-end of the *ADH1* terminator in the plasmid pFA6a-GFP(S65T)-kanMX6.

Name	Sequence
GUPforH	GAGAAGCTTATGTCGCTGATCAGCATCCTG
GUPrevB	AGAGGATCCGCATTTTAGGTAAATCCGTCCT
GFPforB	CACGGATCCTCTAAAGGTGAAGAATTATTC
GFPprevE	GCGGAATTCTAATTTGTACAATTCATCCAT
GUPforB	AGAGGATCCTCGCTGATCAGCATCCTGTCT
GUPrevE	CCGGAATTCTCAGCATTTAGGTAAATCCGTCG
GFPforH	CCCAGCTTATGCTAAAGGTGAAGAATTA
GFPprevB	GAGGGATCCTTTGTACAATTCATCCATACC
5SFH	CAGAGAAGAAAGAAAGAGGCACGGAATTACCTAAATGCCGGATCCCGGGTTAATTA
3SFH	GATAGCAGTGTTATACAATTGATATTCGTAATTTGGCATGAATTCGAGCTCGTTAAAC
pB	ATCCGTGCCTCTTTTCTTC
pC	TTACGAATATCAATTGTATAACTGC
5LFH	TGGTACAGACACGTTTGC
3LFH	CGAGAGGATTTTATCCATC
G1	CCAGAAATTTTGCTACCCA
KAN1	CGATAGATTGTCGCACCTG
KAN2	CCATCCTATGGAACCTGCCTC
G2	AAAGGGACTGTGGATCCAGAA
GFP1	AAAGGATCCATGAGTAAAGGAGAAGAATTTTC
GFP2	AAAGAATCTTTGTATAGTTCATCCATGCC

restreaked on to fresh YPD/G418 plates for further selection. Clones proven to be viable after both selection steps were analysed by qualitative PCR, as described by Paiva et al. [24]. Molecular biology techniques were used according to standard procedures [18].

Preparation and analysis of yeast protein extracts

Extraction of yeast protein was performed as described by Ausubel et al. [17] with the following modifications. Cells (10 ml culture) were grown at mid-exponential phase and harvested by centrifugation at 1500 *g*. Cells were suspended in 2.5 ml of ice-cold extraction buffer (20 mM Tris/HCl, 10 mM MgCl₂, 1 mM Na₂-EDTA, 5 % glycerol, 1 mM dithiothreitol, 0.3 M ammonium sulphate and 1 mM PMSF) supplemented with 50 μ l of protease inhibitor cocktail (Sigma–Aldrich) and sonicated with Branson Sonifier (Model 250-D) by alternating six times, 30 s cycles of burst on ice, with incubation on ice for approx. 1 min. The lysate was centrifuged at 12000 *g* for 20 min and the pellet was suspended and sonicated again as described above. The obtained slurry was added to 2 % (w/v) SDS, incubated under agitation for 1 h at room temperature (25 °C) and finally centrifuged at 12000 *g* for 10 min. After the addition of 2 vol. of ice-cold acetone, the supernatant was centrifuged at 12000 *g* for 10 min and the pellet was suspended in 50 μ l of Laemmli's buffer (0.035 M Tris/HCl, pH 6.8, 1.5 % SDS, 20 % glycerol and 3.5 % 2-mercaptoethanol). Protein samples were resolved by electrophoresis on 12 % (w/v) polyacrylamide gels and Western blots were performed as described by Grieco et al. [25]. Visualization of antigen–antibody complexes was performed by chemiluminescent assay (SuperSignal West Pico; Pierce, Rockford, IL, U.S.A.) following the manufacturer's instructions.

Anti-GFP antiserum production

The sequence of the S65T version of the GFP gene was amplified by PCR using the GFP1/GFP2 primer pair (Table 2). The amplicon obtained was digested with BamHI and EcoRI, ligated into identically cut pGEX-6P-1 plasmid (Amersham Biosciences, Piscataway, NJ, U.S.A.) and cloned in *E. coli* BL21 strain. The

GST–GFP fusion protein (where GST stands for glutathione S-transferase) was expressed and purified as described by Hay et al. [26]. Approximately 300 μ g of GST–GFP fusion protein was dissolved in 500 μ l of PBS (0.02 M sodium phosphate buffer, pH 7.4, and 0.15 M sodium chloride) and emulsified with an equal volume of complete Freund's adjuvant (1 mg/ml inactivated *Mycobacterium tuberculosis* dissolved in 85 % paraffin oil and 15 % mannide mono-oleate) before subcutaneous injection into a rabbit; four weekly booster injections of the same amount of fusion protein, emulsified with incomplete Freund's adjuvant (85 % paraffin oil and 15 % mannide mono-oleate), were given before bleeding. The crude antiserum obtained was routinely used at 1:5000 dilution for Western-blot assays.

Protease protection assay

The method described by Garnier et al. [27] was modified as follows. Yeast cells, transformed with pY-GUPGFP or pY-GFPGUP, were induced with galactose for 4 h. They were then collected, washed with buffer C (50 mM NH₄HCO₃ and 0.5 % 2-mercaptoethanol) and then suspended at 7×10^8 cells/ml in buffer C with 1.4 M sorbitol added. Spheroplasts were obtained by digesting the cells for 30 min at 30 °C in the presence of 150 units/ml Lyticase (Sigma–Aldrich). A 400 μ l aliquot of protoplasts was digested for 10 min at 25 °C with 100 μ g/ml Tos-Phe-CH₂Cl (tosyl-phenylalanylchloromethane; 'TPCK')-treated trypsin (Promega, Madison, WI, U.S.A.). Where indicated, Triton X-100 was included in the reaction mixture at a final concentration of 0.05 %. The reaction mixture was supplemented with trichloroacetic acid to a final concentration of 10 % and the protein extracts were incubated for 20 min on ice and then centrifuged for 15 min at 14000 *g*. Pellets were neutralized with 40 μ l of 1 M Tris base, dissolved by adding 60 μ l of 2-fold concentrated Laemmli's buffer and then heated at 37 °C for 15 min. Aliquots (10 μ l) were then subjected to SDS/PAGE and immunoblotting.

Electron microscopy

To avoid time-consuming centrifugation at each step, cells were collected from the growth medium by filtration and embedded in agar. Cells were fixed in 2 % (w/v) paraformaldehyde and

0.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7), with 1.2 M sorbitol added for 3 h at 4°C. After several washings, samples were incubated in a cell-wall reducing buffer (20 mM Tris/HCl, pH 8, 5 mM Na₂-EDTA, 25 mM dithiothreitol and 1.2 M sorbitol) for 20 min at 6°C. Cells were then incubated for 2 h at 30°C in 200 µl in a cell-wall digestion buffer (0.1 M potassium phosphate/citrate, pH 5.8, and 1.2 M sorbitol) with the addition of 1000 units of β-glucuronidase (MP Biomedicals, Irvine, CA, U.S.A.) and 0.1 mg of zymolyase (Sigma–Aldrich). Samples were washed three times with the cell-wall digestion buffer, fully dehydrated in a graded ethanol series and infiltrated with LR-White (Sigma–Aldrich), which was polymerized for 24 h at 60°C. Ultra-thin sections were cut on a Leica ultra microtome and collected on Formvar-coated grids. Selected grids were preincubated on droplets of 1 % BSA-PBS (3 × 5 min). The grids were then transferred on to droplets of 5 % (v/v) normal goat serum for 45 min and then incubated with anti-GFP antibody diluted to 1:400 with BSA-PBS for 2 h at room temperature in a wet chamber. The sections were washed with BSA-PBS (6 × 5 min) and incubated for 1 h in droplets of 10 nm gold-conjugated goat anti-rabbit IgG. After washing with 1 % BSA-PBS (3 × 5 min) and PBS (3 × 5 min), sections were stained with 1 % uranyl acetate (5 min) and for 2 min in Reynold's lead citrate and (0.025 M lead citrate, 0.120 M sodium citrate and 0.160 M NaOH) and studied using a Philips EM 400T electron microscope. For the immune reaction controls, the procedure was similar but the grids were incubated with either the pre-immune serum or the secondary antibody only. The density of labelling associated with the inner or outer side of the plasma membrane was calculated and expressed as gp (number of gold particles)/µm² ± S.D. For each experiment, the amount of labelling on specific areas was determined by counting the number of gp on 20 different micrographs.

Fluorescence microscopy

Samples from *S. cerevisiae* cells expressing Gup1p itself or in fusion with GFP were directly observed using the Zeiss LSM5 Pascal confocal laser scanning microscope to obtain confocal images (objectives 40.0/1.0, 63.0/1.0 oil). Microscope filter for FITC was employed for detecting GFP fluorescence. For staining of yeast nucleus with DAPI (4',6-diamidino-2-phenylindole), cells were harvested, suspended in 70 % (v/v) ethanol and fixed for 15 min. After two washes with water, the cells were resuspended in mounting medium (90 % glycerol, 1 × PBS, 1 mg/ml *p*-phenylenediamine and 0.25 µg/ml DAPI) and observed with UV filter set. The final images were produced using Adobe Photoshop (Adobe Systems, San José, CA, U.S.A.).

RESULTS

Episomal expression and localization of GFP–Gup1 and Gup1–GFP fusion proteins

The first step of this study was the expression of two Gup1p variants with an N- and C-terminal in-frame fusion with the GFP to detect the subcellular localization of the resulting chimaerical proteins. In order to address this task, both *GUP1*–GFP and GFP–*GUP1* fusion genes were amplified by PCR and separately inserted into the pYES2 vector, under the control of the strong galactose-inducible *GALI* promoter. Two plasmids, called respectively pY-GUPGFP and pY-GFPGUP, were used to transform the wild-type (W303-1A) and the mutant BHY66 strains [7]. The latter strain is deleted in *GUP1* and in *GPD1* and *GUT1*, thus resulting in defective biosynthesis, utilization and uptake of glycerol. To ensure that any observed effect in the transformant

lines was caused by the presence of Gup1p fusions with GFP and not by any undesired effect due to the GFP itself, the BHY66 strain was also transformed with the pYES2 vector containing the GFP gene alone. The obtained transformant strains were collected and tested by PCR analysis to confirm the presence of chimaerical constructs.

In order to evaluate whether chimaeras act as functional proteins, a phenotype analysis under stress conditions was performed on the BHY66 strain, transformed with both the *GUP1*–GFP and GFP–*GUP1* chimaerical genes, on the wild-type (W303-1A) and the BHY66 strains. As control, the BHY66 was also transformed with pYES2 vector containing the GFP gene. All tested strains were grown overnight and then spotted in 10-fold dilution on YP-galactose plates containing 1 M NaCl and supplemented with 10 mM glycerol (Figure 1A). As expected, the untransformed BHY66 strain showed a reduced growth with respect to the wild-type strain, which was not remedied by the presence of 10 mM glycerol. The expression of GFP alone in BHY66 cells did not induce any improvement in the above phenotype. On the contrary, the introduction of chimaerical *GUP1*–GFP and GFP–*GUP1* genes into the BHY66 strain restored wild-type growth in both cases (Figure 1A).

Both the distribution and localization at the subcellular level of Gup1–GFP and GFP–Gup1 fusion proteins in living cells were examined by CLSM (confocal laser scanning microscopy). Strong cellular fluorescence predominantly localized at the cell boundaries and also at the ER (endoplasmic reticulum) level, 4 h after induction in both modified cells (Figures 1B and 1C). The peripheral fluorescent signal was coincident with concanavalin A–Texas Red staining of the cell wall (results not shown). Cells expressing only GFP, used as control, showed a fluorescence signal scattered in the entire cytoplasm compartment. It is remarkable that no vacuolar staining occurred after 4 h of expression, thus indicating that both fusion proteins were very stable in galactose-grown cells. The induction of GFP–Gup1p gave rise to cellular staining as quickly as the Gup1p–GFP, thus suggesting that both fusion proteins did not encounter any problem in their folding and a subsequent delay in their trafficking. Western-blot analysis was performed by probing protein extracts from BHY66 cells expressing Gup1p–GFP and GFP–Gup1p fusions with the anti-GFP serum. The assay allowed identifying, in both the extracts, only the expected 95 kDa band, whereas no free GFP was detectable (Figure 1D). The above findings allowed us to hypothesize that both the Gup1p–GFP and GFP–Gup1p chimaerical proteins were functional and mainly localized in the plasma membrane and ER.

Construction and expression of the genetically fused Gup1p–GFP fusion protein

To study the expression, trafficking and turnover of Gup1p under conditions close to wild-type environment, a construct with the GFP gene fused to the end sequence of the *GUP1* gene, corresponding to the C-terminus of Gup1p, was integrated into the genome of a wild-type strain at the genomic *GUP1* locus. Using PCR, a DNA amplicon, consisting of the GFP–kanMX cassette [21] modified by long flanking regions, was prepared. Initial and terminal regions of the construct were derived from 237 bp of the C-terminal corresponding sequence of *GUP1* gene and 170 bp of the genomic sequence immediately downstream of the gene respectively. The *S. cerevisiae* W303-1A strain was transformed by the above-mentioned PCR fragment using a homologous recombination approach to produce the chimaerical *GUP1*–GFP gene (Figure 2A). The correct targeting of the GFP–kanMX cassette at the selected genomic locus was verified by PCR analysis in a large number of colonies derived from the produced

Figure 1 Episomal expression of Gup1-GFP and GFP-Gup1 fusions

(A) Complementation of the BH66 mutant strain (*gpd1, gut1, gup1*) with Gup1-GFP and GFP-Gup1 fusion proteins. Cells were grown in YPD to $A_{600} \sim 3$ and then diluted to $A_{600} 1$; $10 \mu\text{l}$ of a 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} diluted strains were spotted on to YP-galactose plates containing 1 M NaCl and supplemented with 10 mM glycerol. Lane 1, W303-1A; lane 2, BH66 transformed with pY-GFP-GUP; lane 3, BH66 transformed with pY-GUP-GFP; lane 4, BH66 transformed with pYES2 expressing GFP alone; lane 5, BH66. (B) *In vivo* localization of Gup1-GFP fusion protein by CLSM analysis of the BH66 strain transformed with pY-GUP-GFP after 4 h induction (upper panel). The corresponding DAPI staining of the nuclear DNA is shown (lower panel). (C) *In vivo* localization of GFP-Gup1 fusion protein by CLSM analysis of BH66 strain transformed with pY-GFP-GUP 4 h after induction (upper panel). The corresponding DAPI staining of the nuclear DNA is shown (lower panel). (D) Immunological detection of Gup1-GFP (lane 1) and GFP-Gup1 (lane 2) fusion proteins in BH66 transformed cells. Protein extract from untransformed BH66 cells is probed in lane 3. Molecular-mass standards (in kDa) are on the right of the panel. Arrowhead marks the presence of a protein band at the expected molecular mass of 95 kDa.

kanamycin-resistant line reported as RS80P2. The strategy used and the primers (listed in Table 2) are shown in Figure 2(B). PCR analysis was performed on both recombinant RS80P2 and W303-1A strains by using primer pairs G1 and G2, G1 and KAN1, KAN2 and G2. The amplification assays, of ten different clones of the RS80P2 transformed line, gave clear-cut confirmation that the amplicons bearing the GFP sequence were correctly integrated into the W303-1A genomic *GUP1* locus. The presence of the *GUP1*-GFP fusion gene was also confirmed by Western-blot analysis. The expected 95 kDa band, deriving from the Gup1p-GFP fusion protein, was specifically recognized in the protein extract of RS80P2 probed with an anti-GFP polyclonal antiserum, whereas this band was not detectable in the protein extracts from the untransformed W303-1A strain (Figure 2C).

The behaviour of the RS80P2 strain under osmotic stress was compared with that of the W303-1A and BH54 strains, isogenic to W303-1A but with *GUP1* deleted [7], by testing their growth phenotype on YPD containing 1 M NaCl supplemented with 10 mM glycerol (Figure 2D). The RS80P2 strain showed a phenotype comparable with the W303-1A wild-type strain, thus indicating that the GFP C-terminal tagging did not impair Gup1p functionality under salt stress.

Subcellular localization of the Gup1-GFP fusion protein

The expression and subcellular localization of the Gup1-GFP fusion protein was monitored in living RS80P2 cells by CLSM and by IEM (immunoelectron microscopy). RS80P2 cells were grown overnight in YNB-glucose medium until an absorbance A_{600} of approx. 0.5 was reached, washed twice with sterile distilled water and then transferred on to YNB medium containing glycerol (2%) as the unique carbon and energy source. After moving the cells to the glycerol-supplemented culture medium, the expression and localization of the fusion protein were checked by CLSM over a 24 h extended period. The fluorescence signal was not detected in the first hour; then, gradually, it was clearly localized to the plasma membrane. It reached its maximum intensity 4–6 h after glycerol induction (Figure 3A). The pattern did not show any change up to the 24 h observation period (results not shown).

The *in vivo* localization of the Gup1-GFP fusion protein was confirmed by IEM assay probing of the anti-GFP polyclonal antiserum on an ultra-thin section of 4 h-glycerol-induced RS80P2 cells. As shown in Figure 3(B), immune gold labelling was clearly detectable on the periphery of the cell, mainly associated with the plasma membrane and particularly with the inner side of the membrane. No signal was detected by IEM when preimmune antiserum was used alone or with W303-1A cells incubated with anti-GFP serum.

Comparing the CLSM analysis of RS80P2-induced cells and that of the W303-1A strain, transformed with the pYES2 episomal vector containing a construct consisting of the *GUP1* gene fused to the GFP gene under a galactose-inducible promoter, a similar fluorescence pattern was obtained. This result indicated that the overexpression of the fusion protein did not give any localization artifact.

Gup1p membrane topology

We investigated the Gup1p membrane topology by IEM analysis using the Gup1-GFP chimaeras expressed by the pYES2 vector system. Ultra-thin sections of galactose-induced W303-1A cells, transformed with pY-GFP-GUP and probed with the anti-GFP serum, revealed a sharp immune gold labelling associated with the outer side of the plasma membrane (8.6 ± 1.2 versus $0.6 \pm 0.2 \text{ gp}/\mu\text{m}^2$ found in the inner side), corresponding to the periplasmic space (Figure 4A). The same experiment, performed by using cells expressing Gup1p with a C-terminal fusion with the GFP gene (pY-GUP-GFP), revealed a clear immune signal decoration on the inner (cytoplasm) side of the plasma membrane (9.3 ± 1.4 versus $0.9 \pm 0.3 \text{ gp}/\mu\text{m}^2$ found in the outer side). As expected, this last decoration pattern was also observed in RS80P2 cells, which expressed the Gup1p-GFP fusion protein after C-terminal insertion of the GFP gene into the *GUP1* chromosomal locus (Figure 3B). These results indicate the localization of the Gup1p N- and C-terminal portions with respect to the plasma membrane. To confirm the above evidence on Gup1p membrane topology, yeast protoplasts expressing Gup1p-GFP or GFP-Gup1p fusion proteins were subjected to a protease protection

Figure 2 Strategies followed for the construction of *GUP1*-GFP fusion and properties of the expressed chimaical protein

(**A**) *GUP1*-GFP fusion. Long flanking regions were added to the GFP-kanMX cassette as described in the Materials and methods section. The PCR product obtained was used to transform the W303-1A strain. A strain bearing the *GUP1*-GFP fusion gene at the genomic *GUP1* locus, named RS80P2, was obtained. (**B**) Analytical PCR assay to check the gene fusion by homologous recombination. Template DNA was extracted from RS80P2 and W303-1A cells and used for PCR assay. The amplicons were subjected to agarose gel electrophoresis and the size of the produced fragments is indicated. (**C**) Immunological detection of Gup1-GFP fusion protein in RS80P2 (lane 1) and W303-1A cells. Asterisk marks the presence, in the RS80P2 lane, of a protein band at the expected molecular mass of 95 kDa. Molecular-mass standards (in kDa) are shown on the right of the panel. (**D**) Phenotypes of RS80P2 (lane 1), BHY54 (*gup1*, lane 2) and W303-1A (lane 3) strains on YPD medium containing 1 M NaCl and supplemented with 10 mM glycerol. Cells were spotted and grown as described in Figure 1.

assay. Spheroplasts were digested with trypsin in the presence or absence of Triton X-100 and the protein extracts obtained were probed by immunoblotting using anti-GFP antiserum. When intact protoplasts were exposed to trypsin, the protease should not have access to the GFP tag located in the cytoplasm, whereas it should be able to digest the GFP tag exposed in the periplasmic space. The GFP expressed in N-terminal fusion with the Gup1p was almost completely digested by trypsin treatment (100 µg/ml) of intact protoplasts (Figure 5A, lane 2). The minimal amount of GFP-Gup1p still detectable is likely to consist of the fusion protein localized at the ER level (Figure 1C). A similar assay performed on sealed spheroplasts, obtained from cells expressing the Gup1p-GFP fusion protein, did not lead to GFP degradation (Figure 5B, lane 2). However, when protease protection experiments were performed in the presence of a non-ionic detergent (Triton X-100), both N- and C-terminal GFP tags appeared to be extremely sensitive to trypsin digestion (Figure 5A, lane 3; Figure 5B, lane 3), thus indicating that the GFP in fusion

with the C-terminus of Gup1p was accessible to protease only after the permeability barrier was destroyed by the detergent.

Taken together with the results obtained by the IEM analysis, these results strongly suggested a possible model of Gup1p membrane topology: the extracytosolic N-terminus is separated by an odd number of intramembrane domains from the intracellular C-terminal end (Figure 4C).

Inactivation of Gup1-GFP by glucose

To evaluate whether Gup1p has any turnover regulation with respect to its plasma-membrane location, RS80P2 cells were induced with a YNB medium with 2% glycerol as the unique carbon source for 4 h and, after two washings in sterile water, they were collected and subsequently transferred on to the YNB medium containing 2% glucose. The fluorescence was monitored by CLSM observation during a continuous incubation period at 30°C (Figure 6). In the glucose-treated cells, the fluorescent

Figure 3 Subcellular localization of Gup1-GFP

Localization of Gup1-GFP in RS80P2 cells by CLSM (**A**) and by IEM (**B**), using an anti-GFP serum as primary antibody. Arrowheads point to colloidal gold particles, indicating the localization of the fusion protein. Scale bar, 0.2 μm .

signal was almost totally removed from the plasma membrane and was concentrated in intracellular globular structures 5 min after incubation; 30 min after treatment with glucose, the fluorescence could be detected in spherical fluorescence intracellular aggregates, which were likely to be vacuoles. This identification was helped by their co-localization with vacuoles stained with 7-amino-4-chloromethylcoumaryl-L-arginine amide dihydrochloride (CMAC-Arg; results not shown).

GUP1-GFP protein targeting to the plasma membrane

The secretory pathway involved in Gup1p targeting to the plasma membrane was determined by studying the episomal expression of the Gup1-GFP chimaerical protein in a strain carrying a temperature-sensitive allele of the *SEC6* gene ($\Delta\text{sec6-4}$ strain), which is required for the fusion of post-Golgi vesicles with the plasma membrane. In fact, the mutant strain $\Delta\text{sec6-4}$ shows an accumulation of secreted vesicles in the cytoplasm (at the restrictive temperature of 37°C) [28,29]. Expression of Gup1-GFP protein induced in this strain at a permissive temperature (23°C) resulted in a fluorescent signal clearly associated with the plasma membrane and the ER (Figure 7). When the fusion protein was expressed in the above strain at the restrictive temperature (37°C), a similar fluorescence level was observed in the globular bodies within the cell, i.e. vacuoles, but not associated with the plasma membrane (Figure 7). As reported previously, the *sec6-4* mutation was reversible when cells grown at the restrictive temperature were restored to the permissive temperature [30]. This assay was performed on the $\Delta\text{sec6-4}$ strain, expressing Gup1-GFP fusion, in the presence of cycloheximide to exclude any *de novo* synthesis of the chimaerical protein. The fluorescence pattern of those cells taken back to the permissive temperature showed a distinct signal associated with the plasma membrane and the ER, with the absence of any fluorescence signal at the intracellular level (Figure 7).

Figure 4 Gup1 protein membrane topology

Immunoelectron microscopy detection of GFP-Gup1 (**A**) and Gup1-GFP (**B**) in BHY54 cells transformed with pY-GFP-GUP and pY-GUP-GFP respectively. Arrowheads point to colloidal gold particles, indicating the localization of fusion proteins. Scale bar: (**A**) 0.08 μm and (**B**) 0.1 μm . (**C**) Hypothetical model of Gup1p membrane topology.

Removal of Gup1-GFP protein from the plasma membrane

Strains bearing the *end3-1* defective allele have been demonstrated to show temperature-sensitive defects in endocytosis [30]. Gup1p-GFP episomal expression was induced in the RH1623 (*end3-1*) strain and in the RH1800 (*END3*) wild-type strain by incubating both cultures at the permissive temperature (23°C) for 4 h. As expected, after this period, there was a clear localization of the fluorescence at the plasma membrane level in both the analysed strains. At this time, the two cultures were divided into four aliquots: two were kept at the permissive temperature and the other two were transferred to the restrictive temperature (37°C). At each temperature (23 and 37°C), one of the aliquots was supplemented with glucose at a final concentration of 2% (w/v). All the cultures were examined by CLSM 1 h after incubation (Figure 8). The aliquots of RH1623 (*end3-1*) and RH1800 (*END3*) strains expressing Gup1-GFP fusion protein and not supplemented with glucose showed a sharp fluorescent labelling at the plasma membrane, at both 23 and 37°C incubation

Figure 5 *In vivo* protease protection assay

Trypsin sensitivity of protoplasts prepared from yeast cells transformed with pY-GFPGUP (**A**) or pY-GUPGFP (**B**). Each spheroplast preparation was divided into equal portions, which received trypsin (TRY) and Triton X-100 (TX100), as indicated. The digests were subjected to SDS/PAGE (12% polyacrylamide) and Western blotting using anti-GFP serum. Molecular-mass standards are shown on the right of the panels.

temperatures. The presence of 2% glucose stimulated the accumulation of fluorescence at the intracellular level in both mutant and wild-type strains incubated at the permissive temperature, but only the RH1800 cells grown at restrictive temperature showed internalization of the chimaerical protein. The RH1623 cells, incubated at 37°C after glucose addition, had a clear retention of Gup1–GFP protein in the plasma membrane and the location was constant after prolonged incubation (12 h). These findings were interpreted as indicating that Gup1–GFP fusion protein is removed from the plasma membrane by endocytosis through *END3* and then targeted to the vacuole for inactivation.

DISCUSSION

S. cerevisiae has been reported to have a glycerol/H⁺ symport activity, which allows it to take up glycerol from the outside against a concentration gradient [5]. Holst et al. [7] also demonstrated that the multimembrane-spanning protein encoded by the *GUP1* gene was essential for proper growth on glycerol and for glycerol-mediated recovery from salt stress. However, a recent

Figure 6 Inactivation of Gup1–GFP

Glycerol-induced cells of the RS80P2 strain treated with glucose and examined by CLSM after a continuous incubation period (see the Results section).

Figure 7 Expression of Gup1–GFP in a secretory mutant

Gup1–GFP expression induced in the NY17 strain, a temperature-sensitive mutant strain of the *SEC6* gene, which regulates the fusion of the secretory vesicles with the plasma membrane. Cells were induced in YNB containing glycerol (2%) 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 µg/ml cycloheximide and incubated at 23°C for a further 2 h. The corresponding DAPI staining of the nuclear DNA is shown (lower panels).

Figure 8 Expression of Gup1-GFP in an endocytosis mutant

Gup1-GFP expression was induced for 4 h in both RH1623 and RH1800 strains at 23 °C (see the Materials and methods section). Each culture was then divided into four aliquots: two were maintained at the permissive temperature, and two were transferred to the restrictive temperature (37 °C). Glucose (2 % final concentration) was added to one of the aliquots at each temperature. All the cultures were visualized by CLSM 1 h after incubation.

report [14] has indicated that *GUP1* should not be considered as the gene responsible for glycerol uptake, but that it could be involved in the regulation of the glycerol/H⁺ symport activity. To contribute to a better understanding of the properties and function of Gup1p, various constructs involving *GUP1* gene fusion with the GFP gene were produced and their expression patterns, together with the cellular trafficking of the obtained fusion proteins, were investigated.

Previous analyses of a number of different *S. cerevisiae* plasma-membrane proteins have been performed by tagging them with the GFP, such as Hxt2p [31], Hxt5p [32], Hxt7p [33], Pho84p [34] and Can1p [35]. In all these studies, the fusion proteins produced were proven to retain their biological function, with properties similar to those belonging to the same proteins not fused to any reporter gene. We observed that the Gup1-GFP fusion protein is strongly localized in the plasma-membrane compartment of induced cells. This observation is in agreement both with bioinformatic predictions performed by PSORTII (<http://psort.nibb.ac.jp/>) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) programs and with the evidence of Gup1p presence in the protein fraction containing plasma membranes [7]. Results obtained when the fluorescence signal was analysed in strains expressing the Gup1p fused with GFP at the genome *GUP1* locus directly and those obtained by episomal insertion of the *GUP1* gene fused to the GFP gene in the pYES2 vector under the control of the galactose-inducible promoter *GAL1*, were comparable. However, in the latter case, a stronger signal, similar to that obtained during localization studies of Cyclin Clb2 in yeast, was usually detected [36]. One of the reasons for the stronger fluorescent signal may be found in the high expression efficiency of the *GAL1* promoter, which cannot be compared with the very low cellular expression level of *GUP1* due to its own weak promoter [7,14]. The stronger signal obtained by the episomal expression can also be explained by the fact that, in pYES2 vector, the *GUP1* gene is fused to the yEGFP gene, which is a GFP gene proven to be more efficiently expressed in yeast with respect to the S65T version used for tagging *GUP1* in its genomic locus [19].

When the fluorescent signal was monitored in RS80P2 cells, the maximum expression level of the genetically expressed Gup1-

GFP fusion protein, in terms of fluorescence intensity, was achieved 4–6 h after induction with glycerol and this pattern remained constant over a 24 h period, suggesting that the Gup1-GFP chimera constitutively undergoes a moderate rate of turnover. However, a rapid stress-stimulated turnover of the above fusion protein in glycerol-cultured RS80P2 cells was clearly detectable when treated with glucose. This result appeared as very striking evidence, which adds Gup1p to the list of yeast proteins that are rapidly down-regulated by changing the carbon source in the culture medium. This observation is in agreement with the concept that post-translational modifications triggered by glucose, which could be referred to as an additional example of rapid ubiquitin-dependent down-regulation of a plasma-membrane transporter under defined experimental conditions [37], affects proteins of peripheral functions such as Hxt2p [31] and Jen1p [24], as well as enzymes with central functions [38] and receptors for the mating pheromones [39]. Ubiquitination is used by all eukaryotic organisms for controlling the protein stability and intracellular localization and it is likely to be required for the endocytosis of a number of plasma-membrane proteins studied to date in yeast. As suggested by Horak [40], after covalent attachment of ubiquitin to specific lysine residues in target proteins, a wide range of transporter, channel and receptor proteins can be internalized through the endocytotic pathway and then degraded in the vacuole.

Indeed, after treatment with a high concentration of glucose, Gup1-GFP protein was removed from the plasma membrane, internalized and accumulated in the vacuole compartment for inactivation. In fact, interspersed fluorescent structures, possibly endocytic vesicles, were detected at the early stage of the glucose inactivation process and disappeared when the fluorescence was finally concentrated in the vacuole (Figure 6).

When expressed in a strain defective in endocytic vesicle movement (deleted in the *END3* gene), the Gup1p-GFP could not be internalized after glucose treatment, thus indicating that endocytosis is the mechanism involved in its carbon catabolite inactivation.

In *S. cerevisiae*, most of the peripheral proteins are moved to the plasma-membrane compartment by a secretory pathway, which

requires the *SEC6* gene product for the fusion of post-Golgi vesicles with the plasma membrane [28,29]. Expression of the Gup1–GFP fusion protein in a strain-defective form in the *SEC6* gene indicated that this gene is involved in delivering the chimerical product to the plasma-membrane level. These findings are consistent with the results obtained by studying secretory pathways utilized by a number of yeast proteins such as α -factor, invertase, Pma1 ATPase, Hxt2p and Jen1p [24,28,29,31,41]. Moreover, the expression of Gup1–GFP in the above secretory mutant strain supplied an evidence of Gup1p localization also at the ER level, since in the absence of *de novo* protein synthesis, the Gup1p is also redirected back to this organelle (Figure 7).

The GFP protein has also been used as a reporter molecule for determining the topology of Gup1p. This approach was employed for characterizing the membrane location of Big1p of *S. cerevisiae* [42] and was recently used for topology analysis of *E. coli* inner membrane proteins [43,44]. To improve the discriminative power of this analysis, we used the IEM technique to ascertain the location of Gup1p terminal regions with regard to the plasma membrane. To our knowledge, this approach, which has been previously used for characterizing the human carnitine palmitoyltransferase I [45], is applied for the first time to address the topology of a plasma-membrane protein in *S. cerevisiae*. The IEM assay on ultra-thin sections of cells expressing either Gup1–GFP or GFP–Gup1 fusion proteins allowed the localization of anti-GFP antibodies on the inner and outer sides of the plasma membrane respectively. It is remarkable that the GFP polypeptide fused to the N-terminus of Gup1p was efficiently translocated across the plasma membrane, without undergoing any additional processing, as shown by the absence of free GFP in cells expressing GFP–Gup1p fusion (Figure 1D). These findings, taken together with those obtained by protease protection analysis, suggest that the N-terminus of Gup1p lies in the periplasmic space, whereas its C-terminal tail has an intracellular location (Figure 4C), with a membrane topology similar to that reported for the yeast ammonium Mep2p [46] and copper Ctr1p [47] transporters. However, an extra cytosolic location of the N-terminal tail is not generally predicted or determined in yeast membrane transporters. This feature is a common property of the G-protein-coupled receptors, such as the α -factor receptor [48], and it had also been shown for the human MRP (multidrug resistance protein) [49].

The present study supports previous evidence [7] that Gup1p is localized in the plasma membrane and also gives the first experimental indication of its membrane topology. Additionally, this study made it possible to identify the Gup1p secretory pathway and to add this protein to the list of plasma-membrane proteins discharged into the vacuole for degradation. The biological properties of the Gup1–GFP fusion proteins confirm that Gup1p should be associated with glycerol metabolism. Even though, at present, no evidence of its direct role in glycerol-transport activity can be deduced, our findings indicate, for Gup1p, the typical behaviour of a transporter protein. Studies to determine the biological function of Gup1p and to understand better the control mechanisms of glycerol metabolism are in progress.

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