Functional expression, quantification and cellular localization of the Hxt2 hexose transporter of *Saccharomyces cerevisiae* tagged with the green fluorescent protein

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The Hxt2 glucose transport protein of *Saccharomyces cerevisiae* was genetically fused at its C-terminus with the green fluorescent protein (GFP). The Hxt2-GFP fusion protein is a functional hexose transporter: it restored growth on glucose to a strain bearing null mutations in the hexose transporter genes *GAL2* and *HXT1* to *HXT7*. Furthermore, its glucose transport activity in this null strain was not markedly different from that of the wild-type Hxt2 protein. We calculated from the fluorescence level and transport kinetics that induced cells had 1.4×10^5 Hxt2-GFP molecules per cell, and that the catalytic-centre activity of the Hxt2-GFP molecule *in vivo* is 53 s^{-1} at 30°C . Expression of

Hxt2-GFP was induced by growth at low concentrations of glucose. Under inducing conditions the Hxt2-GFP fluorescence was localized to the plasma membrane. In a strain impaired in the fusion of secretory vesicles with the plasma membrane, the fluorescence accumulated in the cytoplasm. When induced cells were treated with high concentrations of glucose, the fluorescence was redistributed to the vacuole within 4 h. When endocytosis was genetically blocked, the fluorescence remained in the plasma membrane after treatment with high concentrations of glucose.

Key words: endocytosis, glucose, secretion, yeast.

INTRODUCTION

Glucose is the preferred nutrient of *Saccharomyces cerevisiae* for carbon and energy. The concentration of glucose in the yeast's environment also regulates metabolic and cellular activity and gene expression. Transport of glucose and other hexoses is necessary for their metabolism and is also implicated in their regulatory effects. Glucose transport in yeast is mediated by proteins encoded by the *HXT* gene family, of which 20 members have been identified genetically or by sequence homology [1]. This is a remarkably large number of genes for a biochemically simple function in a unicellular organism; mammals express only six homologous glucose transporters [2].

Experimental evidence has implicated seven members of the *HXT* family in metabolically significant hexose transport; these are *GAL2* (the galactose transporter), and *HXT1* to *HXT4*, *HXT6* and *HXT7* (which transport glucose, fructose and mannose) [3,4]. Two other members of the family, *SNF3* and *RGT2*, encode glucose sensors [5,6]; the remaining 11 members are not well characterized [7]. The transport kinetics of the seven metabolically significant Hxt proteins has been measured *in vivo* [4]. Their affinities for glucose vary by two orders of magnitude, whereas the maximal uptake velocity of cells expressing these proteins individually differs only 3-fold. Assuming that the transporters have a similar catalytic-centre activity for glucose, this suggests that they are expressed to approximately the same level.

These *HXT* genes are differentially regulated at the levels of expression and inactivation in response to growth conditions [7]. However, under normal conditions the expression intervals of different *HXT* genes can overlap [8]. The transport kinetics of batch-cultivated cells change constantly during growth on glucose [9]. This reflects the arrival of newly synthesized transporters at the plasma membrane via the secretory pathway and the inactivation of transporters via endocytosis and degradation

[10–13]. The cell must therefore have a mechanism to remove from the membrane selectively only those transporters whose characteristics are inappropriate for the prevailing conditions (for example, low-affinity transporters in a low-glucose environment) while leaving their close homologues with appropriate characteristics in place.

HXT2 encodes a protein with high homology to other yeast sugar transporters [14]. *hxt2*-null mutant strains are partly defective in high-affinity glucose transport and in growth on low concentrations of glucose [3,14,15]. The expression of Hxt2 protein is stimulated by shifting batch-cultured cells from high-glucose to low-glucose medium [15]. These observations suggest that Hxt2 is a high-affinity glucose transporter. However, a strain expressing only *HXT2* displays peculiar transport kinetics: when grown on a low concentration of glucose it displays both high-affinity and low-affinity kinetics, whereas when grown on a high concentration of glucose, it displays transport kinetics of intermediate affinity.

To investigate further the properties and regulation of Hxt2 we have tagged it with the green fluorescent protein (GFP) of *Aequorea victoria*. GFP matures after translation to be an intrinsically fluorescent protein [16]. It has a number of characteristics that make it a useful tool for monitoring biochemical and cellular phenomena. First, genetically constructed chimaeras between GFP and other proteins frequently display functional characteristics that are indistinguishable from those of the native proteins. For example, a chimaera between GFP and the OSCP (oligomycin sensitivity conferring protein) subunit of the yeast mitochondrial ATPase is translocated into mitochondria and assembled into functional ATPase complexes [17]. Secondly, GFP can be a quantitative reporter of protein abundance by the use of fluorimetric and spectrophotometric techniques. The detection threshold of GFP fluorescence in the cytoplasm has been reported to be 200 nM [18]. Assuming a cytoplasmic volume

Abbreviations used: CMAC-Arg, 7-amino-4-chloromethylcoumaryl-L-arginine amide dihydrochloride; conA, concanavalin A; FOA, 5-fluoro-orotic acid; GFP, green fluorescent protein.

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of 500 nl per 10^7 cells [19], this corresponds to 6000 molecules per yeast cell. Thirdly, the fluorescent signal from a GFP-tagged fusion protein can be used to monitor the subcellular localization and dynamics of protein trafficking in living cells. For example, Niedenthal et al. [20] tagged three open reading frames of unknown but essential function in yeast chromosome XIV with GFP. The chimaeric genes complemented null mutations of each locus. One of the fusion proteins was localized to the nucleus, one was localized to the vacuole and one was present in the cytoplasm.

Some integral membrane proteins fused with GFP seem to be targeted properly, despite the constraints imposed on the sequence and structure of these proteins by membrane topogenesis and trafficking. For example, both isoforms of yeast hydroxymethyl glutaryl-CoA reductase fused with GFP are correctly localized to the endoplasmic reticulum membrane; in conditions under which the regulated degradation of yeast hydroxymethyl glutaryl-CoA reductase isoform 2 occurs, the degradation rates of the native and fusion proteins are similar [21]. Another pertinent example is the fusion of the mammalian GLUT4 glucose transporter with GFP. GLUT4 resides in intracellular vesicles in resting cells and is translocated to the plasma membrane on stimulation with insulin. Removal of insulin causes the recycling of GLUT4 to an intracellular pool [22]. Fusions of GLUT4 with GFP display the same insulin-responsive translocation when observed by confocal microscopy. Fusion proteins with GFP at the C-terminus of GLUT4 also undergo reinternalization on the removal of insulin; however, fusions with GFP at the N-terminus do not [23]. This suggests that GFP is not an entirely silent tag in such fusion proteins. Transporter function has not been reported for the GFP-tagged GLUT4.

We have constructed an Hxt2-GFP fusion protein for use as a reporter of Hxt2 expression, abundance and localization within the yeast cell. We find that Hxt2 tagged with GFP is a functional glucose transporter and is localized to the plasma membrane. We exploit the fluorescent properties of the fusion protein to quantify its cellular abundance and to observe its trafficking, in both wild-type cells under various growth conditions and in cells with defects in secretion or endocytosis. Our results demonstrate that this hexose transporter moves to the plasma membrane via the secretory pathway and is removed from the membrane via endocytosis.

MATERIALS AND METHODS

Strains, media and growth

The *E. coli* strains used in this work were DH5 α (F⁻, ϕ 80dlacZ Δ M15, Δ (lacZYA-argF), U169, *deoR*, *recA1*, *endA1*, *hsdR17*(r_K⁻,m_K⁺), *supE44*, λ ⁻, *thi-1*, *gyrA96*, *relA1*) and DM1 (F⁻, *dam-13::Tn9*(Cm^R), *dcm-mcrB*, *hsdR-M*⁺, *gal1*, *gal2*, *ara-*, *lac-thr-*, *leu-*, *ton*^R, *tsx*^R, *Su*⁰) from Life Technologies and BL21(DE3) [F⁻, *ompT*, *hsdSB*, (rB⁻mB⁻), *dcm*, *gal*, (DE3)] from Promega. They were grown in Luria-Bertani medium, and transformed by the CaCl₂ method, as described previously [24]. Transformants were grown in the presence of 60 μ g/ml ampicillin.

The yeast strains used in this study are listed in Table 1. Strain KY73 was constructed as follows: a 3.5 kb *EcoRI*/*SmaI* fragment containing the *GAL2* gene was cloned from plasmid pS25 [25] into pUC18 to create plasmid pAK82. The *EcoRV* fragment of this plasmid (containing the *GAL2* open reading frame) was replaced with the Klenow-treated *HindIII*/*SmaI* fragment of pJR-URA3 [26]. This fragment contains the *URA3* gene flanked by short direct repeats. The resulting plasmid, pAK83, was digested with *EcoRI* and *SmaI* and transformed into strain RE700 [3]. Transformants were selected for uracil prototrophy.

Table 1 Yeast strains used in this paper

Strain	Genotype	Source or reference
KY73	<i>MATa hxt1Δ::HIS3::Δhxt4 hxt5::LEU2 hxt2Δ::HIS3 hxt3Δ::LEU2::hxt6 hxt7::HIS3 gal2Δ::DR* ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL</i>	This study
RE102	<i>MATα hxt2Δ::HIS3 ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL</i>	[3]
RE700	<i>MATa hxt1Δ::HIS3::Δhxt4 hxt5::LEU2 hxt2Δ::HIS3 hxt3Δ::LEU2::hxt6 hxt7::HIS3 ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL</i>	[3]
NY17	<i>MATa sec6-4 ura3-52</i>	[58]
RH1800	<i>MATa his4 leu2 ura3 bar1-1</i>	[37]
RH1623	<i>MATa his4 leu2 ura3 bar1-1 end3-1</i>	[37]
RH1597	<i>MATa his4 leu2 ura3 bar1-1 end4-1</i>	[37]

* Direct repeat.

Ura⁺ isolates were plated on medium containing 5-fluoro-orotic acid (FOA); FOA-resistant isolates were recovered. These were screened for uracil auxotrophy and impaired galactose growth. Southern blotting with probes for the *URA3* and *GAL2* genes showed that the Ura⁻ Gal⁻ isolates had a deletion of the *GAL2* gene and had lost the *URA3* marker from the *gal2 Δ* locus (results not shown). Strain KY73 will be referred to below as the *hxt*-null strain.

The liquid medium used in these experiments consisted of 1.6 g/l Yeast Nitrogen Base (Difco 0335-15-9), 5 g/l (NH₄)₂SO₄, 1 g/l casamino acids (Difco) and 20 mg/l tryptophan. It was supplemented with a low concentration of glucose (0.1 %, w/v), with a high concentration of glucose (5 %, w/v) or with maltose (2 %, w/v). Solid media for strain propagation, yeast transformation and FOA selection were prepared as described previously [27].

The induction experiments described below involved the transfer of exponentially growing cells from non-inducing medium to low-glucose and high-glucose medium, after centrifugation and washing in prewarmed medium. Strain KY73 was grown on maltose medium before induction; other strains were grown on solid medium containing 2 % (w/v) glucose or in liquid medium containing 5 % (w/v) glucose unless indicated otherwise. Strains with temperature-sensitive alleles (NY17, RH1597 and RH1623) were grown at 24 °C as the permissive temperature and 37 °C as the restrictive temperature. Other strains were grown at 30 °C unless indicated otherwise.

Construction of HXT2-GFP plasmid

The plasmid pAK1a, containing the entire *HXT2* gene in YEp352 [28], has been described [14]. An *AscI* restriction site was introduced at the 3' end of the *HXT2* open reading frame via overlap-extension PCR [29] as follows: pAK1a was amplified with *Taq* DNA polymerase in two separate reactions. Reaction 1 included oligonucleotides AK8 and AK9, and reaction 2 included oligonucleotides AK10 and AK11 (Table 2). The products of these reactions were gel-purified, combined with oligonucleotides AK8 and AK11, then reamplified. The product of this secondary reaction was digested with *BclI*. pAK1a was purified from strain DM1, digested with *BclI*, and ligated with the *BclI*-digested PCR product. Three plasmids in which the *HXT2-AscI* cassette was in the correct orientation were identified

Table 2 Oligonucleotides used in this paper

Name	Sequence
AK8	ACCTGTGTTCTTTCTATCAG
AK9	CTCTTACGGCGCGCTTCTCGGAACTCTTTTTC
AK10	GGAAGGCGCGCGCTAAGAGATTACTTAACTAG
AK11	TTAAGTGCAGTCCGTAAG
AK12	CGCGATGGCTTCTATGACCGGTGGTCAACAAATGGGTGG
AK13	CGCGCCACCATTTGTTGACCGGTGATAGAACCCAT
AK22	TTGGCGCGCGATGAGTAAAGGAGAAGAAC
AK23	TTGGCGCGCTTTGTATGATGTCATCCATGC

by DNA sequencing. Each of them had the sequence **GGC-GCGCCG** between the last codon and the stop codon of *HXT2*. This sequence contains the recognition sequence of *AscI* (shown in bold) and encodes glycine, alanine and proline. The remainder of the sequence was unaltered from that previously reported [14]. The *HXT2-AscI* plasmid used below was named pAK121.

An F64L/S65T allele of GFP in plasmid pRSET_B was amplified by PCR with the primers AK22 and AK23 (Table 2); the product was cloned into the *AscI* site of pNEB193. The resulting plasmid, pGFP, was digested with *AscI* and the GFP cassette was gel-purified. pAK121 was digested with *AscI* and ligated with the GFP cassette; *HXT2-GFP* chimaeras were identified by restriction analysis of the resulting recombinant plasmids. The amplification of GFP and subsequent cloning steps were performed in triplicate; products were functionally tested for GFP fluorescence to ensure that the PCR did not introduce any deleterious mutations into the DNA encoding GFP. The resulting plasmid containing the chimaeric *HXT2-GFP* gene was named pAG1-5.

HXT2 was tagged with DNA encoding an epitope tag from the bacteriophage T7 gene 10 protein by the digestion of pAK121 with *AscI* and ligation with annealed oligonucleotides AK12 and AK13 (Table 2). Sequence analysis showed that plasmid pAK125 had the tag sequence in the proper orientation. The sequence encodes the epitope MASMTGGQMG by using the preferred codons of *S. cerevisiae* (M. Cherry, personal communication) except for the threonine codon (ACC); use of this codon (the third most abundant threonine codon) introduces an *AgeI* restriction site into the DNA sequence.

Single-copy vectors bearing *HXT2* and *HXT2-GFP* were constructed by digesting pAK1a and pAG1-5 with *SacI* and *BamHI* and ligating the fragments of interest into *SacI/BamHI*-digested centromeric vector YCplac33 [30]. They were named pAK145 and pAK146 respectively.

Glucose transport assay

Glucose uptake was assayed by using short-term incubations of yeast cell suspensions with D-[U-¹⁴C]glucose at 30 °C essentially as described [9]. The suspension density was approx. 7.5 g wet weight/100 ml. A detailed protocol is available from the authors on request. Kinetic parameters were determined with ENZFITTER software (Elsevier-Biosoft). Total cell protein was measured by the Lowry assay after the digestion of cells overnight in 1 M NaOH; cell number was determined by counting at least 1000 cells with a haemocytometer.

Microscopy

Living cells were examined with a Leitz Aristoplan epifluorescence microscope with filter cube 1001 HQ-FITC (Chroma) for

blue excitation and filter cube N2.1 (Leica) for green excitation. Micrographs were recorded with an UltraPix 12-bit charge-coupled device camera and processed for display with Adobe Photoshop.

Fluorescence spectroscopy

A Hitachi RF-5001PC fluorimeter was used to scan the excitation and emission spectra of whole-cell suspensions in 0.1 M potassium phosphate buffer, pH 6.5. The spectra of cells expressing *HXT2-GFP* were normalized to cell density and were corrected for background by the subtraction of spectra determined for cells expressing *HXT2* cultured under identical conditions. Emission spectra were collected between 500 and 550 nm, with excitation at 489 nm and excitation and emission slit widths of 3 nm. Preliminary experiments showed that the fluorescence signal at the emission peak for GFP F64L/S65T (509 nm [18]) was essentially linear for cell suspensions between D_{600} values of 0.1 and 2. For the experiments on intracellular pH quenching of GFP fluorescence, spectra were recorded with an Aminco DW2000 spectrophotometer and an SLM-Aminco Bowman series 2 luminescence spectrometer.

Immunoblotting

Cells were harvested by centrifugation, washed once in 1 % (w/v) KCl and extracted by abrasion with glass beads in buffer A {50 mM Tris/HCl [pH 8]/10 mM EDTA/5 % [v/v] glycerol/protease inhibitors [1 µg/ml leupeptin/1 µg/ml pepstatin A/0.2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride in DMSO]}. The lysates were cleared by centrifugation at 1300 g for 3 min; total membrane proteins were harvested by centrifugation at 100000 g for 1 h at 4 °C. The membrane pellets were resuspended in buffer A, the protein concentration was determined and the samples were diluted to 1 µg/µl in buffer A. An equal volume of loading buffer was added; the samples were then heated to 40 °C for 15 min and subjected to electrophoresis in an SDS/PAGE Minigel [10 % (w/v) gel]. The proteins were transferred to PVDF membrane by using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) with buffer B [48 mM Tris/39 mM glycine/5 % (v/v) methanol/0.05 % SDS] at 200 V for 1 h at 4 °C. Membranes were incubated with gentle agitation in PBS/0.2 % (v/v) Tween 20 as follows: (1) blocking for at least 1 h in 5 % (w/v) non-fat milk; (2) incubation with primary antibodies for 16 h at 4 °C in 1 % (w/v) non-fat milk, followed by four 10 min washes; (3) incubation with horseradish peroxidase-conjugated secondary antibody for 1 h in 1 % (w/v) non-fat milk, followed by three 5 min washes. The final wash was done without Tween 20 in the buffer. Immunodetection was performed by chemiluminescence, as described previously [31]. The antibodies were diluted as follows: anti-Hxt2 and anti-GFP, 1:40000; anti-T7, 1:10000; and goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugates, 1:10000. Primary antibodies were routinely pre-treated with denatured whole-cell extracts of RE102 before use.

GFP purification

Plasmid pRSET_B:GFP-F64L/S65T was transformed into strain BL21(DE3). Fresh colonies were inoculated into Luria-Bertani/ampicillin medium and grown until the culture reached a D_{600} of 2.5. Cells were lysed by sonication and hexahistidine-tagged GFP was purified by nickel-chelate affinity chromatography as described previously [18]. The purified protein was dialysed against 0.1 M phosphate buffer, pH 6.5. The molar absorption coefficient of GFP F64L/S65T at 489 nm is $55000 \pm 5000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [18]. With the use of a Hewlett Packard 8452A diode array spectro-

photometer, the concentration of the GFP stock solution was determined to be 2.5 μ M.

Chemicals and reagents

The anti-Hxt2 antibody has been described [15,32]. Anti-GFP antibody, Texas Red-conjugated concanavalin A (conA-Texas Red), and 7-amino-4-chloromethylcoumaryl-L-arginine amide dihydrochloride (CMAC-Arg) were from Molecular Probes. The anti-T7 antibody was from Novagen. Electrophoresis and immunoblotting reagents were from Bio-Rad except for pre-stained protein molecular mass markers, which were from Fluka. Restriction enzyme *Asc*I and plasmid pNEB193 were from New England Biolabs. Maltose (M-5885, containing less than 0.3 % glucose) was from Sigma. D-[U- 14 C]Glucose was from Amersham. Custom oligonucleotides were synthesized by Life Technologies and by Pharmacia. All other chemicals were of reagent grade.

RESULTS

Hxt2 fused with GFP is a functional hexose transporter

The *hxt*-null strain KY73 was transformed with single-copy or multicopy plasmids containing the *HXT2* gene or the chimaeric *HXT2-GFP* or *HXT2-T7* genes, or with empty vectors. The transformants were restreaked on glucose medium and growth was assessed after 3 days. As shown in Figure 1, the chimaeric genes restored growth on glucose to the *hxt*-null strain to the same extent as the native *HXT2* gene. Cells containing the empty vector were unable to form visible colonies.

Cells of the null strain carrying single-copy plasmids with *HXT2*, *HXT2-GFP* or the empty vector were grown overnight in maltose medium to mid-exponential phase and were then shifted to low-glucose medium. After 4 h the cells were harvested and zero-*trans* glucose transport was assayed. The results were fitted to a single-component transport system obeying Michaelis-Menten kinetics; fitting the results to a two-component system did not increase the statistical significance of the calculated transport parameters. The K_m and V_{max} are presented in Table 3. The kinetics of glucose transport conferred by *HXT2* and *HXT2-GFP* were not markedly different, although the *HXT2-GFP* gene reproducibly resulted in a slightly lower K_m and higher V_{max} than the native *HXT2* gene.

Table 3 Kinetic parameters of the *hxt*-null strain expressing Hxt2 or Hxt2-GFP

V_{max} is expressed as nanomoles of glucose transported/min per mg of total cell protein.

Gene	V_{max} (nmol/min per mg)	K_m (mM)
<i>HXT2</i>	76 $\pm$ 5	8.2 $\pm$ 0.8
<i>HXT2-GFP</i>	103 $\pm$ 8	5.0 $\pm$ 0.7

Catalytic-centre activity of the Hxt2-GFP glucose transporter

We used the fluorescent signal from Hxt2-GFP to determine the cellular concentration and catalytic-centre activity of the fusion protein, with purified GFP as a fluorescent standard. We first examined the effect of intracellular components on GFP fluorescence. Light absorption and fluorescence emission spectra were recorded from suspensions of cells expressing *HXT2* or *HXT2-GFP*. The suspensions were then subjected to a French press in 50 mM Tris/HCl, pH 8 (GFP fluorescence is maximal at pH 8 [18]); spectra were recorded from the lysates. The fluorescence intensity measured in the lysate, when corrected for the decrease in light absorption at the excitation and emission wavelengths, corresponded to 102 % of the emission from whole cells. Therefore we conclude that GFP fluorescence is not quenched by intracellular components.

To quantify the cellular abundance of the Hxt2-GFP molecules, the Hxt2 and Hxt2-GFP cell suspensions that were used for the glucose transport assays were diluted 1:10 in phosphate buffer to the same attenuation, and fluorescence spectra were recorded. Then fluorescence spectra of the Hxt2 cell suspension were recorded at intervals during titration with purified GFP protein. The concentration of pure protein required to match the fluorescent signal of cells expressing Hxt2-GFP was 19.4 nM ($n = 3$). The density of the cell suspension in the GFP titration was 8.5×10^7 cells/ml. Therefore each cell contained 1.4×10^5 Hxt2-GFP molecules.

The cellular abundance of the Hxt2-GFP chimaera and the glucose transport activity mediated by that protein were used to calculate the catalytic-centre activity for glucose of the transporter. The Hxt2-GFP cell suspension contained 0.61 mg of total cell protein/ml, giving a relationship of 32 pmol of Hxt2-

Figure 1 *HXT2-GFP* restores growth on glucose to the *hxt*-null strain

Strain KY73 was transformed with multicopy plasmids pAG1-5 (*HXT2::GFP*), pAK125 (*HXT2::T7*), pAK1a (*HXT2*) or pRS426 (vector) (A) and with single-copy plasmids pAK145 (*HXT2*), pAK146 (*HXT2::GFP*) or YCplac33 (vector) (B). Transformants were restreaked on YNB medium containing casamino acids, tryptophan and 2 % (w/v) glucose and photographed after 3 days.

Figure 2 Fluorescence emission spectra of cells expressing Hxt2-GFP protein

(A) Strain KY73 (*hxt*-null); (B) strain RE102 (*hxt2Δ*). The traces are labelled as follows: MC, multicopy plasmid (pAG1-5); SC, single-copy plasmid (pAK146); L, low glucose (0.1%, w/v); H, high glucose (5%, w/v). Spectra were recorded as described in the Materials and methods section.

GFP/mg of total cell protein. The $V_{\max}$ for glucose transport of these cells, 103 nmol/min per mg of total cell protein (Table 3) was divided by this value, resulting in an estimate of 53 s^{-1} for the catalytic-centre activity of Hxt2-GFP. All of the Hxt2-GFP in the cells seemed to be at the plasma membrane when examined by fluorescence microscopy (results not shown).

Hxt2-GFP expression is glucose-repressed

We initially observed that cells carrying the *HXT2-GFP* chimaera were fluorescent and that the fluorescence level was glucose-dependent. The time course of expression of fluorescence reached a maximum 4–8 h after cells were shifted to a low concentration of glucose, and 8–12 h after cells were shifted to a high concentration of glucose (results not shown). In subsequent experiments, cells were analysed 4 h after the shift in medium unless otherwise indicated.

The level of Hxt2-GFP expression was assessed by using fluorimetry and immunoblotting. Comparisons were made between uninduced and induced cultures of the *hxt*-null strain and the *hxt2Δ* strain RE102, each carrying single-copy or multicopy plasmids. In the null strain (Figure 2A) the fluorescence due to Hxt2-GFP was increased significantly by growth in low-glucose medium. This was seen for cells bearing *HXT2-GFP* on both single-copy and multicopy plasmids. The absolute level of fluorescence was higher when the *HXT2-GFP* chimaera was borne on the multicopy vector. Similarly, the expression of

Figure 3 Western blot analysis of Hxt2-GFP and Hxt2 protein levels

(A) Detection with anti-GFP antibody. Lane 1, vector pRS426 in RE102; lane 2, pAK1a (*HXT2*) in KY73; lane 3, pAK125 (*HXT2-T7*) in KY73; lane 4, molecular mass markers (116, 84, 58, 48.5, 36.5 and 26.6 kDa); lanes 5–12, *HXT2-GFP*; lanes 5–8, KY73; lanes 9–12, RE102; lanes 5, 6, 9 and 10, multicopy vector; lanes 7, 8, 11 and 12, single-copy vector; lanes 5, 7, 9 and 11, high glucose; lanes 6, 8, 10 and 12, low glucose; lane 13, purified GFP protein. (B) Detection with anti-Hxt2 antibody. Lane 1, vector pRS426 in RE102; lane 2, pAG1-5 (*HXT2-GFP*) in KY73; lane 3, pAK125 (*HXT2-T7*) in KY73; lane 4, molecular mass markers as listed in (A); lanes 5–12, *HXT2*; lanes 5–8, KY73; lanes 9–12, RE102; lanes 5, 6, 9 and 10, multicopy vector; lanes 7, 8, 11 and 12, single-copy vector; lanes 5, 7, 9 and 11, high glucose; lanes 6, 8, 10 and 12, low glucose.

fluorescence by *HXT2-GFP* in the *hxt2Δ* strain was higher when the cells were cultured in low-glucose medium than when they were cultured in high-glucose medium (Figure 2B). The expression level was also higher when the chimaeric gene was present on a multicopy plasmid. However, the absolute level of fluorescence was in all cases lower in the *hxt2Δ* strain than in the *hxt*-null strain [compare Figure 2(B) with Figure 2(A)].

In a separate experiment, the amount of Hxt2-GFP protein was monitored by immunoblotting (Figure 3A). Significantly more protein was expressed by cultivation in low-glucose than in high-glucose media. This was observed for the *hxt*-null strain with multicopy and single-copy *HXT2-GFP*. A similar expression pattern was observed with the *hxt2Δ* strain carrying multicopy and single-copy *HXT2-GFP* plasmids. The relative expression levels were estimated for the *hxt2Δ* strain with single-copy *HXT2-GFP* and for the *hxt*-null strain with multicopy *HXT2-GFP* by using quantitative immunoblotting (Figure 4A). The Hxt2-GFP level in induced cells was more than 16-fold that in uninduced cells of the *hxt2Δ* strain, and in the *hxt*-null strain was approx. 16-fold greater.

Cellular Hxt2 protein levels from the same culture conditions were assessed by immunoblotting (Figure 3B). As with Hxt2-GFP levels, the Hxt2 protein was considerably more abundant in

Figure 4 Quantitative immunoblotting of Hxt2-GFP and Hxt2

(A) Detection of Hxt2-GFP with anti-GFP antibody; (B) detection of Hxt2 with anti-Hxt2 antibody. Membrane protein (5 µg) from cells grown at high or low concentrations of glucose were added in lanes H and L respectively, and a 2-fold dilution series of the low glucose extract was loaded in the subsequent four lanes. CEN and 2-micron refer to the yeast origin of DNA replication used in that plasmid.

low-glucose cells than in high-glucose cells; indeed, in the exposure shown, the Hxt2 protein could not be detected in the high-glucose samples. Moreover, the Hxt2 protein was over-expressed in cells containing multicopy *HXT2*. However, the levels of Hxt2 protein were similar in *hxt2Δ* and *hxt*-null strains in each condition. The level of Hxt2 induction after cells were shifted to a low concentration of glucose was assessed by quantitative immunoblotting (Figure 4B). For the *hxt2Δ* strain with single-copy *HXT2* the amount of Hxt2 protein in induced cells was more than 16-fold that in repressed cells. For the null strain with multicopy *HXT2* the protein level was induced approx. 16-fold by glucose. Hxt2 protein was approx. 2-fold more abundant in the null strain with the multicopy plasmid than in RE102 with the single-copy plasmid.

The expression of Hxt2 and Hxt2-GFP was consistently higher in the *hxt*-null strain than in the *hxt2Δ* strain after being shifted to high-glucose or low-glucose medium. We examined the possibility that the *HXT2* and *HXT2-GFP* genes were up-regulated in the *hxt*-null strain as a compensatory response to the absence of other functional *HXT* genes. Instead, we found that *HXT2* is partly derepressed by cultivation in the pre-culture medium containing maltose (results not shown); this medium

Figure 5 Subcellular localization of Hxt2-GFP

Cells of the *hxt*-null strain (KY73), transformed with multicopy *HXT2-GFP* (pAG1-5), were induced by growth for 4 h on low-glucose medium. Left panel, fluorescence of *HXT2-GFP*; right panel, fluorescence of conA-Texas Red decorating the cell wall.

was used for the *hxt*-null strain carrying *HXT2* or *HXT2-GFP* plasmids, because it does not grow in high-glucose medium.

The anti-Hxt2 antibody was raised against a peptide corresponding to the C-terminal 13 residues and the terminal carboxy group of Hxt2 [15,33]. We found that this antibody did not recognize the Hxt2-GFP fusion protein (Figure 3B, lane 2). This might have been due to the absence of the native carboxy group, because the anti-Hxt2 antibody also does not recognize an Hxt2-T7 fusion protein (Figure 3B, lane 3), even though this fusion protein is readily detected by the anti-T7 antibody (results not shown).

Hxt2-GFP is localized to the plasma membrane

The distribution of the Hxt2-GFP fusion protein in living cells was examined by fluorescence microscopy. Strong cellular fluorescence was observed after induction; it was predominantly localized to the cell periphery, coincident with conA-Texas Red staining of the cell wall (Figure 5). We interpret this location as being the plasma membrane. The signal in uninduced cells was very weak and did not seem to be localized to the plasma membrane; the autofluorescence of cells expressing Hxt2 was nil (results not shown). Cells containing multicopy *HXT2-GFP* plasmids were more fluorescent than cells with single-copy plasmids; however, no differences in the distribution of the fluorescence were apparent as a result of overexpression of the protein (results not shown).

Induced cells were treated with 5% (w/v) glucose and examined after continued incubation. The fluorescent signal was gradually lost from the plasma membrane. Early in the incubation it appeared in punctate structures close to the plasma membrane (Figure 6, left panel). With longer incubation it accumulated in single large globular structures (Figure 6, middle panel). To characterize this localization, glucose-treated cells were incubated with CMAC-Arg, a coumarin-based compound that, on hydrolysis by vacuolar peptidases, becomes fluorescent and unable to permeate the vacuolar membrane. The GFP fluorescence in cells incubated for long periods with a high concentration of glucose clearly co-localized with vacuoles stained by CMAC-Arg (Figure 6, right panel) and was distinct from mitochondria and nuclei stained with 4,6-diamidino-2-phenylindole (results not shown).

It was occasionally observed that a proportion of cells in a culture (up to 5%) did not fluoresce and that the fluorescence intensity varied from cell to cell. We do not know whether this

Figure 6 Inactivation of Hxt2-GFP

A culture of RE102 containing single-copy *HXT2-GFP* was shifted to low-glucose medium for 4 h, then treated with glucose to a final concentration of 5% (w/v). Left panel: cells were examined after 2 h of high-glucose treatment. Middle and right panels: cells were examined after 7 h of high-glucose treatment and staining with CMAC-Arg. The same cells are shown in the middle panel (GFP fluorescence) and the right panel (CMAC-Arg fluorescence).

Figure 7 Hxt2-GFP localization in a secretory mutant

Cells of NY17 (*sec6-1*) containing pAG1-5 (multicopy *HXT2-GFP*) were induced by growth in 0.1% glucose for 4 h, at the permissive temperature (23 °C) (left panel) or at the restrictive temperature (37 °C) (middle panel). Induced cells from the restrictive temperature were treated with 1 µg/ml cycloheximide and incubated at 24 °C for 2 h (right panel).

was due to plasmid loss or to an impaired ability of some cells to respond to induction. Heterogeneity of GFP expression in yeast has also been observed by others [20].

Hxt2-GFP is transported via the secretory pathway

The role of the secretory pathway in traffic of Hxt2-GFP to the plasma membrane was assessed by examining the distribution of the fusion protein in a strain that has a temperature-sensitive allele of the *SEC6* gene. This gene is required for the fusion of post-Golgi secretory vesicles with the plasma membrane. At the restrictive temperature in a *sec6-4* strain the secretory vesicles and their integral and luminal proteins accumulate in the cytoplasm [34,35]. When Hxt2-GFP expression was induced in this strain grown at the permissive temperature, the plasma membrane was clearly labelled by GFP (Figure 7, left panel), although more fluorescence was seen in the cytoplasm of this strain than in *SEC6* wild-type strains. 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 7, middle panel).

The *sec6-4* phenotype has been reported to be reversible; when cells are restored to the permissive temperature, a proportion of

the invertase accumulated in the vesicles is secreted to the periplasm [36]. After *sec6-4* cells expressing Hxt2-GFP had been shifted from the restrictive temperature to the permissive temperature for 2 h, their plasma membranes were distinctly labelled by fluorescence (Figure 7, right panel). This phenomenon was observed both in mother cells and in buds. Moreover, bright spots of GFP fluorescence were often seen in the vicinity of the bud neck. The labelling occurred even in the presence of the protein synthesis inhibitor cycloheximide. However, the fluorescent signal that had accumulated within the cells was only partly redistributed to the plasma membrane, even after 8 h of incubation at the permissive temperature and supplementation of the medium with 0.1% glucose (results not shown). The incorporation of Hxt2-GFP into the plasma membrane from secretory vesicles also occurred in the presence of a high concentration of glucose: if cultures were treated with 5% glucose upon being shifted to the permissive temperature, a proportion of the GFP signal could be detected in the plasma membrane (results not shown).

Endocytosis is involved in the removal of Hxt2-GFP from the plasma membrane

Strains with the *end3-1* or *end4-1* alleles display temperature-sensitive defects in endocytosis [37]. *HXT2-GFP* expression was

Figure 8 Hxt2-GFP is retained in the plasma membrane of cells blocked in endocytosis

Cells induced by low-glucose treatment at the permissive temperature were treated with 5% (w/v) glucose and incubated at the restrictive temperature for 4 h. Right panel, RH1800 (*END3 END4*); left panel, RH1623 (*end3-1*); middle panel, RH1597 (*end4-1*).

induced for 4 h in strains RH1623 (*end3-1*) and RH1597 (*end4-1*) and in the wild-type strain RH1800 (*END3 END4*) at the permissive temperature. At this time all strains had strong GFP staining in the plasma membrane. The cultures were then divided into four aliquots: two were maintained at the permissive temperature and two were shifted to the restrictive temperature of 37 °C. One of the aliquots at each temperature was supplemented with 5% (w/v) glucose. The cultures were examined periodically by fluorescence microscopy. After 2 h the *end3* and *end4* strains maintained at the permissive temperature in a low concentration of glucose had strong fluorescent staining of the plasma membrane, but in a high concentration of glucose they had accumulated a large proportion of their fluorescence in internal structures (results not shown). At the restrictive temperature both the low-glucose-treated (results not shown) and high-glucose-treated (Figure 8, left and middle panels) mutant cells had strong fluorescent staining of the plasma membrane. In the wild-type strain a high concentration of glucose stimulated the internalization of the fluorescence from the plasma membrane to the vacuole (Figure 8, right panel); this internalization was more rapid at 37 °C (results not shown). The retention of Hxt2-GFP in the plasma membrane of *end3* and *end4* cells persisted with prolonged incubation at the restrictive temperature, at which time all of the fluorescence in wild-type cells had disappeared from the plasma membrane (results not shown).

DISCUSSION

We have shown that a chimera between the Hxt2 glucose transporter of *S. cerevisiae* and GFP is a functional transport protein with a glucose transport capacity similar to that of the wild-type protein. As a solute transporter, Hxt2 is inferred to be targeted to the plasma membrane, and indeed it has been identified in the plasma membrane fraction of cell lysates [15]. Our results are in agreement: the Hxt2-GFP protein in induced cells was strongly localized at the plasma membrane.

By quantifying the emission from GFP in the fusion protein we were able to determine a value for the catalytic-centre activity of Hxt2-GFP *in vivo*. We estimate that under inducing conditions each *hxt*-null cell transformed with single-copy *HXT2-GFP* has 1.4×10^5 Hxt2 molecules in the plasma membrane and that the transporter has a catalytic-centre activity at 30 °C of 53 s^{-1} . To our knowledge this is the first empirical estimate of a catalytic-centre activity for a yeast hexose transport protein. It assumes

that all Hxt2-GFP molecules were actively transporting glucose and is therefore a minimum estimate. Catalytic-centre activities for other solute transporters have been estimated *in vivo* by using ligand binding and transport velocity measurements. For example, the catalytic-centre activities of the GLUT1 and GLUT3 human glucose transporters at 37 °C have been calculated to be 123 and 853 s^{-1} respectively [38]. The catalytic-centre activity of the yeast purine-cytosine permease is approx. 1 s^{-1} [39]. The phosphate/triose phosphate translocator of the chloroplast envelope has a catalytic-centre activity *in vivo* of 83 s^{-1} at 20 °C [40], the phosphate/ H^+ co-transporter of heart mitochondria has a catalytic-centre activity of $350\text{--}1000 \text{ s}^{-1}$ at 25 °C [41], and the ADP/ATP translocase of heart mitochondria has a catalytic-centre activity of $8\text{--}10 \text{ s}^{-1}$ at 18 °C [42]. Serrano [43] suggested that the number of hexose transporters in an actively fermenting wild-type yeast cell is $10^5\text{--}10^6$, and from the measured fermentative capacity of yeast he calculated the catalytic-centre activity of the hexose transporters to be $10\text{--}100 \text{ s}^{-1}$. Using a fluorescent non-transported glucose analogue, *N*-dansyl-D-glucosamine, Kotyk and co-workers estimated that there are up to 10^6 glucose transporters per yeast cell [44]. Thus the values that we have determined for Hxt2 are in general agreement with those in the literature for similar proteins.

The canonical secretory pathway carries secreted proteins such as invertase and α -factor to the periplasm, and other proteins such as the Pma1 ATPase and solute transporters to the plasma membrane; the *SEC1* and *SEC6* genes act at a late step in this pathway [34,35,45]. It has previously been shown that Gal2 [12] and high-affinity glucose transport activity [10] reach the plasma membrane via this pathway and require *SEC1* for functional expression at the plasma membrane. We show that *SEC6* is involved in delivering Hxt2-GFP to the membrane. We found that Hxt2-GFP that had accumulated in secretory vesicles at the restrictive temperature in a *sec6-4* strain was able to reach the plasma membrane when the cells were shifted to the permissive temperature. Targeting to the plasma membrane at the permissive temperature occurred even in the presence of a high concentration of glucose, demonstrating that *HXT2* gene expression, but not outward trafficking of the Hxt2 protein, is under glucose control. These observations agree with those of Bisson [10], who found that a high concentration of glucose did not prevent the expression of high-affinity glucose transport activity after it had been blocked in a *sec1-1* mutant at the restrictive temperature.

After treatment with a high concentration of glucose, Hxt2-

GFP expression ceases and the protein is removed from the plasma membrane by endocytosis. The *END3* and *END4* gene products are required for internalization; the destination of the protein is the vacuole. It has been recognized that this pathway acts to internalize and inactivate a number of plasma membrane proteins such as transporters of galactose [46], maltose [47], inositol [48], amino acids [49], nucleobases [50], drugs [51] and mating pheromones [52,53], as well as receptors for the mating pheromones [54]. We have been able to discern Hxt2-GFP-containing structures that are formed at an early stage of this process; these might be endocytic vesicles. We have also observed that these structures disappear once the fluorescence becomes localized in the vacuole.

In cells that are competent for endocytosis, Hxt2-GFP fluorescence declines markedly after prolonged growth of low-glucose cells or after high-glucose treatment. When growth is arrested owing to glucose limitation or temperature sensitivity, it is clear that this decline is due not to dilution of the GFP chromophore into new biomass but to degradation. This is in contrast with reports that describe the GFP chromophore as protease-resistant [16]. Sensitivity of the GFP chromophore to vacuolar proteases is advantageous for the use of GFP to tag membrane proteins because it allows the fluorescent signal to be 'chased' by vacuolar targeting of the fusion protein.

Fusions of GFP to yeast hexose transporters will be useful in addressing some of the outstanding questions about this family of proteins. By taking advantage of alleles of GFP with different absorption and emission maxima we shall be able to monitor two or more transporters simultaneously. It has been suggested that some of these transporters might interact physically [14,55,56]. Such an interaction could be detected by fluorescence resonance energy transfer between different GFP alleles [57]. In addition, the ability to monitor the proteins in living cells, where both the genotype (with respect to protein trafficking and *HXT* gene expression, for example) and the environment (such as nutrients, temperature) can be controlled, will be exploited to detect the location and movement of these proteins in three dimensions over time.

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