Physiological Characterization of Putative High-Affinity Glucose Transport Protein Hxt2 of Saccharomyces cerevisiae by Use of Anti-Synthetic Peptide Antibodies

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Characterization and quantification of the Hxt2 (hexose transport) protein of Saccharomyces cerevisiae indicate that it is one of a set of differentially expressed high-affinity glucose transporters. The protein product of the HXT2 gene was specifically detected by antibodies raised against a synthetic peptide encompassing the 13 carboxy-terminal amino acids predicted by the HXT2 gene sequence. Hxt2 migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a broad band or closely spaced doublet with an average Mr of 47,000. Hxt2 cofractionated with the plasma membrane ATPase, Pma1, indicating that it is a plasma membrane protein. Hxt2 was not solubilized by high pH or urea but was solubilized by detergents, which is characteristic of an integral membrane protein. Expression of the Hxt2 protein was measured under two different conditions that produce expression of high-affinity glucose transport: a medium shift from a high (2.0%) to a low (0.05%) glucose concentration (referred to below as high and low glucose) and growth from high to low glucose. Hxt2 as measured by immunoblotting increased 20-fold upon a shift from high-glucose to low-glucose medium, and the high-affinity glucose transport expressed had a strong HXT2-dependent component. Surprisingly, Hxt2 was not detectable when S. cerevisiae growing in high glucose approached glucose exhaustion, and the high-affinity glucose transport expressed under these conditions did not have an HXT2-dependent component. The role of Hxt2 in growth during aerobic batch culture in low-glucose medium was examined. An hxt2 null mutant grew and consumed glucose significantly more slowly than the wild type, and this phenotype correlated directly with appearance of the Hxt2 protein.

High-affinity (low-$K_m$) uptake of the hexoses glucose, fructose, and mannose is a glucose-repressible kinetic component of hexose transport in Saccharomyces cerevisiae. It is maximally expressed when yeast cells grown with a high (2.0%) glucose concentration approach glucose exhaustion or are shifted to medium containing a low (0.05%) hexose concentration or a nonfermentable carbon source (3, 4).

The high-affinity glucose transport system appears to be very complex genetically, involving SNF3, HXT1, HXT2, HXT3, HXT4, and possibly yet undiscovered genes (18). A snf3 mutant is defective in fermentative growth on 2.0% sucrose, 2.0% raffinose, 0.05% glucose, and 0.05% fructose and is almost completely lacking high-affinity hexose transport (5). The HXT genes, on high-copy-number plasmids, suppress the growth defect caused by snf3. The HXT genes, which form a gene family within the sugar transporter superfamily, have high sequence similarity and identity to each other and to other sugar transporters in fungi, mammals, and bacteria (2, 18). The high-affinity galactose transporter gene GAL2 is also a member of this family (2). For example, the similarities of the predicted amino acid sequence of Hxt2 and Hxt1 are 78% to Hxt1, 58% to Snf3, 79% to Gal2, 51% to Kluyveromyces lactis Lac12, 55% to human GLUT1, and 60% to Escherichia coli xylE (2, 20).

Deletion of either HXT2 or HXT1 results in the partial reduction of high-affinity hexose transport under certain conditions (20, 23). An hxt2::LEU2 null mutant shows greater than 50% reduction in velocity of high-affinity glucose transport relative to the wild type when shifted to a low glucose concentration (20). The transport kinetics of other mutants have not been determined.

The presence of so many genes raises questions about their individual roles in the process. Are they all transporters? Are they all used by the cell at the same time? A model has been proposed in which some members of the group have regulatory or sensing roles and some are catabolic transporters (2). Suppression of snf3 by a clone on a high-copy-number plasmid does not necessarily mean that it encodes a transport protein; snf3 can be suppressed by a clone that clearly does not encode a protein (2). To understand the HXT genes, they must be studied independent of their role as snf3 suppressors.

Kinetic analysis of glucose transport is also very complex. The estimates of a low-affinity $K_m$ of 10 to 20 mM and a high-affinity $K_m$ of 1 to 2 mM were obtained by analysis of Eadie-Hofstee plots (3). (Note that the term affinity is used even though actual binding constants are not known.) Fuhrmann et al. disagree with these estimates (11, 35) on the basis of the use of computer-assisted nonlinear regression analysis of the same data. While they reach the same conclusions regarding high-affinity transport and the effect of snf3 and hxt2 mutations (though they arrive at more numerically precise estimations of high-affinity $K_m$s), they concluded that the apparent low-affinity glucose transport can be fit to a first-order model (35), and if it is transport, the low-affinity $K_m$ is in the molar range (11). A molar low-affinity $K_m$ is not outlandish given that grape juice, one of the natural environments of S. cerevisiae, contains on average 1.5 M combined glucose and fructose and that S. cerevisiae can grow at sugar concentrations even higher than this. A method of kinetic analysis can only be as conclusive as the data allow, and transport by whole live cells or even membrane vesicles does not warrant such precise analysis. Before kinetic models are refined, more must be learned about the individual components of this complex system.

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As part of such work, we are currently examining the product of HXT2 genetically and biochemically to understand its role in the high-affinity hexose transport system. To facilitate this analysis antibodies were raised against a synthetic peptide representing the carboxyl-terminal 13 amino acids predicted by the cloned HXT2 gene, by using established methods (22, 34). Peptide anti-synthetic peptide antibodies have been used with great success to investigate both human and E. coli sugar transport proteins (7, 8, 17, 28, 36, 38).

We report here that Hxt2 is an integral plasma membrane protein. This protein is highly expressed in fresh medium containing a low glucose concentration, a condition in which it is required for a component of high-affinity glucose transport. However, it is not expressed and does not contribute to high-affinity glucose transport under other conditions in which high-affinity glucose transport is observed. Therefore, the Hxt2 protein is responsible for a component of high-affinity uptake under specific, but limited, growth conditions.

MATERIALS AND METHODS

Reagents. All reagents, unless otherwise indicated, were obtained from Fischer and are reagent grade.

Buffers. Phosphate-buffered saline (PBS) was as previously described (15). PBN is 150 mM NaCl–20 mM potassium phosphate buffer (pH 7.4), PBNT is PBN plus 0.2% Tween 20, Blotto is PBNT plus 10% Lucerne nonfat dry milk, and PBNG is PBN plus 20 mM glycerol. Sodium azide (0.05%) was added to all primary antibody solutions. TE is 10 mM Tris–HCl (pH 7.4)–0.2 mM EDTA–0.2 mM dithiothreitol (31), and TEDG is TED plus 20% glycerol.

Strains. S. cerevisiae LBY410 (MATa HXT2) and LBY413 (MATa hxt2::LEU2) are isogenic strains differing only at the HXT2 locus and have been described previously (20).

Media. All cultures were grown in synthetic rich medium (SR) (6.7 g of yeast nitrogen base without amino acids [Difco] per liter, 2.0 g of Casamino Acids [Difco] per liter, 25 mg of tryptophan per liter, 30 mg of adenine [Sigma] per liter, 25 mg of uracil [Sigma] per liter, 40 mM sucinate–KOH, pH 6.2 to 6.5) with either a high (2.0%) or a low (0.05%) glucose concentration (referred to below as high and low glucose).

Culture conditions. Yeast cells were grown aerobically at 30°C in baffled flasks with the culture volume between one-third and one-fifth of the vessel volume in a New Brunswick Instruments Psychrotherm incubator with agitation at 250 oscillations per min.

Medium shift. Cultures were grown in SR with high glucose for 15 doublings (initial optical density at 580 nm of 0.000015) to an optical density at 580 nm of 0.5 (early log phase and glucose concentration of 1.5 to 2.0%). Cells were collected by centrifugation and washed twice in ice-cold SR without a carbon source. Cultures were split and suspended to the same density in SR containing either high or low glucose. The cells were then cultured for 2 h. For protein analysis, cells were collected by centrifugation, frozen in liquid nitrogen, and stored at −70°C until homogenization. For glucose transport assays, cultures were handled as previously described (20).

Assay of glucose transport. Zero time influx assays were performed as previously described (3, 20). Transport due to the high-affinity (low-\(K_m\)) system was estimated by measuring velocity at a concentration of 0.5 mM glucose in triplicate for each experiment. Experiments were performed in duplicate.

Development of antigen. The amino acid sequence of the open reading frame of HXT2 was analyzed by the database PEPTIDESTRUCTURE (13, 19). The polypeptide spep-HXT2.529-541 was synthesized by Multiple Peptide Systems, San Diego, Calif., and provided both in free form and conjugated to keyhole limpet hemocyanin. The sequence of the peptide is acetyl-GGSWISKEKRVSSE-aminocaproic acid. The acetylated peptide residue is not from the amino acid sequence of Hxt2 but is added to serve as a substrate for coupling reactions.

Nomenclature for the synthetic peptide. The prefix spep indicates synthetic peptide. The capitalized letters and number before the period are the name of the gene from which the sequence is taken (here, HXT2). After the period are numbers indicating the amino acid residues constituting the synthetic peptide.

Immunization of rabbits. spepHXT2.529-541 conjugated to keyhole limpet hemocyanin was used for immunization of rabbits. All injections, animal care, and bleedings were performed by Antibodies Incorporated, Davis, Calif. Three rabbits were used; the following amounts are per animal: Peptide (1.0 mg) was injected in PBS and Freund’s complete adjuvant. Boosters of 200 μg of peptide in PBS and Freund’s incomplete adjuvant were injected at days 14, 21, 35, and 49. Serum samples (20 μl) were taken at days 28 and 56 and assayed for anti-spezpHXT2.529-541 titer by enzyme-linked immunosorbent assay (ELISA). Serum from day 56 were characterized for the ability to specifically recognize the Hxt2 protein on immunoblots. Boosters of 100 μg of peptide were given at days 110 and 120. At day 134 the animals were sacrificed, and 70 ml of serum was collected.

Immunoblotting. Protein samples were prepared for electrophoresis by incubation at 40°C for 10 min in 50 mM sodium carbonate (pH 11.0)–4% sodium dodecyl sulfate (SDS)–4 M urea–1 mM EDTA–1% β-mercaptoethanol. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDSPAGE) and blotted to a polyvinylidene difluoride membrane (Schleicher & Schuell Western) with a Bio-Rad Mini Protean II electrophoresis unit. Gels for routine detection of HXT2 were 7.5% acrylamide (Bio-Rad), as this concentration gave better separation of Hxt2 from some major background proteins that appear in the long (1- to 3-min) film exposures needed to detect low levels of Hxt2. For molecular weight determination, proteins were resolved in 8.5% acrylamide with molecular weight standards (Sigma). The proportion of cross-linking (bisacrylamide) used was 25% lower than is conventional; i.e., the acrylamide stock solution was 30% total acrylamide with 2.0% cross-linking. This lower proportion of cross-linking did not affect the relative mobility of any protein, including HXT2, but did appear to increase the efficiency of protein transfer to the blot (data not shown). Affinity-purified anti-spezpHXT2.529-541 antibody was incubated with blots in PBNT at 0.2 μg/ml. Blots were probed with antibody as described by Harlow and Lane (16) with the following exceptions. Primary antibody was incubated for 16 to 24 h. After antibody incubations, blots were washed extensively six times for 10 min each with PBNT. For immunoblots using serum or impure antibodies, doing the first 10-min wash in Blotto increased clarity of the chemilumigram. Horseradish peroxidase-detect was detected by a chemiluminescent product (Amersham ECL).

To quantify antigen, chemilumigrams were scanned with a Shimadzu model CS-9000 scanning densitometer. Variable amounts of protein were loaded to ensure that the signals were linear with respect to antigen. Bands producing densitometric values of between 10,000 and 400,000 were in the linear range of this immunoassay.

Immunofluorescence purification of antibodies. Rabbit immunoglobulin G was precipitated from serum as described by Harlow and Lane (14) except that antibodies were precipitated
FIG. 1. (A) Immunoblot (SDS-PAGE in 7.5% acrylamide) of homogenates (20 µg per lane) of LBY410 (HXT2 wild type) (+) and LBY413 (hxt2 null mutant) (−) cultures shifted to high glucose. Blots were probed with either preimmune serum, day 56 immune serum, or affinity-purified antibodies. Where indicated, primary antibody was incubated with 1.0 µg of synthetic peptide per ml added 1 h prior to incubation with the blot. Hxt2 protein is indicated by the arrow on the right. (B) Immunoblot (8.5% polyacrylamide SDS-PAGE) of whole-cell homogenates (4 µg per lane) of cultures of LBY413 (hxt2 null mutant) (lane 1) and LBY410 (HXT2) (lane 2) shifted to low glucose. Molecular weight standards (thousands) are indicated on the left. The 47-M, Hxt2 protein is indicated by the arrow on the right.

by 40% rather than 50% saturated ammonium sulfate (9) and precipitates were resuspended in PBNG.

spepHXT2.529-541 was coupled to Pierce Sulfolink agarose beads via its acetylcysteine at 3.15 mg of peptide per ml of gel bed volume. A 2-ml (bed volume) column of spepHXT2.529-541 agarose (height, 1 cm; diameter, 1.6 cm) was packed at 2 ml/min (1 ml/min/cm²) in degassed 0.05% sodium azide. All further operation was at 1 ml/min (0.5 ml/min/cm²). The column was equilibrated with PBNG. Forty-one milliliters of ammonium sulfate-precipitated day 56 antibodies at 7.1 mg/ml (intended to be antibody excess) was circulated through the column for 25 h at room temperature (26°C). The column was then flushed with 10 column volumes of PBNG, 5 column volumes of PBNG containing 1.0 M NaCl, and 5 column volumes of PBNG while 2-ml fractions were collected. The column was washed with 10 column volumes of 1.0 M NH₄OH, pH 11.2 (33), and 700-µl fractions were collected into tubes containing 560 µl of 1.0 M MES (morpholineethanesulfonic acid)-NaOH, pH 5.0. Fractions containing material that adsorbed at 280 nm were pooled, dialyzed in PBNG, and adjusted to a final protein concentration of 2 mg/ml.

Subcellular fractionation. Subcellular fractionation was a one-fifth-scale version of the method of Serrano (31). Samples of intermediate steps were saved for analysis. Homogenates were made by vortexing with glass beads for 2 min in 20-s pulses with cooling on ice-water between pulses. The centrifugation for one-fifth scale is as follows. The homogenate was centrifuged at 20,000 × g for 20 min in a Sorvall T-865 rotor to produce a membrane-enriched pellet (fraction 1-P) and a membrane-depleted supernatant (fraction 1-S). A discontinuous sucrose gradient was made with 1.5 ml of 53% (wt/wt) sucrose and 3 ml of 43% (wt/wt) sucrose, and fraction 1-P was applied to the gradients. Protein from 1.3 g of starting cells was applied per tube. The gradients were spun at 84,000 × g for 6 h in an SW50.1 rotor. The visible band on top of the 43% sucrose layer (fraction 2-UB) and the visible band at the 43%-53% sucrose interface (fraction 2-1B) of the gradient were collected with a Pasteur pipette and centrifuged at 80,000 × g in the T-865 rotor for 20 min. The pellets were resuspended at 200 µg/ml (fresh weight) of starting material.

NADPH-cytochrome c reductase was measured by the method of Kubota et al. (21). Mitochondrial ATPase was measured as sodium azide-sensitive ATPase activity at pH 9.0 (29). Calcium-dependent GDPase was measured under the conditions described by Yanagisawa et al. (37), except that Pi was detected by the same method used for ATPase assays (31) and Ca²⁺-dependent GDPase was determined as activity in 10 mM CaCl₂ minus activity in 1 mM EGTA [ethylene glycol(b-aminooethyl ether)-N,N,N',N'-tetraacetic acid] and no added calcium. For all enzymes, the unit of activity is micromoles per minute at 30°C. Specific activities are expressed as units per milligram of protein.

Yeast plasma membrane ATPase, Pma1, and Hxt2 were detected by immunoblotting and quantified by scanning densitometry (see above). Pma1 antigen was used to assay the plasma membrane ATPase marker because the protein is constitutively expressed but the enzyme activity is regulated by glucose (30). Anti-Pma1 antibodies were a generous gift from Ramon Serrano.

Whole-cell homogenates. For expression experiments, cells were homogenized with glass beads by the method of Blumer et al. (6), but the lysates were not fractionated.

Miscellaneous methods. Protein was assayed by a modified Lowry method (25). Antibody capture ELISA was performed as described by Harlow and Lane (15). Glucose concentrations in media were measured with a Yellow Springs Instruments model 27 Industrial Analyzer.

RESULTS

Production of anti-Hxt2 antibodies. Immunization of rabbits with spepHXT2.529-541 produced an antipeptide titer, determined by antibody capture ELISA, at day 28 that was approximately 300-fold greater than that with preimmune serum
FIG. 2. Immunoblot of fractions from subcellular fractionation of LBY410 (HXT2 wild type) (+) and LBY413 (hxt2 null mutant) (−) probed for Hxt2. Quantitation of antigen is in Table 1. H, homogenate; 1-S, 20,000 × g supernatant; 1-P, 20,000 × g pellet; 2-UB, upper band of sucrose step gradient; 2-IB, interface band of sucrose step gradient.

(data not shown). This titer did not increase with further injections. Before the rabbits were sacrificed, the serum was evaluated for the ability to recognize Hxt2. Immunoblotting showed that the day 56 serum recognized a band that was present in the HXT2 wild-type strain LBY410 but not in the hxt2::LEU2 null mutant strain LBY413 (Fig. 1A). This band was competed against for antibody by free spepHXT2.529-541. At high antibody concentrations and/or long film exposures, many other bands were seen, but they were present in the preimmune serum and not affected by incubation of antibodies with the synthetic peptide.

At least one lower-molecular-weight band cross-reacted with the anti-spepHXT2.529-541 antibodies. It migrated at the dye front of 7.5% acrylamide gels. In 8.5% acrylamide gels, it migrated at approximately 29 kDa. This band was diminished by spepHXT2.529-541 (Fig. 1A) and was present in both LBY413 and LBY410, demonstrating that it was not a degradation product of Hxt2. Like Hxt2, it cofractionated with Pma1 (see Fig. 2). The cross-reacting material did not vary in response to glucose, though it did increase upon approach to stationary phase (see Fig. 5).

Anti-Hxt2 antibodies were purified on a spepHXT2.529-541 column, which greatly increased specific activity (Fig. 1A). The cross-reacting band was still detected by affinity-purified antibodies.

Mobility of Hxt2 in SDS-PAGE. Hxt2 migrated in SDS-PAGE as a broad band or closely spaced doublet (depending on the resolution of the gel) with an average Mr of 47,000 (Fig. 1B). One possible cause of this broad electrophoretic mobility is N-linked glycosylation, since the Hxt2 sequence has two consensus N-linked glycosylation sites (20). However, shifting yeast cells from high glucose to low glucose plus 10 μg of tunicamycin per ml did not affect the Mr or distribution of Hxt2 on immunoblots, though it did block glycosylation of invertase (data not shown).

Subcellular location of Hxt2. Yeast cells shifted to low glucose for 2 h, conditions which yield maximum expression of Hxt2, were homogenized and subjected to subcellular fractionation. In sucrose density gradient centrifugation of a membrane-enriched fraction, Hxt2 cofractionated with the plasma membrane ATPase, Pma1. Both Hxt2 and Pma1 were enriched approximately threefold in fraction 2-IB relative to fraction 2-UB (Fig. 2 and Table 1). Mitochondrial ATPase, calcium-dependent GDPase, and NADPH-cytochrome c reductase were each depleted at least sixfold in fraction 2-IB relative to fraction 2-UB (Table 1). Thus, Hxt2 is a plasma membrane protein.

A total protein stain of the plasma membrane fraction showed that Hxt2 is a major membrane protein under conditions in which it is highly expressed. It was visible by Coomassie blue stain as a diffuse band (Fig. 3).

When the purified membrane fraction (fraction 2-1) was treated with high pH or urea, treatments which disrupt protein-protein interactions, Hxt2 remained associated with the membrane (Fig. 4). Observation of total protein showed that

FIG. 3. Coomassie blue-stained gel (10 μg of protein per lane, 8.5% acrylamide) of fractions from subcellular fractionation of LBY413 (hxt2 null mutant) (−) and LBY410 (HXT2 wild-type) (+). Hxt2 is indicated by the arrow on the right. Fractions are as given in the legend of Fig. 2. Numbers on the left are molecular weights in thousands.

### TABLE 1. Subcellular fractionation of LBY410

| Fraction | Hxt2 (relative antigen)* | Pma1 (relative antigen)* | NADPH-cytochrome c reductase (U/mg) | ATPase, pH 9 (U/mg) | ATPase, pH 9, with 5 mM NaF (U/mg) | GDPase with 10 mM Cyp (U/mg) | GDPase with 10 mM EGTA (U/mg) | Protein (mg) |
|----------|-------------------------|--------------------------|-------------------------------------|-------------------|----------------------------------|----------------------------|----------------------------|-------------|
| Homogenate | 1.00                    | 1.00                     | 0.09                                | 0.16              | 0.12                             | 0.48                       | 0.36                      | 178         |
| 1-S      | 0.20                    | 0.20                     | 0.08                                | 0.15              | 0.12                             | 0.47                       | 0.38                      | 162         |
| 1-P      | 21.50                   | 8.90                     | 0.19                                | 0.29              | 0.10                             | 0.49                       | 0.23                      | 10.2        |
| 2-U      | 9.00                    | 4.20                     | 0.17                                | 0.56              | 0.12                             | 0.88                       | 0.30                      | 2.1         |
| 2-1      | 27.30                   | 11.10                    | 0.03                                | 0.04              | 0.03                             | 0.14                       | 0.08                      | 1.1         |

* Proteins were detected by immunoblot and quantified by scanning densitometry. Relative antigen was measured by dividing densitometric area by total protein loaded and normalizing fraction values to the densitometric area per protein of the homogenate. In this experiment the Hxt2 peak area of 2.0 μg of homogenate was 80,700, and the Pma1 peak area of 0.5 μg of homogenate was 98,200. Units are comparable for the same antigen in different fractions, but the two antigens are not quantitatively comparable to each other.
FIG. 4. Extraction of Hxt2 from the plasma membrane. LBY410 fraction 2-IB (450 µg) was incubated with either TEDG buffer, 2.5 M urea in TEDG buffer, 100 mM sodium carbonate (pH 11.0), 1.0% Triton X-100 in TED buffer, or 1.0% octylglucoside in TED buffer for 30 min on ice. Samples were centrifuged at \( R_{	ext{max}} = 150,000 \times g \) for 1 h. Supernatant (S) and pellet (P) were collected. Equal amounts of total sample volume from each treatment were loaded for SDS-PAGE, and Hxt2 (arrow) was detected by immunoblot. The high-\( M \) species is not HXT2 related.

Treatment with high pH or urea did solubilize discrete protein species (data not shown) but not Hxt2. When the purified membrane fraction was treated with detergents, Hxt2 was released into the soluble fraction. Hxt2 was only slightly released by Triton X-100 but was strongly solubilized by octyl glucoside. Thus, Hxt2 is an integral membrane protein.

Expression of Hxt2 in response to glucose concentration. Expression of Hxt2 was measured under two different conditions that derepress high-affinity glucose transport (4). Hxt2 as a fraction of total protein increased approximately 20-fold after a shift from high glucose to low glucose for 2 h (Fig. 5). This is a condition which produces high expression of high-affinity glucose transport and under which the hxt2 transport phenotype has been observed (20). Hxt2 protein was expressed at or below repressed levels during the entire course of growth on 2.0% glucose. Even though net high-affinity glucose transport increases as yeast cells growing in high glucose reach glucose exhaustion (4), Hxt2 as a fraction of total protein actually decreased from the level present in yeast cells in 2.0% glucose and was undetectable when glucose was exhausted (Fig. 5).

Because of this disparity in expression of Hxt2 and high-affinity glucose transport, glucose transport was assayed in HXT2 wild-type and null mutant strains in a shift to low glucose and after growth from high glucose to glucose exhaustion. The glucose transport velocity at 0.5 mM can be used as a quantitative estimate of high-affinity glucose transport. The contribution of low-affinity transport is small, as 0.5 mM is approximately 1/30 of the low-affinity \( K_m \) (4). In addition, the low-affinity system is believed to be constitutive, and the strains compared do not differ in low-affinity transport (20). Thus, even if the data contain some low-affinity component, the variation in velocity at 0.5 mM between HXT2 and hxt2 strains can be assumed to be due to variation in high-affinity transport. Consistent with previous work (20), deletion of the HXT2 gene resulted in at least a 50% reduction in high-affinity glucose transport by this estimate (Table 2). hxt2::LEU2 disruption did not affect the high-affinity glucose transport that was expressed as yeast cells reach glucose exhaustion (Table 2). Deletion of HXT2 does not affect growth rate over the course of aerobic batch culture on 2.0% glucose (20), and it is shown here that Hxt2 is never highly expressed under such conditions. It is reasonable to expect that deletion of HXT2 would affect growth rate under conditions in which it is highly expressed.

Effect of HXT2 deletion on growth rate on low glucose. Because Hxt2 was found to be maximally expressed in fresh medium containing low glucose as the only carbon source, we

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**TABLE 2. Expression of high-affinity glucose uptake and Hxt2 protein**

| Growth condition       | Strain      | Glucose in medium (%) | Uptake velocity\(^a\) (0.5 mM glucose) | Relative antigen\(^b\) |
|------------------------|-------------|-----------------------|---------------------------------------|------------------------|
| Shifted to high glucose| LBY413 (null) | 1.700                 | 1.3                                   | 0                      |
|                        | LBY410 (wild type) | 1.810                 | 1.1                                   | 0.04                   |
| Shifted to low glucose | LBY413     | 0.038                 | 5.2                                   | 0                      |
|                        | LBY410     | 0.013                 | 12.4                                  | 1.00                   |
| Grown from high to low glucose | LBY413 | 0.030                 | 7.7                                   | 0                      |
|                        | LBY410     | 0.046                 | 7.4                                   | ND\(^c\)               |
| Grown to glucose exhaustion | LBY413 | 0.005\(^e\)               | 10.5                                  | 0                      |
|                        | LBY410     | 0.005\(^e\)               | 11.3                                  | ND                     |

\(^a\) Data are averages of duplicate experiments.

\(^b\) Nanomoles per minute per milligram (dry weight); average of triplicate assays.

\(^c\) Hxt2 signal on immunoblots was quantified by scanning densitometry. Data were normalized to the signal from the shift to low glucose as described in Table 1, footnote a. Here, relative antigen of 1.00 = 121,200 densitometric units per 4.0 µg.

\(^d\) ND, not detectable.

\(^e\) This reading is probably due to interfering substances in the glucose assay; true value is zero.
measured growth and glucose consumption of wild-type and hxt2::LEU2 strains during aerobic batch culture on 0.05% glucose. Cultures were inoculated at a low density (optical density at 580 nm = 0.035) so that they would go through several doublings before exhausting glucose. Deletion of HXT2 reduced growth and glucose consumption rates under these conditions (Fig. 6A). There was no effect on lag time or final density, and the hxt2::LEU2 strain was able to completely exhaust the glucose. The greater growth rate of the wild-type strain persisted only as long as glucose was available.

Timing of expression of Hxt2 corresponded directly with the growth effect (Fig. 6B). Hxt2 as a fraction of total protein increased as the yeast cells began exponential growth, reached a maximum level equal to that seen in a medium shift to low glucose, and began to decline upon exhaustion of glucose. Thus, although Hxt2 was not expressed at all times when net high-affinity glucose transport was observed, there was an excellent correlation between the level of protein as detected by antibody and the impact of the loss of Hxt2 protein on growth or glucose uptake.

**DISCUSSION**

Previous analysis of the sequence of HXT2 and transport kinetics of an hxt2::LEU2 mutant led to the prediction that HXT2 encodes a high-affinity glucose transporter (20). The pattern of expression and subcellular location of Hxt2 described in this work support that model.
Antibodies raised against the carboxyl-terminal 13 amino acids of Hxt2 recognize Hxt2 protein in immunoblots. Immunofinity purification was necessary to reduce nonspecific background. There is at least one species that does cross-react with the anti-sppHXT2.529-541 antibodies, and it too is an integral plasma membrane protein, but it can easily be separated from Hxt2 by electrophoresis.

Hxt2 migrates as a broad band or closely spaced doublet with a relative mobility corresponding to 47 kDa, much greater than what is predicted by the sequence (59.8 kDa). An anomalously high relative mobility in SDS-PAGE is a common characteristic of proteins with high hydrophobic amino acid contents (26), such as glucose transporters (1, 12, 24, 36). For example, human erythrocyte glucose transporter cDNA predicts a protein of 54.1 kDa, but in deglycosylated form the protein migrates at 46 kDa (24). The E. coli galactoside/H+ transporter is predicted by its gene, lacY, to be 46.5 kDa, but the protein migrates at 51 kDa (36). The fact that Hxt2 migrates as a doublet is probably due to its high hydrophobicity, which allows it to have secondary structure even in the presence of SDS. A human erythrocyte membrane protein was observed to migrate as a sharp, closely spaced doublet in SDS-PAGE with 0.1% SDS but to be resolved as one band in a gel containing 1.0% SDS (10). Long exposure of a chemilumigam containing a large Hxt2 signal shows multiple minor bands of descending relative mobility (Fig. 5 and 6B). A decrease in mobility can be caused by glycosylation, as is the case with mammalian glucose transporters (24), and the Hxt2 sequence has two consensus N-linked glycosylation sites, one of which is in the same topological position as the glycosylated site of GLUT1 (20). However, we are unable to detect glycosylation of Hxt2.

Hxt2 is demonstrated to be a plasma membrane protein by its cofractionation with the yeast plasma membrane ATPase, Pma1. The method used is an adaptation of a method for purification of yeast plasma membranes. Though Hxt2 and Pma1 are both present in a fraction greatly depleted of all membranes except the plasma membrane, they are also present in the fraction that contains all nonplasma membranes, including the secretory system. The method used here cannot determine whether that is due to plasma membrane contamination of fraction 2-UB or due to Hxt2 and Pma1 in the secretory pathway en route to the plasma membrane. The method used here is sufficient to prove that Hxt2 is a plasma membrane protein but cannot address the dynamics of its transit through the secretory pathway.

Treatment of plasma membranes with reagents that disrupt protein-protein or protein-lipid interactions showed that Hxt2 is an integral membrane protein. Hxt2 is solubilized only weakly by Triton X-100 but strongly by octyl glucoside. Octyl glucoside is a very powerful and useful nonionic detergent because of its ability to solubilize membrane protein without denaturation and its relative ease of removal due to its high critical micelle concentration (27).

When Hxt2 is highly expressed, high-affinity glucose transport and growth are dependent on its activity. Yeast cells shifted from high to low glucose display a 20-fold increase in Hxt2 as a fraction of total protein and at the same time express high-affinity glucose transport that requires HXT2. When yeast cells growing in high glucose reach derepressing concentrations and glucose exhaustion, high-affinity glucose transport is expressed, but Hxt2 is not detectable. The high-affinity glucose transport expressed does not have an HXT2-dependent component. This explains the previous observation that hxt2::LEU2 does not affect growth rate on high glucose, even as yeast cells reach glucose exhaustion (20). When Hxt2 is highly expressed, such as during batch culture on initially low glucose, hxt2::LEU2 does affect growth rate.

Two different derepressing conditions, batch culture from high glucose to near glucose exhaustion and a shift to fresh medium containing only glucose, kinetic radically different with respect to Hxt2 expression. Thus, caution must be taken when comparing glucose transport from different derepressing conditions. Preliminary studies suggest that both growth and the presence of low glucose are required for Hxt2 expression. Future experiments will address the nature of this regulation. In addition to supporting a proposed role for Hxt2 as a genuine high-affinity glucose transporter, these results suggest that the model of high-affinity glucose transport in S. cerevisiae being catalyzed by multiple transporters. The simple kinetic view of high-affinity glucose transport being conducted by a single species is invalid, and kinetic constants represent a composite of the kinetics of the individual species present. Within this group, there is differential and subtle regulation. This work sets the stage for biochemical analysis of Hxt2 by purification and reconstitution and analysis of posttranslational modification. The antibodies developed here can be used for assay of Hxt2 in further physiological and genetic experiments.

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