Properties of the Human Erythrocyte Glucose Transport Protein Are Determined by Cellular Context†

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ABSTRACT: Human erythrocyte hexose transfer is mediated by the glucose transport protein GLUT1 and is characterized by a complexity that is unexplained by available hypotheses for carrier-mediated sugar transport [Cloherty, E. K., Heard, K. S., and Carruthers, A. (1996) *Biochemistry 35*, 10411−10421]. The study presented here examines the possibility that the operational properties of GLUT1 are determined by host cell environment. A glucose transport-null strain of *Saccharomyces cerevisiae* (RE700A) was transfected with the p426 GPD yeast expression vector containing DNA encoding the wild-type human glucose transport protein (GLUT1), mutant GLUT1 ($GLUT1_{338-A3}$), or carboxy-terminal hemagglutinin-polyHis-tagged GLUT1 (GLUT1-HA-H6). GLUT1 and GLUT1-HA-H6 are expressed at the yeast cell membrane and restore 2-deoxy-D-glucose, 3-*O*-methylglucose, and D-glucose transport capacity to RE700A. GLUT1-HA-H6 confers GLUT1-specific sugar transport characteristics to transfected RE700A, including inhibition by cytochalasin B and high-affinity transport of the nonmetabolized sugar 3-*O*-methylglucose. $GLUT1_{338-A3}$, a catalytically inactive GLUT1 mutant, is expressed but fails to restore RE700A sugar uptake capacity or growth on glucose. In contrast to transport in human red cells, $K_{m(app)}$ for 2-deoxy-D-glucose uptake equals $K_{i(app)}$ for 2-deoxy-D-glucose inhibition of 3-*O*-methylglucose uptake. Unlike transport in human red cells or transport in human embryonic kidney cells transfected with GLUT1-HA-H6, unidirectional sugar uptake in RE700A-GLUT1-HA-H6 is not inhibited by reductant and is not stimulated by intracellular sugar. Net uptake of subsaturating 3-*O*-methylglucose by RE700A-GLUT1-HA-H6 is a simple, first-order process. These findings support the hypothesis that red cell sugar transport complexity is host cell-specific.

The facilitated diffusion of glucose across cell membranes is mediated by a family of integral membrane proteins named glucose transporters (GLUTs).[1] Twelve mammalian glucose transport proteins have been identified, differing in primary structure, tissue distribution, and substrate specificity (*1*). GLUT1, the human erythrocyte glucose transport protein, mediates sugar transport in red blood cells and in endothelial cells lining the vasculature and contributes to basal glucose transport in adipose and cardiac muscle (*2*−*5*). Membrane-associated GLUT1 is multimeric (*6, 7*), and cytoplasmic ATP interacts directly and cooperatively with GLUT1 to modify transporter affinity for substrate and net sugar flux through the transport complex (*8*−*10*).

Red cells are uniquely suited to steady-state sugar transport determinations because they provide a physically uniform population of cells in which to measure sugar fluxes. The human red blood cell is also an unusually rich source of GLUT1, with the transporter representing 6% or more of total erythrocyte integral membrane proteins. Erythrocyte-resident GLUT1 is not directly amenable to mutagenesis and in situ expression. Manipulation of GLUT1-mediated sugar transport through mutagenesis has required the development of heterologous expression systems. GLUT1 has been successfully expressed in mammalian cells [CHOK1 (*6*), Cos-7, and HEK293 (*11*)], *Xenopus* oocytes (*12*), and insect cells (*13*). None of these systems has achieved an efficiency of protein expression that yields sufficient GLUT1 to facilitate detailed biochemical analysis. Only the oocyte presents a relatively uniform cell geometry and thereby permits detailed kinetic analysis of GLUT1-mediated sugar transport.

Previous heterologous expression studies have suggested that *Saccharomyces cerevisiae* can express a relatively homogeneously glycosylated form of human GLUT1 (*14*). Yeast contains a superfamily of sugar transporters (35 genes)

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[1] Abbreviations: GLUTs, glucose transporters; GLUT1, human erythrocyte glucose transporter; 2DG, 2-deoxy-D-glucose; 3MG, 3-*O*-methyl-D-glucopyranoside; C-Ab, rabbit polyclonal antiserum raised against a synthetic peptide comprised of GLUT1 residues 480−492; CCB, cytochalasin B; DMEM, Dulbecco's modified Eagle's medium; Dpm1p, dolichol phosphate mannose synthase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FOA, 5-fluoroorotic acid; Glc, D-glucose; GPD, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; HA-Ab, mouse monoclonal antibody directed against the hemagglutinin sequence LYPYNVPNYA; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; OST1, oligosaccharyltransferase1; RBC, red blood cell; RE700A, HXT1−7 null yeast strain; SCM, synthetic complete medium; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Ste2p, *S. cerevisiae* α-factor receptor.

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that includes maltose, inositol, and glycerophosphoinositol transport proteins (*15*). *S. cerevisiae* contains a subfamily of 18 hexose transporters [HXT 1−17 and galactose permease (Gal2)] which, like the GLUTs, catalyze the facilitated diffusion of sugars across cell membranes (*16*). Sequence alignment indicates that the HXT proteins are similar to GLUT1, containing 12 putative transmembrane domains and intracellular amino and carboxyl termini (*17*). Yeast may, therefore, present a suitable expression system in which to produce wild-type and mutant recombinant human red blood cell glucose transport proteins.

Deletion of *S. cerevisiae* HXT 1−7 produces a strain of yeast (RE700A) that is unable to grow on glucose media (*18*). This suggests that HXT 1−7 are the major hexose transporters in *S. cerevisiae*. Wieczorke et al. (*16*) have suggested that a minimum of 20 hexose transporter genes (hxt 1−17, Gal2, and maltose permeases Y9DL247w and YJR160c) must be eliminated in *S. cerevisiae* to produce a strain completely lacking in sugar transport capacity. However, in contrast to RE700A, the yeast strain used in their study (CEN.PK2-1C) appears to possess a greater capacity for respiration and, consequently, may be able to grow by utilizing residual levels of sugar present in the cells (*19*). Gal2 (a galactose permease) remains in the RE700A strain where it is tightly controlled by the presence of galactose and is inhibited by a high level of intracellular glucose (*20*). Expression of the maltose permeases (YDL247w and YJR160c), which may modulate glucose transport in *S. cerevisiae*, is also repressed by glucose (*21*).

This study characterizes human GLUT1 expression and function in RE700A. When transfected with exogenous human GLUT1, RE700A can be selected because of its ability to grow on and transport glucose. Expressed GLUT1 interacts with human GLUT1-directed C-terminal antisera, colocalizes with yeast plasma membrane markers, displays a functional stereochemistry similar to that of erythrocyte-resident GLUT1, and is inhibited by GLUT1-specific sugar transport inhibitors. This heterologous expression system may provide a rapid screening vehicle for mutant GLUT1 proteins. Significant kinetic differences between sugar transport in human red cells and GLUT1-expressing RE700A cells suggest that the complexity of human erythrocyte sugar transport (*22*) may be determined by the host cell environment.

EXPERIMENTAL PROCEDURES

Materials. [^{14}C]-D-Glucose, [^{14}C]-3-*O*-methylglucose, and [^{3}H]-2-deoxyglucose were purchased from Dupont NEN (Wilmington, DE). Complete protease inhibitor tablets were purchased from Roche (Mannheim, Germany). Supersignal ECL reagents were obtained from Pierce (Rockford, IL). Centricon concentrators were acquired from Millipore (Bedford, MA). SCM-URA dropout mix was purchased from Bufferad (Newark, NJ). Nitrocellulose and Immobilon-P were obtained from Fisher Scientific (Pittsburgh, PA). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA).

Red Cells. De-identified, washed red blood cells and whole blood stored in CPDII AS-1 preservative solution were obtained from Biological Specialties Corp. (Colmar, PA). Red cells were isolated by repeated washing and centrifugation cycles in ice-cold saline. One volume of whole blood was mixed with ≥3 volumes of saline and centrifuged at 10000*g* for 5 min at room temperature. Serum and the buffy coat were aspirated, and the washing and centrifugation cycle was repeated until the supernatant was clear and the buffy coat was no longer visible. Cells were resuspended in 20 volumes of saline and were incubated for 30 min at 37 °C to deplete intracellular sugar levels.

HEK293 Cells. HEK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 37 °C humidified 5% CO_2 incubator.

Antisera. A peptide corresponding to GLUT1 residues 480−492 was synthesized by the University of Massachusetts Medical School Peptide Synthesis facility. This peptide was conjugated to keyhole limpet hemocyanin using a kit purchased from Pierce. Rabbit antisera (C-Ab) against this GLUT1 peptide were obtained from Animal Pharm Services Inc. (Healdsburg, CA). The goat anti-rabbit IgG−horseradish peroxide conjugate was purchased from Bio-Rad (Hercules, CA). Anti-yeast dolichol phosphate mannose synthase (Dpm1p) antiserum was obtained from Molecular Probes (Eugene, OR). Rabbit anti-yeast Ste2p antiserum was a generous gift from D. Jenness (Department of Microbiology and Molecular Genetics, UMass Medical School).

Solutions. Phosphate-buffered saline (PBS) contained 140 mM NaCl, 10 mM Na_2HPO_4, 3.4 mM KCl, 1.84 mM KH_2PO_4 (pH 7.3), and 5 mM EDTA. Spheroplasting buffer (2×) contained 2.8 M sorbitol, 0.1 M potassium phosphate buffer (pH 7.4), and 10 mM NaN_3. Membrane buffer was composed of 0.8 M sorbitol, 1 mM EDTA, and 25 mM HEPES. Sample buffer (2×) contained 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 50 mM DTT. Phosphate buffer (PB) (pH 6.5) included 0.329 mM KH_2PO_4. Lysis buffer consisted of 10 mM Tris-HCl (pH 7.5), 0.5 mM $MgCl_2$, and 0.5% Triton X-100.

Reagents. Restriction enzymes were purchased from New England Bioloabs. The glucose transport-null strain of *S. cerevisiae*, RE700A, was generously provided by M. Johnston (Washington University, St. Louis, MO). p426 and p416 yeast expression vectors, as described in ref *23*, were provided by C. Hirschberg (Department of Molecular and Cell Biology, Goldman School of Dentistry, Boston University, Boston, MA).

Genetic Engineering. A 1.7 kb fragment of GLUT1 cDNA was subcloned into the BamHI site of the high-copy number yeast expression vector p426. Removal of the 5′-untranslated region of the GLUT1 sequence was accomplished by using PCR to insert a unique SpeI restriction site 3 bp prior to the GLUT1 start codon. This construct was subcloned into the SpeI−BamHI restriction site of the p426 yeast expression vector. The triple alanine GLUT1 mutant, $GLUT1_{338-A3}$, in which GLUT1 residues 338−340 were each replaced with alanine, was engineered using the Stratagene Quickchange Site-Directed Mutagenesis Kit and confirmed by sequence analysis.

Transfection of RE700A with GLUT1. Stable expression of GLUT1 in yeast was accomplished using yeast strain RE700A (MATa hxt1::HIS3::hxt4 hxt5::LEU2 hxt2::HIS3 hxt3::LEU2::hxt6 hxt7::HIS3) (*18*). Seven of the 20 hexose transporters of *S. cerevisiae* are disrupted in this strain (*18*). This results in a yeast strain incapable of using glucose as a

primary carbon source and subsequently unable to grow on glucose media. Briefly, cells were prepared for electroporation using the following protocol. RE700A cells (250 mL) grown to an OD_{600} of 1.3−1.5 in YP-maltose were pelleted by spinning at 1500*g* for 5 min at 4 °C. Following two washes in ice-cold sterile water (250 mL followed by 125 mL), the cells were washed in 20 mL of ice-cold 1 M sorbitol and then resuspended in 1 mL of 1 M sorbitol. A portion of the cell slurry (80−100 μL) was mixed with 5−20 μg of DNA, equilibrated on ice for 10−20 min, and then transferred to a prechilled 0.2 cm electroporation cuvette. The yeast were pulsed at 1.5 kV, 25 μF, and 200 Ω. Immediately, 1 mL of ice-cold sorbitol was added to the cuvette, and the cells were plated onto SCM-URA maltose selective media. Growth was observed after 2−4 days at 30 °C.

Transient Expression of GLUT1 in HEK Cells. GLUT1-HA-H6 was subcloned into the BamHI site of the pcDNA3.1+ mammalian expression vector as described previously (*24*). Subconfluent HEK923 cells were transfected with GLUT1-HA-H6 cDNA using the Lipofectamine 2000 transfection reagent system. Following transfection, cells were maintained in DMEM for 24 h, at which time 0.72 mM G418 (Geneticin) was added to the media and the cells were allowed to continue growing for an additional 24 h.

Membrane Isolation. Yeast grown to late log phase were washed in an equal volume of cold 10 mM NaN_3 and resuspended in 20 mL each of 10 mM NaN_3 and 2× spheroplasting buffer. Zymolase 20T was added (25 mg/15 mL), and the suspension was incubated at 30 °C for 1−2 h. Successful cell wall removal was monitored by measuring yeast optical density periodically throughout the incubation period. Yeast were centrifuged at 3000*g* for 10 min, and the pellet was resuspended in a small volume of membrane buffer containing protease inhibitors. Yeast were lysed by repetitive pipetting and were then diluted to 100 mL by addition of membrane buffer and protease inhibitors. Lysed cells were spun at 3000*g* for 10 min to isolate the P1 fraction, at 8000*g* for 20 min to pellet the P2 fraction, and at 100000*g* for 30 min to sediment the plasma membrane-containing P3 fraction. Membranes were resuspended in membrane buffer with protease inhibitors and stored at −70 °C until they were used.

To harvest HEK923 membrane proteins for SDS−PAGE, culture plates of cells (150 mm) were washed twice with PBS and 5 mM EDTA, and the cells were scraped into 20 mL of hypotonic lysis buffer mixed with Roche protease inhibitor cocktail and 0.5% Triton X-100. Following incubation on ice for 20 min, the suspension was centrifuged at 100000*g* for 30 min to sediment insoluble material. The clear supernatant was collected and used immediately for SDS−PAGE.

Renografin Density Gradients. Crude yeast membranes were resolved on Renografin gradients as previously described in ref *25*. Briefly, the P3 fractions isolated from RE700A and RE700A-GLUT1-HA-H6 were combined with an equal volume of Renografin-76. The Renografin gradient was constructed on top of 0.8 mL of a sample (38% Renografin) by sequentially layering 0.8 mL of 34, 30, 26, and 22% Renografin-76 diluted in 50 mM Tris-HCl (pH 7.4). Gradients were centrifuged in a 50.1Ti rotor at 40 000 rpm for 20 h at 4 °C. Samples were isolated from the top down in aliquots of 500 μL, and stored at −70 °C until they were used. Fractions containing GLUT1, the yeast endoplasmic reticulum marker Dpm1p, and the yeast plasma membrane marker Ste2p were identified by Western blotting.

Polyacrylamide Gel Electrophoresis and Western Blotting. Proteins were resolved on 10% polyacrylamide gels as described previously in ref *26*. Recombinant human GLUT1, Dpm1p, and Ste2p were detected by Western blot analysis (*11*, *27*, *28*). Peptides separated by SDS−PAGE were transferred electrophoretically to nitrocellulose membranes, which were subsequently blocked for 1 h in PBS-T (PBS and 0.1% Tween detergent) with 20% Carnation nonfat dry milk. Following four washes of 5 min in PBS-T, membranes were incubated for 1 h in primary antibody. C-Ab was diluted 1:10000 in 3% nonfat dry milk and PBS-T, anti-Dpm1p diluted to 4 μg/mL in 3% nonfat dry milk and PBS-T, and anti-Ste1p diluted 1:2000 in 3% nonfat dry milk and PBS-T. Following four wash cycles to remove the primary antibody, membranes were exposed for 45 min to secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG) diluted 1:5000 in PBS-T containing 3% nonfat dry milk. Detection of antigen−antibody complexes was achieved by chemiluminescence using Pierce Supersignal ECL reagents.

Sugar Transport Measurements. Zero-Trans Uptake. RE700A and RE700A-GLUT1 (wild-type and mutant 338) were grown to an OD_{600} of 0.6 in YP-maltose and SCM-URA-maltose, respectively; 100 mL of the culture was centrifuged at 5000*g* and then washed twice in phosphate buffer (PB). Samples were resuspended in 1 mL of PB. Aliquots (100 μL) of cells at room temperature were exposed to 2 volumes of PBS containing [^{3}H]-D-glucose at room temperature. Uptake of 100 μM D-glucose was assessed at 5 min intervals, and then 10 volumes of ice-cold PBS and 500 mM maltose was added to the samples to stop the reaction. The yeast suspension was sedimented by centrifugation (14000*g* for 30 s); the supernatant was aspirated, and the wash/centrifugation/aspiration cycle was repeated. The resulting pellet was extracted in 500 μL of 3% perchloric acid. The acid extract was centrifuged, and duplicate samples of the clear supernatant were counted. Zero-time points were prepared by addition of ice-cold PBS to cell suspensions prior to addition of medium containing sugar and radiolabel. Cell suspensions were immediately processed. In experiments in which the total uptake was corrected for zero-time uptake, radioactivity associated with yeast suspensions at time zero was subtracted from the activity associated with cell suspensions following the uptake period. Uptake assays were performed using solutions and tubes pre-equilibrated to 20 °C.

2-Deoxy-D-glucose (2DG) uptake in yeast was carried out as described for D-glucose uptake. Aliquots (100 μL) of cells at room temperature were exposed to 2 volumes of room-temperature PBS containing [^{3}H]-2-deoxy-D-glucose. Uptake of 100 μM 2DG was assessed at 2 min intervals and quenched by addition of 10 volumes of ice-cold PBS buffer containing 500 mM maltose.

Zero-trans sugar uptake experiments in intact human red blood cells were carried out at 4 °C (to slow transport and thereby permit accurate determinations of transport) as described previously (*22*). Briefly, sugar-free cells or erythrocyte ghosts (at ice temperature) were exposed to 5 volumes of saline (ice temperature) containing variable concentrations

of unlabeled sugar and labeled sugar. Uptake was permitted to proceed over intervals of 30 s, and then 50 volumes (relative to cell volume) of stopper solution (PBS containing 10 μM CCB and 100 μM phloretin) was added to the cell suspension. Cells were sedimented by centrifugation (14000*g* for 30 s), washed once in stopper, collected by centrifugation, and extracted in 500 μL of 3% perchloric acid. The acid extract was centrifuged, and duplicate samples of the clear supernatant fluid were counted. Zero-time uptake points were prepared by addition of stopper to cells prior to addition of medium containing sugar and radiolabel. Cells were immediately processed. Radioactivity associated with cells at time zero was subtracted from the activity associated with cells following the uptake period. Uptake assays were performed using solutions and tubes pre-equilibrated to 4 °C. Triplicate or quadruplicate samples were processed at each time point. Cell counts were obtained from a calibration curve relating red cell absorbance at 417 nm to cell numbers (obtained using a hemocytometer).

Michaelis−Menten parameters for sugar uptake by recombinant human GLUT1 expressed in yeast were calculated by measuring the concentration dependence of 2DG transport (from 100 μM to 100 mM 2DG). 3MG inhibition of 2DG transport in wild-type yeast and RE700A-GLUT1 was analyzed by assessing 2DG uptake (100 μM) in the presence or absence of increasing concentrations (from 100 μM to 100 mM) of 3MG. 2DG inhibition of Glc transport in wild-type yeast and RE700A-GLUT1 was analyzed by assessing Glc uptake (100 μM) in the presence or absence of increasing concentrations (from 100 μM to 100 mM) of 2DG.

FOA Induced Vector Dropout. Clones of RE700A-GLUT1-HA-H6 that have lost the URA3-based p426-GPD plasmid were selected by plating on media containing 5-fluoroorotic acid (5-FOA; 0.1% for 2−3 days).

RESULTS

Yeast strain RE700A is capable of growth on maltose (a disaccharide imported by maltose-H+ symport and then hydrolyzed to yield two glucose molecules) but not on glucose (*18*). RE700A were transfected with epitope-tagged GLUT1 cloned into the yeast expression vector p426 GPD and were subsequently plated sequentially onto selective (URA-free) medium containing maltose only (SCM-URA maltose) and then medium containing glucose only (SCM-URA glucose). Transfection with either an empty vector (p426 GPD) or p426-GLUT1-HA-H6 results in growth on selective maltose medium. Only p426-GLUT1-HA-H6-containing yeast grow on selective glucose medium (Figure 1). In the absence of vector, RE700A fails to grow on selective medium (Figure 1). RE700A was also transfected with the $GLUT1_{338-3A}$ mutant (residues 338−340 each replaced with alanine). Mammalian cells (Cos-7) transiently transfected with $GLUT1_{338-3A}$ exhibit sugar transport rates indistinguishable from that of untransfected control cells, yet GFP-tagged $GLUT1_{338-3A}$ is expressed at high levels at the plasma membrane, suggesting that $hGLUT1_{338-3A}$ is targeted correctly but is catalytically impaired (*24*). Figure 1 demonstrates that RE700A-$GLUT1_{338-3A}$ grows on maltose but lacks the ability to survive on glucose. This result is consistent with previous observations in mammalian cells suggesting that GLUT1 mutant $GLUT1_{338-3A}$ lacks the capacity to transport D-glucose.

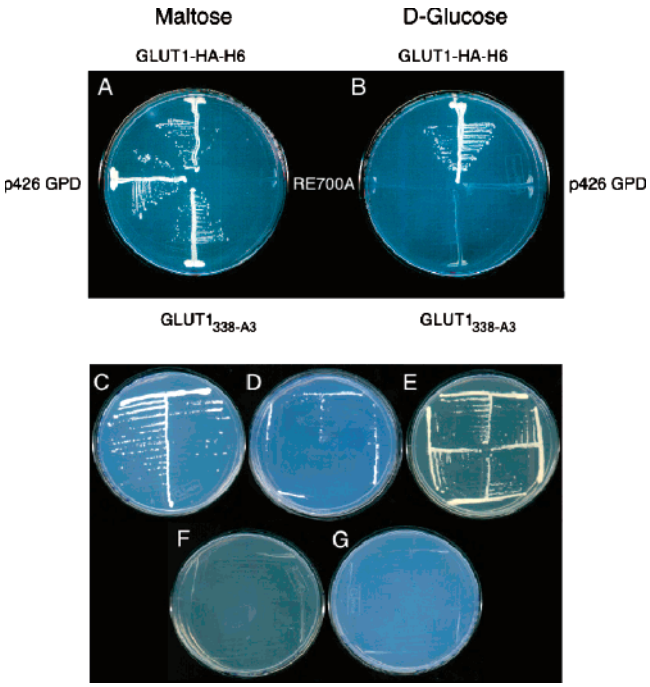

FIGURE 1: GLUT1-HA-H6 expression in *S. cerevisiae*. RE700A cells were transfected with HA-H6-tagged wild-type GLUT1 (GLUT1-HA-H6), with mutant GLUT1 ($GLUT1_{338-A3}$), with an empty vector (p426 GPD), or without a vector (RE700A) and plated on SCM-URA maltose (A) or SCM-URA glucose (B). Plates were photographed after 3 days. (C) RE700A-GLUT1-HA-H6 grown on SCM-URA maltose. (D) RE700A-GLUT1-HA-H6 streaked onto 5-FOA-containing media. (E) RE700A-GLUT1-HA-H6 restreaked post-5-FOA exposure (3 days) onto YP-maltose. (F) RE700A-GLUT1-HA-H6 restreaked post-5-FOA exposure (3 days) onto YP-dextrose. (G) RE700A-GLUT1-HA-H6 restreaked post-5-FOA exposure (3 days) onto SCM-URA dextrose.

To determine whether growth on glucose results directly from the GLUT1-HA-H6 sequence carried on the p426 GPD vector, cells containing p426 GLUT1-HA-H6 were plated onto medium containing 5-fluoroorotic acid (FOA). FOA is toxic to yeast expressing the URA-3 gene. This medium thus selects clones that have lost their URA-3-containing plasmid (p426 GLUT1-HA-H6). Following exposure of RE700A-GLUT1 to FOA, growth was observed on YP maltose but not on YP glucose, SCM-URA maltose, or SCM-URA glucose (Figure 1C−G). This suggests that yeast which lose the GLUT1-containing vector (p426 GLUT1-HA-H6) also lose the capacity to survive on glucose.

hGLUT1 Expression in RE700A. GLUT1-transfected RE700A were screened for protein expression by immunoblot analysis. Initial attempts at RE700A transfection resulted in yeast with slow growth rates (doubling time of >8 h on glucose medium) and undetectable GLUT1 expression by Western analysis of total yeast membranes (lane 6 of Figure 2A). In an effort to improve human GLUT1 expression, we re-engineered the transporter construct by deleting the mammalian 5′-untranslated region (5′UTR). A unique SpeI site 3 bp 5′ to the GLUT1 start codon was introduced by PCR. Excision, using SpeI and BamHI, permits ligation of the resulting 1.5 kb fragment of epitope-tagged GLUT1 (5′UTR-GLUT1-HA-H6) into the SpeI/BamHI restriction site of the p426 GPD expression vector. RE700A was transfected with p426 5′UTR-GLUT1-HA-H6 and plated as described previously. These yeast grow more rapidly on glucose medium (doubling time of ≈2 h).

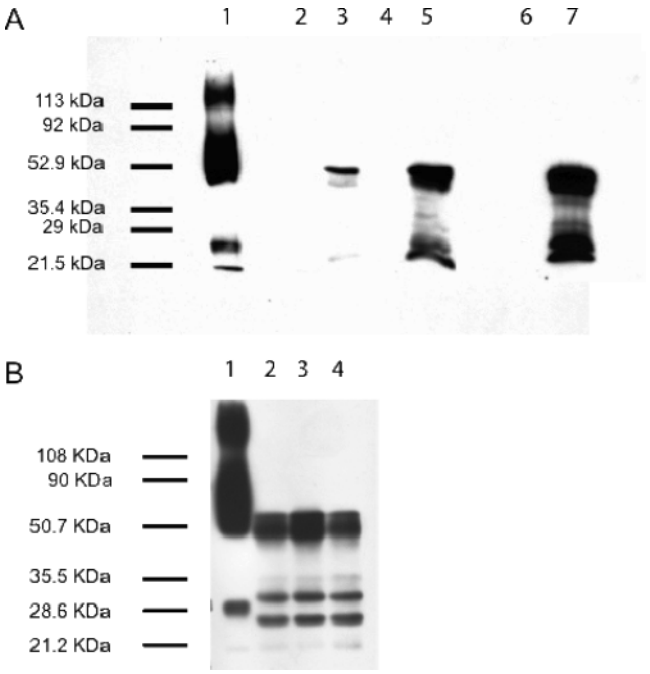

FIGURE 2: Human GLUT1 expression in *S. cerevisiae*. (A) Western blot using GLUT1 carboxyl-terminal antibodies (C-Ab) of GLUT1 expressed in RE700A. Membranes were isolated by differential centrifugation, resolved by 10% SDS−PAGE, and transferred to nitrocellulose for Western blotting. Lanes 2−7 each contained 35 μg of total membrane protein. Lane 1 contained 1 μg of purified erythrocyte GLUT1. Two fractions were obtained upon yeast lysis. The F2 fraction (lanes 2 and 3) was collected as the low-speed (8000*g*) pellet. The F3 membrane fraction (lanes 4−7) was obtained as the high-speed (100000*g*) pellet. Lane 2 contained membranes from the RE700A hxt-null mutant. Lane 3 contained membranes from 5′UTR-GLUT1-HA-H6-transfected RE700A. Lane 4 contained membranes from the RE700A hxt-null mutant. Lane 5 contained membranes from 5′UTR-GLUT1-HA-H6-transfected RE700A. Lane 6 contained membranes from RE700A transfected with GLUT1-HA-H6 containing the 5′UTR. Lane 7 contained membranes from 5′UTR-GLUT1-HA-H6-transfected RE700A. Lanes 6 and 7 were obtained from a separate gel. The migration and mass of molecular weight markers are shown to the left of lane 1. (B) Western blot using GLUT1 carboxyl-terminal IgGs (C-Ab) of GLUT1 expressed in RE700A. Membranes were isolated by differential centrifugation, resolved by 10% SDS−PAGE, and transferred to nitrocellulose for Western blotting. Lanes 2−4 each contained 35 μg of fraction F3 membrane protein. Lane 1 contained 0.25 μg of purified erythrocyte GLUT1. Lanes 2−4 contained membranes from RE700A-5′UTR-GLUT1-HA-H6 grown on glucose (lane 2), maltose (early passage, lane 3), or maltose (late passage, lane 4). The migration and mass of molecular weight markers are shown to the left of lane 1.

C-Ab immunoblot analysis of yeast membranes indicates the presence of a 50−55 kDa C-Ab reactive protein in RE700A-5′UTR-GLUT1-HA-H6 (Figure 2A, lanes 3 and 5; Figure 2B, lanes 2−4) which is absent in untransfected cells (Figure 2A, lanes 2 and 4). Thus, removal of the 5′UTR appears to improve GLUT1 expression in RE700A. Densitometric analysis suggests that recombinant GLUT1 [present in both the F2 (8000*g*) and F3 (100000*g*) membrane fractions of RE700A-5′UTR-GLUT1-HA-H6] approaches 1% of the total protein. GLUT1 expressed in yeast is characterized by a relatively narrow electrophoretic mobility. In contrast, purified human red blood GLUT1 is heterogeneously glycosylated and is resolved as a broader smear (Figure 2A, lane 1). C-Ab reactive peptides with a relatively low M_r (21−29 kDa) are detected in the F3 fraction of RE700A-GLUT1-5′UTR (Figure 3A, lane 5), suggesting that GLUT1 proteolytic fragments or truncated versions of the transporter may also be present. GLUT1-HA-H6 expression is unaffected by growth medium and passage number (Figure 2B).

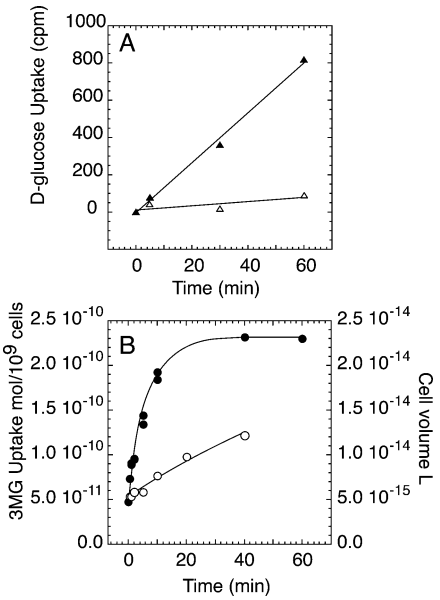

FIGURE 3: Sugar transport by RE700A expressing or lacking hGLUT1-HA-H6. (A) D-Glucose transport in RE700A transfected with GLUT1 DNA containing the 5′UTR. D-Glucose uptake was assessed in transfected (▲) and untransfected (△) cells. Each point represents the mean of four or more separate determinations. (B) 3MG uptake in RE700A transfected with GLUT1 DNA lacking the 5′UTR. 3-*O*-Methyglucose uptake (100 μM sugar) was assessed in transfected (●) and nontransfected (○) cells. Each point represents the mean of four or more separate determinations. The alternate *y*-axis indicates the 3MG space of the cells (liters). The curve drawn through the full time course was computed by nonlinear regression by assuming first-order uptake kinetics according to the expression $3MG_t = 3MG_0 + 3MG_\infty(1 - e^{-kt})$, where $3MG_t$ is the accessible 3MG space of RE700A-GLUT1-HA-H6 at time *t*, $3MG_0$ is the instantaneously accessible 3MG space of RE700A-GLUT1-HA-H6 (5.3 ± 0.5 fL), $3MG_\infty$ is the slowly exchangeable equilibrium 3MG space of RE700A-GLUT1-HA-H6 (17.8 ± 6.8 fL), and *k* is the rate constant for equilibration of the space (0.15 ± 0.02 min^{-1}; $R^2 = 0.9943$). Curve fitting assuming multiple (e.g., fast and slow) components of 3MG exchange results in compartments characterized by identical rate constants (0.15 min^{-1}), indicating that only a single exchangeable compartment is necessary to explain the uptake data.

GLUT1-HA-H6 thus promotes growth on glucose and is expressed in RE700A cells, but does it promote glucose uptake by RE700A? The level of net uptake at 24 °C of 100 μM [^{3}H]-D-glucose by RE700A-5′UTR-GLUT1-HA-H6 is 12-fold greater than the level of uptake in nontransfected RE700A (Figure 3A). The level of uptake of [^{14}C]-3-*O*-methylglucose (3MG, a transported but nonmetabolizable sugar) is 10.5-fold greater in RE700A-5′UTR-GLUT1-HA-H6 than in RE700A (Figure 3B). Uptake of 3MG by human red cells is characterized by multiphasic import kinetics (*10*, *29*−*31*). 3MG uptake by RE700A-5′UTR-GLUT1-HA-H6 is a simple, first-order process (Figure 3B) characterized by an instantaneously accessible 3MG space of 5.3 ± 0.5 fL per cell, an exchangeable 3MG (water) space of 17.8 ± 6.8 fL per cell, and a rate constant for equilibration of this space of 0.15 ± 0.02 per min. Analysis of the time course of 3MG uptake assuming multiple (fast and slow) exchangeable compartments results in multiple compartments characterized

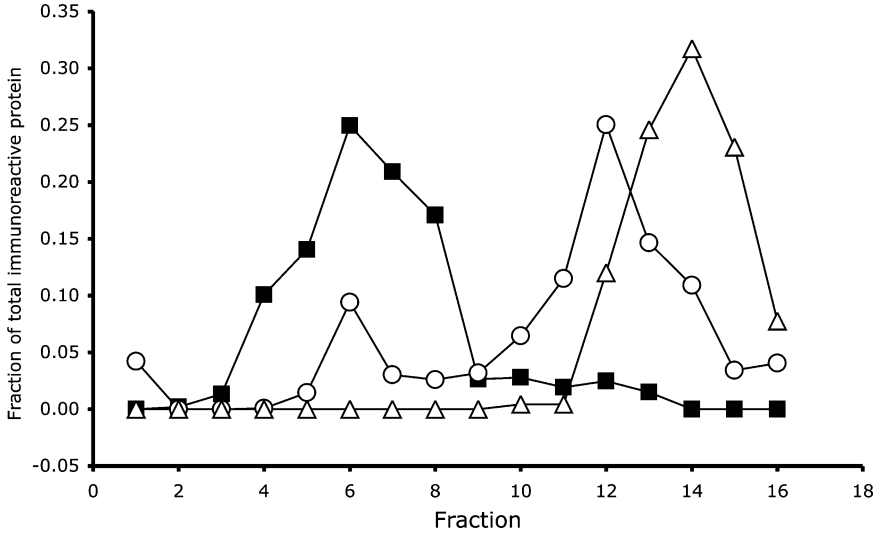

FIGURE 4: Renografin gradient centrifugation of yeast membranes. F3 fractions isolated from RE700A and RE700A-GLUT1 were combined with an equal volume of Renografin-76. The Renografin gradient was constructed over 0.8 mL of sample (38% Renografin) by sequentially layering 0.8 mL of 34, 30, 26, and 22% Renografin-76 diluted in 50 mM Tris-HCl (pH 7.4). Gradients were centrifuged in a 50.1Ti rotor at 40 000 rpm for 20 h at 4 °C. Samples were isolated from the top down in aliquots of 500 μL, and stored at −70 °C until they were used. Fractions containing GLUT1 (△), yeast endoplasmic reticulum marker Dpm1p (■), and yeast plasma membrane marker Ste2p (○) were determined by Western blot analysis. The distributions of Dpm1p and Ste2p are unchanged by GLUT1 expression (nontransfected RE700A yeast give identical results for Dpm1p and Ste2p; not shown).

Table 1: Specificity of GLUT1-Mediated Sugar Transport

		RE700A-GLUT1-HA-H6[a]			wild-type *S. cerevisiae*[a]	human erythrocytes[b]		
sugar	temp (°C)	$K_{m(app)}$ (mM)	V_{max}[c]	$K_{i(app)}$[d]	$K_{i(app)}$[d]	$K_{m(app)}$ (mM)	V_{max}[c]	$K_{i(app)}$[d]
2DG	4					0.59 ± 0.27	19.1 ± 2.6	1.4 ± 0.4
	24	2.89 ± 0.42	2.35 ± 0.09	2.05 ± 0.57	5[e]	1.8 ± 0.2[f]		3.2[g]
3MG	4					0.38 ± 0.13	11.9 ± 1.3	1.9 ± 0.3
	24			10.1 ± 4.2	64.4 ± 3.8	4.6 ± 0.3[g]		13[g]
					250[e]	3.1 ± 0.8[h]		
						2[i]		
						15−23[j]		
CCB	4							126 ± 16
	24			142 ± 42	>10000			110[k]

[a] Sugar uptake was assessed at 24 °C in GLUT1-HA-H6-transfected RE700A yeast and in wild-type (baker's) yeast. [b] Sugar uptake in red cells was assessed at 4 °C. $K_{m(app)}$ is that concentration at which uptake is $0.5V_{max}$. [c] V_{max} is the maximal rate of sugar uptake in nanomoles per 10^9 cells per minute. [d] $K_{i(app)}$ is that concentration of sugar (millimolar) or cytochalasin B (nanomolar) that reduces sugar uptake by 50% of the maximal inhibition. In experiments where 2-deoxy-D-glucose (2DG) was the inhibitory sugar, uptake of 66 μM D-glucose was assessed. In experiments where 3MG or CCB was the inhibitor, uptake of 66 or 100 μM 2DG was assessed. [e] From ref *61*. [f] From ref *62*. [g] From ref *43*. [h] From ref *63*. [i] From ref *64*. [j] From refs *30* and *31*. [k] From ref *65*.

by identical rate constants for exchange (0.15 per min). This indicates that only a single first-order process is necessary to describe the time course of 3MG uptake by RE700A-5′UTR-GLUT1-HA-H6.

Sites of hGLUT1 Expression in RE700A. Renografin gradient separation of cellular membrane compartments was used to identify the cellular location of recombinant GLUT1-HA-H6 expression in RE700A. Membrane fractions from yeast with and without 5′UTR-GLUT1-HA-H6 were layered under a Renografin-76 gradient. Samples were centrifuged for 20 h at 40 000 rpm to allow proteins to migrate to regions of equivalent buoyant density. Fractions were then isolated in order of increasing density, and were immunoblotted by using GLUT1 C-Ab and antibodies directed against yeast plasma membrane (Ste2p) and endoplasmic reticulum (Dpm1p) markers. Figure 4 indicates that the largest fraction of RE700A-5′UTR-GLUT1-HA-H6 cofractionates with the yeast plasma membrane marker Ste2p. Little transporter is detected in the less dense, ER-dominated (Dpm1p) membrane fractions.

Characterization of Sugar Transport in RE700A GLUT1. The concentration dependence of 2DG uptake by RE700A-5′UTR-GLUT1-HA-H6 at 20−24 °C shows saturation kinetics with a $K_{m(app)}$ of 2.9 ± 0.4 mM and a V_{max} of 2.4 ± 0.1 nmol per 10^9 cells per minute (Table 1 and Figure 5A). 2DG inhibition of D-glucose uptake by RE700A-5′UTR-GLUT1-HA-H6 at 24 °C is saturable with a $K_{i(app)}$ of 2.1 ± 0.6 mM (Figure 5A and Table 1). In human red cells, the $K_{i(app)}$ for 2DG inhibition of D-glucose uptake is 1.4 ± 0.4 mM while the $K_{m(app)}$ for 2DG uptake by red cells is 0.6 ± 0.2 mM (Table 1).

3MG inhibition of 2DG uptake by RE700A-5′UTR-GLUT1-HA-H6 is saturable with a $K_{i(app)}$ of 10.1 ± 4.2 mM (Table 1). In contrast, the $K_{i(app)}$ for 3MG inhibition of 2DG uptake by wild-type *S. cerevisiae* (baker's yeast) is 64.4 ± 3.8 mM (Table 1). 2DG transport rates in baker's yeast are at least 10-fold greater than in GLUT1-transfected RE700A (Figure 5B,C). Cytochalasin B, an inhibitor of human red blood cell sugar transport, also inhibits 2DG uptake by RE700A-5′UTR-GLUT1-HA-H6 with a $K_{i(app)}$ of 142 ± 42

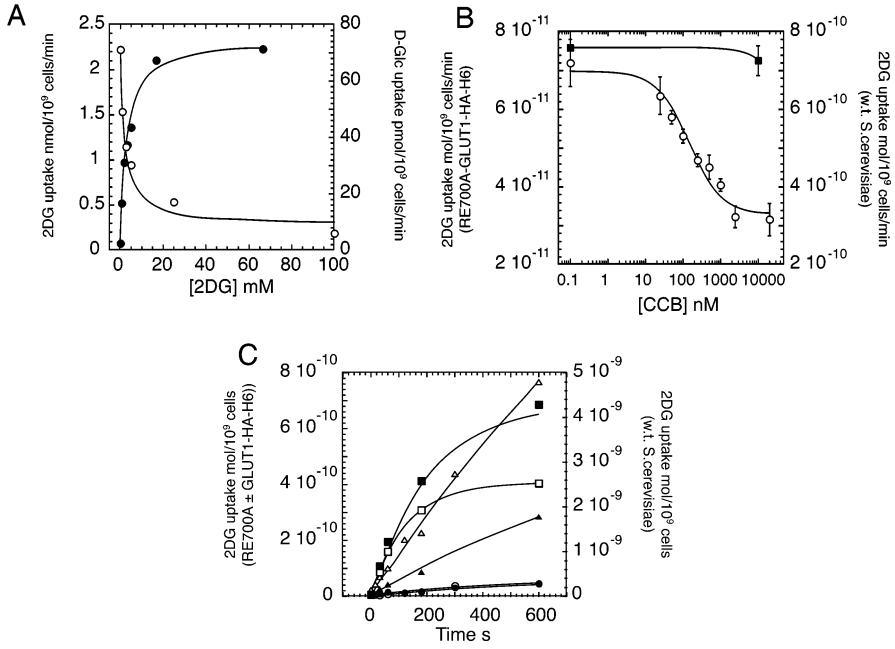

FIGURE 5: (A) Concentration dependence of 2-deoxy-D-glucose uptake and inhibition of D-glucose uptake by RE700A-5′UTR-GLUT1-HA-H6. Left ordinate: 2DG uptake [nmol/10^9 cells/min (●)]. Right ordinate: uptake of 100 μM D-glucose [pmol/10^9 cells/min (○)]. Abscissa: [2DG] (mM). Each point represents the mean of at least four separate determinations in triplicate. The curve drawn through the 2DG uptake data points (●) was computed by nonlinear regression assuming simple Michaelis−Menten sugar uptake kinetics and takes the form $v = V_{max}[2DG]/[K_{m(app)} + [2DG]$, where v is the rate of uptake at any [2DG], V_{max} is the maximum rate of sugar uptake obtained at a saturating [2DG], and $K_{m(app)}$ is that [2DG] that results in uptake of $0.5V_{max}$. The curve has the following constants: $V_{max} = 2.35 \pm 0.09$ nmol $(10^9$ cells$)^{-1}$ min^{-1}; $K_{m(app)} = 2.9 \pm 0.4$ mM 2DG; $R^2 = 0.985$. The curve drawn through the D-Glc uptake data points (○) was computed by nonlinear regression assuming simple Michaelis−Menten competitive inhibition of D-Glc uptake by 2DG and takes the form $v = v_o - v_i[2DG]/[K_{i(app)} + [2DG]]$, where v_o is the rate of D-Glc uptake in the absence of 2DG, v_i is the extent of inhibition produced at a saturating [2DG], and $K_{i(app)}$ is that [2DG] that results in 50% of the maximum inhibition of D-Glc uptake. The curve has the following constants: $v_o = 72.9 \pm 4.5$ pmol $(10^9$ cells$)^{-1}$ min^{-1}; $v_i = 64.2 \pm 5.0$ pmol $(10^9$ cells$)^{-1}$ min^{-1}; $K_{i(app)} = 2.1 \pm 0.6$ mM 2DG; $R^2 = 0.983$. (B) Concentration dependence of CCB inhibition of 2DG uptake by RE700A-5′UTR-GLUT1-HA-H6 and baker's yeast. Left ordinate: 2DG uptake in RE700A-GLUT1-HA-H6 [mol/10^9 cells/min (○)]. Right ordinate: 2DG uptake in baker's yeast [mol/10^9 cells/min (■)]. Abscissa: [CCB] (nM). Each point represents the mean of four triplicate determinations of 2DG uptake at 100 μM sugar. The curve drawn through the RE700A-GLUT1-HA-H6 data points was computed by nonlinear regression assuming simple Michaelis−Menten inhibition of sugar uptake and takes the form $v = v_o - v_i[CCB]/[K_{i(app)} + [CCB]]$, where v is the rate of uptake at any [CCB], v_o is the rate of uptake in the absence of CCB, v_i is the maximum decrease in sugar uptake produced by a saturating [CCB], and $K_{i(app)}$ is that [CCB] that inhibits uptake by 50% of v_i. The curve has the following constants: $v_o = (6.99 \pm 0.24) \times 10^{-11}$ mol $(10^9$ cells$)^{-1}$ min^{-1}; $v_i = (3.68 \pm 0.27) \times 10^{-11}$ mol $(10^9$ cells$)^{-1}$ min^{-1}; $K_{i(app)} = 142 \pm 42$ nM CCB; $R^2 = 0.969$. (C) Inhibition of 2DG uptake in RE700A-GLUT1-HA-H6 and in baker's yeast by cytochalasin B. Ordinate: moles of 2-deoxy-D-glucose imported by 10^9 cells. Left ordinate: uptake in nontransfected RE700A (● and ○) or in RE700A-GLUT1-HA-H6 (▲ and △). Right ordinate: uptake in baker's yeast (■ and □). Abscissa: time in seconds. 2DG uptake was assessed at 100 μM sugar in the presence (■, ●, and ▲) or absence (□, ○, and △) of cytochalasin B. RE700A-5′UTR-GLUT1-HA-H6 cells were exposed to 10 μM CCB, while wild-type *S. cerevisiae* cells were exposed to 100 μM CCB. Cells received CCB 10 min prior to 2DG uptake initiation. The curves drawn through the points were computed by nonlinear regression assuming uptake is described by the expression $2DG_t = 2DG_\infty(1 - e^{-kt})$, where $2DG_t$ is the amount of 2DG associated with the cells at time t, $2DG_\infty$ is the amount of 2DG associated with the cells at equilibrium, and k is the first-order rate constant describing the rate of 2DG uptake. The following constants were obtained: $2DG_\infty = 2.5 \pm 0.2$ nmol/10^9 cells, $k = 0.007 \pm 0.001$ s^{-1}, and $R^2 = 0.999$ for baker's yeast; $2DG_\infty = 4.3 \pm 0.4$ nmol/10^9 cells, $k = 0.0048 \pm 0.001$ s^{-1}, and $R^2 = 0.975$ for baker's yeast and CCB; $2DG_\infty = 2.1 \pm 0.2$ nmol/10^9 cells, $k = 0.00076 \pm 0.00033$ s^{-1}, and $R^2 = 0.992$ for RE700A-GLUT1-HA-H6; $2DG_\infty = 2.1 \pm 0.3$ nmol/10^9 cells, $k = 0.00025 \pm 0.00001$ sec^{-1}, and $R^2 = 0.985$ for RE700A-GLUT1-HA-H6 and CCB; $2DG_\infty = 2.1$ nmol/10^9 cells, $k = (4.1 \pm 0.4) \times 10^{-5}$ s^{-1}, and $R^2 = 0.835$ for RE700A; and $2DG_\infty = 2.1$ nmol/10^9 cells, $k = (4.2 \pm 0.5) \times 10^{-5}$ s^{-1}, and $R^2 = 0.770$ for RE700A and CCB. With RE700A cells, $2DG_\infty$ was assumed to be identical to RE700A-GLUT1-HA-H6.

nM (Figure 5B). Maximum inhibition of unidirectional uptake is 58%. The remaining uptake is similar to that observed in the presence of saturating concentrations of 3MG or D-glucose and is comprised of a nonmediated (leakage) component plus the instantaneously accessible (zero time) space associated with RE700A observed during the time course of 3MG uptake (Figure 3B). The instantaneously accessible space may result from nonspecific sugar binding to the yeast cell. The rate of 2DG uptake in baker's yeast is not significantly affected by 100 μM CCB (Figure 5C). In two separate experiments, k (the first-order rate constant for 2DG uptake) equaled 0.0073 and 0.007 s^{-1} in the absence of CCB and 0.0057 and 0.005 s^{-1} in the presence of CCB. The equilibrium 2DG space of baker's yeast was 3.0 and 2.5 nmol/10^9 cells in the absence of CCB and 3 and 4.3 nmol/10^9 cells in the presence of CCB. 3MG and 2DG uptake by nontransfected RE700A cells is significantly slower than uptake by GLUT1-transfected RE700A cells in the presence of CCB or maltose. This suggests that GLUT1-HA-H6 expression increases nonmediated sugar permeability or CCB/maltose-insensitive sugar association with yeast cells.

Human red cell unidirectional sugar import is inhibited 5-fold by an extracellular reductant (*6*) and is stimulated by 10−100-fold by intracellular sugar (32−35). 2DG uptake at 24 °C by RE700A-5′UTR-GLUT1-HA-H6 is not inhibited by DTT exposure (10 mM for 30 min at 30 °C; Table 2).

Table 2: Effect of DTT on Sugar Uptake by Human Red Cells and RE700A

cell type	2DG uptake[a] control[b]	DTT[b]	CCB[c]	n[d]	P[e]
RE700A-GLUT1-HA-H6[f]	101 ± 13	124 ± 9	42 ± 6	5	0.264
HEK293[g]	0.39 ± 0.02	0.33 ± 0.03	0.04 ± 0.003	4	0.153
HEK293 + GLUT1-HA-H6[g]	0.59 ± 0.02	0.46 ± 0.01	0.04 ± 0.01	4	0.002
GLUT1-HA-H6-mediated uptake[g,h]	0.20 ± 0.02	0.13 ± 0.01		4	0.013

[a] 2DG uptake at 100 μM sugar was assessed in RE700A-GLUT1-HA-H6 cells and control (HEK293) or GLUT1-HA-H6-transfected HEK cells (HEK293+GLUT1-HA-H6). [b] 2DG uptake was assessed at 20 °C following a 30 min preincubation at 30 °C in the absence (control) or presence (DTT) of 20 mM DTT at pH 7.4. [c] 2DG uptake by HEK cells (control and transfected) is inhibited by >90% by 20 μM CCB. [d] Number of separate measurements made in duplicate. [e] Probability that there is no difference between control and DTT conditions in each experiment calculated using the Student's *t*-test. This two-tailed test assumes that paired samples have equal variance. [f] Uptake is expressed as picomoles per 10^9 cells per minute and is shown as mean ± standard error of the mean. [g] Uptake was assessed as picomoles per microgram per minute and is shown as mean ± standard error of the mean. [h] GLUT1-HA-H6-specific 2DG was calculated as the difference between uptake in transfected versus nontransfected HEK293 cells.

Unidirectional uptake of 100 μM 3MG by RE700A-5′UTR-GLUT1-HA-H6 is unaffected by preloading cells with 10, 20, or 40 mM unlabeled 3MG (Figure 6A). Introduction of the carboxy-terminal HA-H6 tag into GLUT1 could modulate GLUT1-mediated sugar transport. We therefore expressed GLUT1-HA-H6 in human embryonic kidney cells (HEK923) and determined whether reductant or intracellular sugar influences sugar transport in these cells. GLUT1-HA-H6 catalyzes DTT-inhibitable 2DG uptake in HEK cells (Table 2). Intracellular 3MG stimulates GLUT1-HA-H6-mediated 2DG unidirectional uptake in HEK cells (Figure 6B).

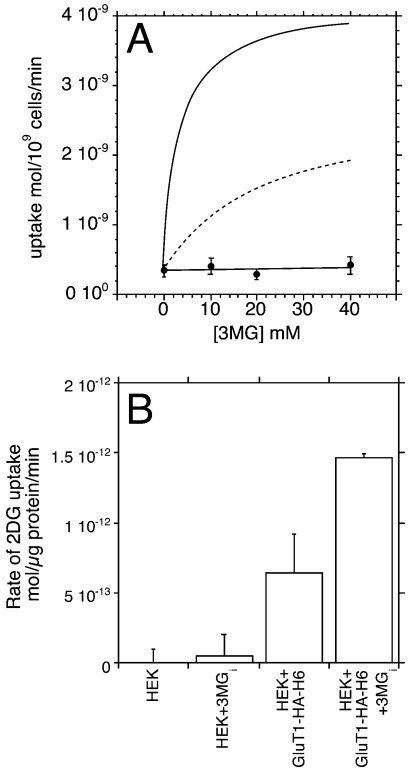

FIGURE 6: Lack of hGLUT1-HA-H6-mediated transacceleration in RE700A. Ordinate: mol of 2-deoxy-D-glucose imported by 10^9 cells per min. Abscissa: intracellular [3MG] in mM. 2DG uptake (100 μM) was assessed in RE700A-5′UTR-GLUT1-HA-H6 cells (●) that were preloaded for 1 h with 0, 10, 20, or 40 mM 3MG. The rate of 2DG uptake was then measured over a 5 min interval. Results are shown as means ± the standard error of the mean of at least three separate determinations made in triplicate. The straight line drawn through the data points was computed by linear regression. The rectangular hyperbolae illustrate the fold stimulation and the 3MG concentration dependence of transport stimulation that is expected (*22*) if transport were assessed in human red cells at 4 °C (—) or at 20 °C (- - -). (B) Transacceleration is observed in HEK cells expressing GLUT1-HA-H6. Ordinate: rate of 3MG uptake in mol per μg of cell protein per min. Absissa: experimental condition. Uptake of 100 μM 3MG was assessed in control (HEK) or 3MG (25 mM)-loaded HEK cells (HEK+3MG$_i$) or in HEK cells transiently transfected with GLUT1-HA-H6 and preloaded with 0 (HEK-GLUT1-HA-H6) or 40 mM 3MG (HEK-GLUT1-HA-H6+3MG$_i$). These results represent the mean ± standard error of the mean of four separate measurements. Uptake in nontransfected HEK cells [1.62 pmol (μg of protein)$^{-1}$ min^{-1}] was subtracted from the data.

DISCUSSION

Sugar Transport in S. cerevisiae. A recent analysis suggests that it is necessary to disrupt all 17 yeast hxt transporter genes and three maltose transporter genes to prevent yeast growth on glucose media (*16*). The study presented here confirms earlier observations that yeast strain RE700A, which lacks Hxt1−7, is unable to grow on glucose as the sole carbon source (*18*, *36*, *37*). We further demonstrate that expression of human GLUT1 in RE700A restores the ability of the organism to grow on glucose.

GLUT1 is directly involved in promoting glucose transport in transfected RE700A cells since subsequent exposure to 5-FOA selects for cells that have lost the URA-3-containing plasmid and results in clones capable of growth on YP-maltose but not on YP-dextrose or SCM-URA dextrose. Sugar transport measurements also indicate that FOA-treated yeast have lost the ability to import 2-deoxy-D-glucose. This establishes that GLUT1 expression, sugar transport, and growth on glucose are inexorably linked.

RE700A might respond to GLUT1 transfection by inducing expression or activation of an endogenous yeast hexose transport protein. Ozcan et al. (*38*) have shown that two hxt-like genes in *S. cerevisiae* (rgt2 and snf3) are not transporters but rather are glucose sensors that control expression of several members of the hxt family of genes. Furthermore, Wieczorke et al. (*16*) demonstrate that deletion of snf3 in the background of the 20-hexose transporter gene deletion partially restores growth on hexoses, suggesting the existence of even more yeast proteins that are capable of transporting hexoses. Thus, recombinant GLUT1 could serve as a glucose sensor in *S. cerevisiae*, introducing a signal for transcriptional induction of previously inactive or inhibited sugar carriers.

This seems unlikely for several reasons. (1) Glucose transport by wild-type yeast is characterized by a lack of sensitivity to the sugar transport inhibitor cytochalasin B (see here and ref *14*). Expression of cytochalasin B-sensitive,

human GLUT1 confers acute CCB sensitivity to RE700A sugar transport. If GLUT1 induces expression of an unrecognized sugar transporter, this transporter must differ from other yeast hexose transporters in being CCB-inhibitable. CCB inhibition of sugar transport in transfected RE700A is unlikely to result from CCB inhibition of transcriptional activation by GLUT1 because the actions of the inhibitor are fully developed within 10 min of CCB addition. (2) Wild-type yeast shows only very low affinity [$K_{i(app)} = 65$ mM] for the nonmetabolized but transported sugar 3-*O*-methylglucose, while human GLUT1 and RE700A yeast expressing GLUT1-HA-H6 are characterized by 6-fold greater affinity for the sugar (see ref *39* and here). (3) The transport-deficient GLUT1 mutant $GLUT1_{338-A3}$ does not catalyze sugar transport when expressed in mammalian cells or in RE700A. (4) Identical results have been obtained with GLUT1 (K. L. Levine and A. Carruthers, unpublished findings) using the yeast strain employed by Wieczorke et al. (*16*) in which all 20 hexose transporter genes were eliminated. Thus, if GLUT1 serves as a transcriptional activator, it must induce the expression of other, unrecognized glucose transporters. We conclude, therefore, that human GLUT1 directly catalyzes glucose, 2-deoxy-D-glucose, and 3-*O*-methylglucose transport when expressed in yeast.

Previous studies have suggested that GLUT1 expression is limited to the endoplasmic reticulum in yeast (*14*, *40*). Our study shows not only that GLUT1 expression enhances sugar import by RE700A but also that expressed GLUT1 comigrates with yeast plasma membrane and endoplasmic reticulum markers upon membrane fractionation by Renografin gradient density centrifugation. Similar results have been described by Wieczorke et al. (*41*). However, in this case, GLUT1 was expressed at the cell surface but was either inactive or misfolded (*41*). These workers demonstrated that GLUT1-mediated transport could be activated in one of two ways: (1) by mutagenesis of a cluster of amino acids (human GLUT1 residues 63 and 67−69) in putative transmembrane domain 2 or (2) by selection of a Δhxt strain containing a genomic mutation (fgy1−1) that now permits GLUT1 function. Use of the yeast expression vector p426 GPD and elimination of the hGLUT1 5′UTR must result in sufficiently robust GLUT1 expression to saturate ER-sequestering mechanisms permitting leakage to the yeast plasma membrane. GLUT1 overexpression may saturate the mechanism (fgy1−1) that suppresses GLUT1 transport function. It is also possible that RE700A contains the fgy1−1 genomic mutation (*41*).

Properties of GLUT1 in S. cerevisiae and Human Red Cells. 2DG uptake in RE700A cells expressing hGLUT1 is a saturable process characterized by a $K_{m(app)}$ of 2.9 ± 0.4 mM. 2DG uptake is competitively inhibited by 3MG [$K_{i(app)} = 10$ mM], and D-glucose uptake is competitively inhibited by 2DG [$K_{i(app)} = 2.1 \pm 0.6$ mM]. These measures of the apparent affinity of hGLUT1 for 2DG compare with $K_{i(app)}$ and $K_{m(app)}$ values for 2DG in human red cells at 4 °C of 1.4 and 0.6 mM, respectively.

These observations in yeast illuminate a long-standing puzzle in the field of human erythrocyte sugar transport. The Michaelis parameters for D-glucose and 3MG uptake by human red cells are 1.6 and 5 mM, respectively (*42*). However, $K_{i(app)}$ values for glucose and 3MG inhibition of L-sorbose uptake by human red cells are 7 and 14 mM, respectively (*43*). These discrepancies between $K_{i(app)}$ and $K_{m(app)}$ are unexpected (*39*) and have led some to suggest that sugar transport measurements in human red cells are technically flawed because of complications arising from interactions of transported sugar with intracellular sugar binding species and/or the extremely high sugar transport capacity of red cells which makes accurate estimates of initial rates of transport difficult to attain (*22*, *35*, *44*, *45*). Support for this hypothesis is available from recent measurements of 3MG transport by human red cells at 22 °C made using a rapid quench device that permits millisecond resolution of transport. These studies indicate $K_{m(app)}$ for net 3MG uptake by red cells is 15−25 mM (*30*, *31*).

The transport properties of RE700-5′UTR-GLUT1-HA-H6 differ from those of human red blood cells (*6*, *10*, *30*, *39*, *46*) in several interesting ways. (1) $K_{m(app)}$ for net transport of sugar is indistinguishable from $K_{i(app)}$ for sugar inhibition of transport of a second sugar. (2) Sugar uptake is not inhibited by extracellular reductant. (3) Net sugar uptake at a subsaturating 3MG concentration is a simple monoexponential process ($\tau = 400$ s). (4) CCB inhibition of sugar uptake shows simple, saturable (Michaelis) inhibition. (5) GLUT1-mediated sugar uptake does not show trans-acceleration.

Properties of GLUT1 in Other Cells. The possibility that transporter phenotype could be determined by cellular environment is not unexpected. GLUT1-mediated sugar transport in human red cells is asymmetric [V_{max} and $K_{m(app)}$ for net sugar exit are greater than V_{max} and $K_{m(app)}$ for net sugar entry], shows transacceleration [intracellular sugar stimulates unidirectional uptake of radiolabeled sugar (*32*)], and is inhibited by extracellular reductant (*6*). GLUT1-mediated sugar transport in rabbit red cells and metabolically depleted avian erythrocytes is symmetric, does not display transacceleration (*47*−*50*), but is inhibited (in avian red cells) by reductant (*48*, *50*). These differences may result from subtle differences between the primary structures of human and avian GLUT1 (85% identical) or human and rabbit GLUT1 (97% identical). Rat erythrocyte GLUT1 is kinetically symmetric and has been reported to lack (*51*) or to display (*52*, *53*) accelerated exchange transport. Rat GLUT1 is 98% identical to human GLUT1. Human GLUT1 expressed in *Xenopus* oocytes displays transacceleration (*54*) but is not inhibited by extracellular reductant (*6*). Primary sequence differences cannot account for the divergent behavior of human GLUT1 in yeast, *Xenopus* oocytes, and human red cells. The absence of transacceleration in RE700A-GLUT1-HA-H6 (Figure 6A) is unrelated to the introduction of a modified GLUT1 carboxy-terminal region because the same construct catalyzes transacceleration when expressed in HEK cells (Figure 6B).

Accelerated exchange and kinetic asymmetry are not obligate properties of carrier-mediated sugar transport. Rat GLUT4, the insulin-sensitive sugar transporter (*1*), is 65% identical and 83% similar to GLUT1 but is kinetically symmetric and does not display accelerated exchange (*55*). Passive sugar transport in axons of *Loligo forbesi* is asymmetric but shows transinhibition (*56*). Passive sugar transport in isolated, striated muscle cells of *Balanus nubilis* is symmetric and lacks transacceleration or inhibition (*57*). Cytoplasmic factors and temperature profoundly influence the GLUT1 phenotype. Transport in human red cell ghosts

lacking ATP at 4 °C is symmetric (vs asymmetric in the presence of ATP) but continues to display accelerated exchange (*58*). At 37 °C, ATP-containing red cells lose kinetic asymmetry and transacceleration (*59*). Transacceleration is also lost in rat red cells at 40 °C (*52*).

A Model of GLUT1-Mediated Transport. We have proposed that human GLUT1 forms a reductant-sensitive GLUT1 homotetramer in the red cell membrane (*6*). When reduced, the transporter complex dissociates into GLUT1 dimers. Tetrameric GLUT1 interacts allosterically with cytoplasmic ATP (*11*, *24*, *60*). This interaction promotes conformational changes in GLUT1 cytoplasmic domains (*8*), forming a "cytoplasmic cage" that encloses the exit site of the translocation pathway and introduces a noncatalytic sugar binding site within the cage, thereby delaying the release of sugar into the cytosol (*10*). Reductant causes dissociation of tetrameric GLUT1, loss of GLUT1 ATP binding capacity (*11*), and loss of the cytoplasmic cage. In the absence of ATP, the cytoplasmic cage is relaxed, the noncatalytic sugar binding site is lost, and the level of transport asymmetry (which is proposed to result from substrate recycling within the cage) is reduced (*10*, *30*, *31*). We therefore propose that RE700A-GLUT1-HA-H6 exists predominantly as a "reduced" GLUT1 homodimer. While the so-called "cage hypothesis" can account for ATP and reductant sensitivity of GLUT1-mediated sugar transport, it does not account for the lack of human GLUT1 sensitivity to reductant when expressed in *Xenopus* oocytes and *S. cerevisiae*, tissues in which GLUT1 displays transacceleration and no transacceleration, respectively.

The RE700A GLUT1 heterologous expression system may provide a useful model for systematic analysis of specific GLUT1 primary structure and environmental factors that influence GLUT1-mediated sugar transport. RE700A may prove to be useful in the development of genetic and pharmacodynamic screens for molecules that modulate GLUT1 activity and may also serve as a rich source of recombinant hGLUT1.

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