Expression of the human erythrocyte glucose transporter in Escherichia coli

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ABSTRACT The gene encoding the human erythrocyte glucose transporter, cloned from HepG2 hepatoma cells, was expressed in Escherichia coli by introducing a prokaryote-type ribosome binding site, subcloning the gene into the T7 promoter/T7 polymerase expression system, and transforming a strain that is defective in glucose transport. Cells bearing plasmids with the transporter gene take up 2-deoxy-D-glucose and D-glucose, unlike cells bearing plasmids without the transporter gene. Moreover, 2-deoxy-D-glucose uptake is inhibited by unlabeled D-glucose, cytochalasin B, or mercuric chloride but not by lactose. The glucose transport protein is inserted into the membrane of E. coli, as evidenced by immunoblotting experiments with two site-directed polyclonal antibodies, one directed against the COOH terminus of the glucose transporter and the other directed against a synthetic peptide containing amino acid residues 225-238. As detected with both antibodies, the protein migrates with apparent molecular mass of 34 kDa in sodium dodecyl sulfate/12% polyacrylamide, a size similar to that of the unglycosylated glucose-transport protein synthesized in vitro.

Most mammalian cells catalyze glucose transport by means of specific integral membrane proteins, and two such systems have been characterized (see refs. 1-6 for reviews). The glucose transporter present in nonilethelial cells catalyzes facilitated diffusion, a carrier-mediated equilibration process that is driven by the concentration gradient of glucose and that is not ion-coupled. In contrast, the Na⁺-dependent glucose transporters present in the brush border of renal and intestinal epithelial cells utilize free energy released from the translocation of Na⁺ down its electrochemical gradient to drive glucose uphill against a concentration gradient. Human erythrocytes have unusually high glucose-transport activity, and the glucose transporter in these cells has been solubilized, purified, and identified as an integral membrane glycoprotein of molecular mass approximately 55 kDa (3). Furthermore, proteoliposomes reconstituted with purified erythrocyte glucose transporter catalyze facilitated diffusion of D-glucose specifically (1-5).

Both murine and human glucose transporter mRNAs encode a protein of 38 kDa as judged by in vitro translation or metabolic labeling in the presence of an inhibitor of N-linked glycosylation (7). Mueckler et al. (8) cloned the complementary DNA (cDNA) encoding the glucose transporter from HepG2 hepatoma cells. Comparison of the amino acid sequence of the transporter deduced from the cDNA with the partial sequence obtained by structural analyses of the glucose transporter purified from human erythrocytes confirmed the identity of the cloned DNA and demonstrated that the glucose transporters in HepG2 cells and erythrocytes are probably identical. Independently, Birnbaum et al. (9) cloned a cDNA encoding the glucose transporter from adult rat brain and demonstrated >97% amino acid sequence identity between the rat brain and human erythrocyte glucose-transport proteins. Finally, Flier et al. (10) showed that mRNAs encoding the glucose transporter are present in various tissues and cultured cell lines.

Analysis of the primary structure of the glucose transporter shows that it lacks a cleavable NH₂-terminal signal sequence (8). In addition, the primary structure and biophysical evidence (11) suggest that the protein consists of 12 hydrophobic segments in α-helical conformation that traverse the membrane in zigzag fashion and are connected by more hydrophilic loops. The NH₂ terminus, the COOH terminus, and a highly hydrophilic domain in the middle of the protein are all predicted to lie on the cytoplasmic face of the membrane. The single N-linked oligosaccharide is attached to the exoplasmic loop between transmembrane helices 1 and 2 (8, 12). The glucose transporter is unusual because it can insert into endoplasmic reticulum membranes after its synthesis (13).

The Escherichia coli arabinose and xylose permeases, which catalyze substrate-ß-H⁺ symport, exhibit a high degree of sequence homology with each other (14). Although these proteins exhibit no significant homology with two other symporters from E. coli [i.e., lactose permease (lacY gene product) and melibiose permease (melIB gene product), which exhibit no homology with each other (15)], they exhibit about 40% sequence homology with the glucose transporter cloned from HepG2 cells. However, glucose transport in E. coli occurs by vectorial phosphorylation by the phosphoenolpyruvate phosphotransferase system (PTS; refs. 16 and 17) with enzyme IIßLeu (encoded by ptsG) or enzyme IIßMan (encoded by ptsH) as substrate-specific components (see ref. 18 for a review), and neither of the enzymes II exhibits sequence homology with the erythrocyte glucose transporter (19, 20). In addition, glucose may be transported by the galactose permease in E. coli (21).

In this communication, we show that the human glucose transporter can be synthesized in vivo in E. coli and inserted into the cytoplasmic membrane in a functional state. Thus, expression of the gene encoding the glucose transporter in a strain of E. coli that is severely defective in glucose transport yields cells with 2-deoxy-D-glucose- and D-glucose-uptake properties similar to those observed in erythrocytes. Furthermore, immunoblotting experiments with antibodies directed against the HepG2 glucose transporter revealed the presence of a membrane protein with molecular mass similar to that of the unglycosylated glucose transporter.

MATERIALS AND METHODS

Materials. Bacteriophage T4 DNA ligase and restriction enzymes BamHI, HindIII, and Sma I were from New England BioLabs. The Klenow fragment of DNA polymerase and the replicative-form (RF) DNA of phage M13 mp19 were

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Abbreviation: RF, replicative form.

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from Bethesda Research Laboratories. Plasmid pGEM-3 was from Promega Biotec (Madison, WI) and plasmid pGPl-2 was a gift from S. Tabor (Harvard Medical School). Deoxy- and dideoxynucleoside triphosphates were obtained from Pharmacia. 2-Deoxy-d-[U-14C]glucose (300 mCi/mmol; 11 GBq/mmol) was purchased from Amersham. 2-Deoxy-d-glucose, d-glucose, and L-glucose were from Sigma, and cytochalasin B was from Aldrich. All other materials were reagent-grade and obtained from commercial sources.

The following strains of *E. coli* were used: SR425 [gal, thi, Tfr, endA, hsdR, sbcB, ptsM, ptsG/F'] (22), JM101 [supE, thi, Δ(lac-proAB)/traD36, proA^-B^, lacZD/ΔM15] (23), and BMH7-18 Δ(lac-proA, supE, thi, proA^-B^, lacZD/ΔM15/ mutL::Tnl0] (24). *E. coli* SR425 was the generous gift of N. Williams and S. Roseman (The Johns Hopkins University).

**Oligonucleotide Synthesis.** Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) model 381B DNA synthesizer and purified by polyacrylamide gel electrophoresis as described in the Applied Biosystems manual.

**Recombinant DNA Techniques and Site-Directed Mutagenesis.** Unless stated otherwise, recombinant DNA techniques were carried out according to Maniatis *et al.* (25). The plasmid pJ12 contains the glucose transporter gene from *HepG2* cells in the *Bam*HI site of pGEM-3. The gene was excised from plasmid pJ12 by using restriction enzymes *Bam*HI and *Hind*III and cloned into M13 mp19 RF DNA that had been linearized with the same restriction enzymes. A portion of the ligation mixture was used to transfect JM101, and the single-stranded DNA containing the transporter gene was isolated as described (26).

Oligonucleotide-directed, site-specific mutagenesis was performed essentially as described (26, 27) with the following modifications to improve the frequency of mutant recovery: closed circular heteroduplex DNA with the desired mutations was synthesized in vitro as described (27) and *E. coli* BMH7-18 mutL was transfected with the heteroduplex DNA to minimize mismatch repair (24). Phage harboring the mutations were identified initially by colony-blot hybridization using the appropriate 32P-labeled mutagenic primer (28). Phage from positive colonies were plaque-purified, and the mutations were verified by oligodeoxynucleotide sequencing (29, 30).

Double-stranded glucose-transporter DNA was excised from the M13 mp19 RF DNA by using restriction enzymes *Bam*HI and *Hind*III and ligated into pGEM-3 plasmid DNA that had been digested with the same restriction enzymes. The resulting plasmid (pGTSD12) and plasmid pGEM-3 were used to transform *E. coli* SR425 that was previously transformed with pGP1-2 [which contains the gene for phase T7 RNA polymerase (under the control of the heat-inducible P7 promoter of phage λ) and the kanamycin-resistance gene (see ref. 31)]. The resulting strains are termed S12B (SR425/pGP1-2/pGTSD12) and S3 (SR425/pGP1-2/pGEM-3).

**Transport Assays.** *E. coli* S3 and S12B were grown at 30°C in Luria broth containing ampicillin at 40 μg/ml and kanamycin at 20 μg/ml (150-200 ml cultures) to an OD620 of about 0.8. Synthesis of T7 polymerase was induced by transferring the cultures to a water bath at 44°C and shaking for 30 min. The cultures were then transferred back to 30°C, and growth was continued for an additional 1.5-2 hr. Cells were harvested by centrifugation for 15 min at 4°C, washed twice with 0.1 M potassium phosphate, pH 7.5/10 mM MgSO4, and resuspended in the same buffer to an OD620 of about 50 (an OD620 of 10 corresponds to about 1.1 mg of protein per ml).

Transport assays were carried out at 25°C with adding 0.2 mM (final concentration) 2-deoxy-d-[U-14C]glucose (5 mCi/mmol) to 50-μl cell suspensions and incubating for a given period of time. Reactions were terminated by diluting the sample with 3.0 ml of 0.1 M potassium phosphate, pH 5.5/0.1 M LiCl/1 mM MgCl2 and immediately filtering through Whatman GF/F glass-fiber filters (25-mm diameter). Each filter was then washed once with 3.0 ml of the same solution, and the radioactivity retained was assayed by liquid scintillation spectrometry.

**Treatment of Cells with Ethylenediaminetetraacetic Acid (EDTA).** Cells grown and washed as described above were resuspended in 0.1 M potassium phosphate (pH 7.5) to 1/20th of the original culture volume. The suspension was equilibrated to 37°C, adjusted to 1 mM EDTA (sodium salt) by adding an equal volume of 0.1 M potassium phosphate (pH 7.5) containing 2 mM sodium EDTA, and incubated for 3 min. EDTA treatment was terminated by adding 100 volumes of 0.1 M potassium phosphate, pH 7.5/10 mM MgSO4 and harvesting the cells by centrifugation at 4°C for 15 min. Cells were then resuspended in the same buffer to an OD620 of about 50, treated with cytochalasin B as indicated, and assayed for transport.

**Preparation of Site-Directed Polyclonal Antibodies Against the Glucose-Transport Protein.** Peptides corresponding to the COOH terminus (amino acid residues 477-492) and loop 6 (amino acid residues 225-238) of the glucose transporter, as deduced from the cDNA (8), were prepared by Peter Kim (Whitehead Institute) by a solid-phase method (32) on an automated Applied Biosystems 430 peptide synthesizer and coupled to keyhole limpet hemocyanin by using N-maleimidobenzoyl N-hydroxysuccinimide ester or succimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate as described (33). The conjugates were then emulsified with complete Freund's adjuvant and injected subcutaneously into New Zealand White rabbits. Rabbits were given a booster injection with peptide emulsified in incomplete Freund's adjuvant.

Prior to use in immunoblot analyses, the antibodies were absorbed with an *E. coli* lysate. Cells from a 10-ml sample of an overnight culture of *E. coli* SR425 grown and washed as described above were harvested by centrifugation and resuspended in 1 ml of 2% (wt/vol) sodium dodecyl sulfate (NaDodsO4), heated in boiling water for 5 min (34), and diluted 1:200 in Tris-buffered saline (TBS: 10 mM Tris HCl, pH 7.4/130 mM NaCl) containing 5% (wt/vol) bovine serum albumin (BSA) and 0.2% Nonidet P-40. Antiserum directed against given synthetic peptides were then diluted 1:200 with the cell lysate and incubated at room temperature for 2 hr before use.

**Membrane Preparation and Immunoblot Analysis.** Membranes were prepared from *E. coli* S3 and S12B by the method of Davis and Model (35). Cell suspensions prepared as described were mixed rapidly with a 5-fold excess of ice-cold 0.1 M NaOH, and the mixtures were agitated vigorously on a Vortex mixer. The membrane fraction was harvested by centrifugation at 27,000 × g at 4°C, washed once with ice-cold 5% (wt/vol) trichloroacetic acid and resuspended in 1.0 M Tris/5% NaDodsO4. The sample was then mixed with an equal volume of 160 mM Tris HCl, pH 6.8/10% NaDodsO4/10% 2-mercaptoethanol/20% (vol/ vol) glycerol and incubated at room temperature for 1 hr. Samples were subjected to NaDodsO4/12% polyacrylamide gel electrophoresis (36) and the protein bands were electroblotted onto nitrocellulose (37). The nitrocellulose filter was washed in distilled water for 10 min at room temperature and then in TBS/5% BSA/0.1% Tween 20 for 20 min at room temperature. The filter was incubated in TBS/5% BSA/0.2% Nonidet P-40 for 1 hr at 37°C, to block nonspecific binding sites, and then incubated with preadsorbed antiserum in TBS/5% BSA/0.2% Nonidet P-40 for 2 hr at room temperature. After incubation with antibody, the filter was sequentially washed with TBS/5% BSA/0.2% Nonidet P-40 (20 min at room temperature), TBS/0.2% Nonidet P-40 (twice, 20 min each at room temperature), and TBS/0.1% Tween 20 (twice, 20 min each at 37°C).
RESULTS

Construction of Plasmids. The role of a specific ribosome binding site, the Shine–Dalgarno sequence (39), in translation initiation in prokaryotic systems is well established. The average spacing between the last base of the Shine–Dalgarno sequence and the first base of the initiation codon is 7 nucleotides and spacings of less than 5 or more than 9 nucleotides are rare (39). Since the gene encoding the glucose transporter is eukaryotic, a prokaryote-type ribosome binding site was introduced upstream of the initiation codon by carrying out oligonucleotide-directed, site-specific mutagenesis with M13 mp19 single-stranded DNA into which the glucose transporter gene had been inserted. Fig. 1 shows the sequence of wild-type and mutated DNA upstream from the initiation codon (ATG) of the glucose transporter gene. By changing the cytosine at position –13 to an adenine and the cytosine at –11 to a guanine, a purine-rich, Shine-Dalgarno-like sequence was created 8 bases upstream from the initiation codon. The mutated gene was then excised from M13 mp19 RF DNA with BamHI and HindIII and ligated back into pGEM-3 that had been digested with the same restriction enzymes. The recombinant plasmid (pGTS121) containing the glucose transporter gene and a prokaryote-type ribosome binding site was then transformed into E. coli SR425 that had been transformed with pGPI-2, a plasmid encoding T7 RNA polymerase under the control of the heat-inducible A. P. promoter (31).

Immunoblot Analysis. Fig. 2 shows an immunoblot analysis of membrane fractions prepared from E. coli that do (S12B) or do not (S3) contain the glucose transporter cDNA. The blots were probed with two site-directed polyclonal antibodies (Ab 6183 and Ab 6184) against the glucose transporter. These were raised against synthetic polyepitopes corresponding to the COOH-terminal 16 amino acid residues and loop 6 (amino acids 225-238) of the glucose transporter, respectively, coupled to limpet hemocyanin (29). Both antibodies recognized a protein that migrates with an apparent molecular mass of 33-34 kDa and is expressed specifically in the membrane fraction from heat-induced E. coli S12B. [35S]Methionine-labeled glucose-transport protein synthesized in vitro from HepG2 mRNA in a rabbit reticulocyte system and immunoprecipitated with Ab 6183 migrated to the same position (~36 kDa) (data not shown). No immunoreactive 34-kDa protein was expressed in cells not subjected to heat induction (data not shown).

Functional Expression. Heat-induced E. coli S12B, which express the glucose-transport protein, take up 2-deoxy-d-[U-14C]glucose, a nonmetabolizable glucose analogue, at least 4 times better than similarly treated E. coli S3, which contain the same plasmids without the gene encoding the glucose transporter (Fig. 3). Although not shown, similar results were obtained with d-[U-14C]glucose. Furthermore, when excess unlabeled 2-deoxy-d-glucose was added after a steady state was attained, at least half of the radioactivity taken up was released by the cells. Uptake of 2-deoxy-d-[U-14C]glucose was almost completely inhibited by addition of a 100-fold excess of unlabeled 2-deoxy-d-glucose or D-glucose but, importantly, not by addition of L-glucose (Fig. 4A). Thus, the uptake system expressed in E. coli exhibits the stereospecificity typical of the glucose transporter in erythrocytes (40).

Cytochalasin B and mercuric chloride are well-known inhibitors of the human erythrocyte chloride transporter (41).

![Fig. 2. Immunoblot analyses of the E. coli S12B and S3 membranes by glucose transporter antibodies Ab 6183 and Ab 6184. Membrane fractions (50 μg of protein) from S12B (lanes 2 and 4) and S3 (lanes 1 and 3) were subjected to NaDodSO4/polyacrylamide gel electrophoresis and the protein bands were electroblotted onto nitrocellulose. The nitrocellulose was subsequently treated with antibody Ab 6183 (lanes 1 and 2) or Ab 6184 (lanes 3 and 4) and 125I-labeled protein A. Positions of protein size standards (Bethesda Research Laboratories and Bio-Rad) are shown at left. Arrow indicates the position of the glucose transporter.](image-url)

![Fig. 3. 2-Deoxy-d-[U-14C]glucose uptake by E. coli S12B (○) and S3 (●). S12B contains the hepatoma HepG2 glucose transporter gene and the T7 promoter/T7 polymerase expression system, and S3 contains the control plasmids pGPI-2 and pGEM-3. Uptake studies were carried out with a final concentration of 0.2 mM 2-deoxy-d-[U-14C]glucose (5 μCi/mm). In an independent experiment S12B and S3 were incubated with 2-deoxy-d-[U-14C]glucose (0.2 mM), and at a given time (arrow) 20 mM nonradioactive 2-deoxy-d-glucose was added. Aliquots were taken at given times and amounts of 2-deoxy-d-[U-14C]glucose retained were assayed (broken line).](image-url)
There is a high degree of sequence similarity between the human erythrocyte glucose transporter and the arabinose-H\textsuperscript{+} and xylose-H\textsuperscript{+} symporters of E. coli. Although none of these proteins has significant sequence homology with the E. coli lactose permease or melibiose permease, all exhibit similar hydropathy profiles (14). Thus, it is predicted that each protein consists of at least 12 transmembrane hydrophobic domains in \(\alpha\)-helical conformation that traverse the membrane in zigzag fashion and are connected by hydrophilic loops containing most of the charged amino acid residues. Like many bacterial integral membrane proteins, the HepG2 glucose transporter lacks a cleavable signal sequence (8), and it has been shown (13) that the glucose transporter can insert into microsomal membranes posttranslationally. Many bacterial membrane proteins, but no other known eukaryotic protein with multiple membrane-spanning domains, insert into membranes after their synthesis (43). On the other hand, the mammalian glucose transporter, unlike the bacterial symporters, catalyzes downhill translocation of glucose across the membrane in a manner that is not coupled to ion translocation (i.e., uniport).

In this context, it is noteworthy that recent application of site-directed mutagenesis to the lactose permease has shown that Arg-302, His-322, and Glu-325, neighboring residues in putative helices IX and X, play an important role in permease activity, possibly as components of a catalytic triad like that postulated for the serine proteases (44). More specifically, when Glu-325 is replaced with alanine, lactose translocation by the permease becomes uncoupled from H\textsuperscript{+} translocation, and the permease catalyzes downhill lactose influx without H\textsuperscript{+} translocation, equilibrium exchange, and counterflow. In other words, lactose permease with Ala-325 behaves phenom-

entially like the facilitated-diffusion glucose transporter.

In addition to similarities in hydropathy profiles, the lactose permease and the glucose transporter each have four histidine residues, but in the latter, none of the histidine residues is followed by a glutamic or aspartic acid within the next 3–4 residues. In particular, there is a histidine residue at position 337 followed by a glycine at position 340. By site-directed mutagenesis, Gly-340 was replaced with glutamic acid with the intention of determining whether or not the glucose transporter might be converted to a glucose–H\textsuperscript{+} symporer by means of this single amino acid alteration. Although the mutation had no apparent effect on the activity of the glucose transporter, as judged by 2-deoxy-D-glucose uptake in E. coli, it was this notion that led to the experiments described here.

The results presented in this communication provide strong evidence that the HepG2 gene encoding the human erythrocyte glucose transporter can be expressed functionally in E. coli. Thus, when E. coli SR425, a strain defective in all known pathways for glucose transport (i.e., defective in \(ptsG\), \(ptsM\), \(gal\) (19)), is transformed with plasmids containing the HepG2 gene with an upstream prokaryote-type ribosome binding site in the T7 promoter/T7 polymerase expression system, the cells transport 2-deoxy-D-glucose and D-glucose 4–5 times better than cells from the same strain of E. coli transformed with the same plasmids devoid of the HepG2 gene. Furthermore, our data indicate that the glucose-transport activity expressed in E. coli S12B has properties similar to those observed in erythrocytes, in that transport is inhibited by 2-deoxy-D-glucose and D-glucose but not by 1-glucose. In addition, as observed in erythrocytes, transport is inhibited by cytochalasin B (after the outer membrane is permeabilized with EDTA) or by mercuric chloride. Finally, as evidenced by immunoblotting experiments with two different site-directed polyclonal antibodies against the glucose transporter, membranes from S12B cells induced to express the HepG2 gene manifest a protein of mating 34 kDa, a size very similar to that of the unglycosylated glucose-transport protein synthesized in vitro.

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DISCUSSION

![Graph](image-url)