Identification of a Hydrophobic Residue as a Key Determinant of Fructose Transport by the Facilitative Hexose Transporter SLC2A7 (GLUT7)*

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Until recently, the only facilitated hexose transporter GLUT proteins (SLC2A) known to transport fructose were GLUTs 2 and 5. However, the recently cloned GLUT7 can also transport fructose as well as glucose. Comparison of sequence alignments indicated that GLUTs 2, 5, and 7 all had an isoleucine residue at position “314” (GLUT7), whereas the non-fructose-transporting isoforms, GLUTs 1, 3, and 4, had a valine at this position. Mutation of Ile-314 to a valine in GLUT7 resulted in a loss of fructose transport, whereas glucose transport remained completely unaffected. Similar results were obtained with GLUTs 2 and 5. Energy minimization modeling of GLUT7 indicated that Ile-314 projects from transmembrane domain 7 (TM7) into the lumen of the aqueous pore, where it could form a hydrophobic interaction with tryptophan 89 from TM2. A valine residue at 314 appeared to produce a narrowing of the vestibule when compared with the isoleucine. It is proposed that this hydrophobic interaction across the pore forms a selectivity filter restricting the access of some hexoses to the substrate binding site(s) within the aqueous channel. The presence of a selectivity filter in the extracellular vestibule of GLUT proteins would allow for subtle changes in substrate specificity without changing the kinetic parameters of the protein.

The facilitative glucose transporters (SLC2A) belong to the facilitated transporter super gene family, which all appear to have a core structure of 12 transmembrane helices clustered in two sets of six between which there is a central aqueous pore (1). The recent crystal structures solved for LacY and GlpT suggest that during the transport cycle, these two clusters undergo an alteration in their tilt such that their binding site moves from an outward- to an inward-facing conformation (2, 3). In the case of the GLUT proteins, scanning mutagenesis studies and computer modeling indicate that their pore is formed by TMs 5, 7, 8, 10, and 11, whereas other helices may influence the pore structure, i.e. TMs 1, 2, 3, and 4 (4 – 6). What is less clear is the shape of the outer- or inner-facing vestibules allowing entry of the substrates into the pore and access to the proposed substrate binding site. Mueckler et al. (7) initially reported that a naturally occurring conservative mutation of valine 197 to isoleucine (V197I) in GLUT2 resulted in a greatly reduced transport capacity when the protein was expressed in Xenopus oocytes. Subsequently, they also reported that a comparable mutation in GLUT1 (valine 165 to isoleucine) abolished glucose transport (8). Valine is a smaller residue than isoleucine, and this raised the possibility that, if these residues in TM5 faced into the pore, the larger isoleucine might hinder the passage of substrate. These results did not explain why such a conservative substitution could have such a profound effect on the function of these proteins. However, it has been proposed that one or more hydrophobic residues projecting into the pore can influence substrate access to the binding site through steric hindrance (8).

GLUTs 1, 3, and 4 can transport glucose and galactose but not fructose, whereas GLUT2 can transport all three, and GLUT5 can only recognize fructose and possibly 2-deoxyglucose. Sequence alignments using this limited number of isoforms revealed the QLS motif in TM7 as a likely substrate selectivity filter (9). However, recently, the SLC2A family has been expanded to 14 possible members (10, 11), of which we have shown that GLUT7 is another glucose/fructose transporter (12). Comparison of the sequence alignments of the full family showed another possible hydrophobic residue, which might be related to fructose specificity, namely Ile-314 found in TM7 of GLUT7. GLUTs 2 and 5, which recognize fructose, also express Ile at the equivalent position. In contrast, the non-fructose-transporting proteins, GLUTs 1, 3, and 4, have a valine at the equivalent position. This led us to hypothesize that this hydrophobic residue might play a role in the recognition of fructose.

We have employed site-directed mutagenesis to investigate this possibility by changing this isoleucine residue to valine in GLUTs 7, 2, and 5. Our results indicated that Ile-314 is critical for the transport of fructose by GLUT7 but has no influence on the recognition of glucose. Computer modeling of the docking of glucose and fructose in GLUT7 indicated that Ile-314 projects into the lumen of the vestibule directly above the putative initial substrate binding site. Our results provided clear evidence that this residue in TM7 plays a key role in determining the ability of fructose to be transported by GLUT7 proteins.

MATERIALS AND METHODS

Site-directed Mutagenesis—A 4597-bp human wild type GLUT7-pGEM-HE construct was used as a template for creating a GLUT7 mutant in which we substituted isoleucine for valine 314 (I314V). Site-directed mutagenesis was performed using a QuikChange II site-directed mutagenesis kit (Stratagene). The forward and reverse primers were 5’-CCG GCA TCA ATG CGT TCA ACT ACT ATG CGG AC-3’ and 5’-GTC CGC GTA ATG GTA GGT GAA CGC ATT GAT GCC CG-3’. A second mutant was created using the same strategy but replacing Val with Ser. These primers were: forward, 5’-TCG GGC ATC AAT GCC AGC AAC TAT TAT GCG GAC ACC-3’, and reverse, 5’GTT GTC

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The atomic coordinates and structure factors (codes 1YG1, 1YG7, and 15UK) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: GLUT, facilitated glucose transporter proteins; TM, transmembrane domain; h, human.
Determinants of Fructose Transport in GLUT7

We have shown previously that human GLUT7 can transport both glucose and fructose with high affinity ($k_m$ of $-0.2 \text{ mM}$) (12). Comparing the sequence alignments of GLUTs 1, 2, 5, and 7 revealed that at the homologous position of the Val-290 in GLUT1, GLUTs 2, 5, and 7 contain an Ile (Ile-306, Ile-321, and Ile-314, respectively) (Fig. 1 C). Given that GLUTs 2, 5, and 7 transport fructose and all have this conserved Ile, whereas the GLUTs, which transport glucose, but not fructose, have a Val at the equivalent site, we focused on the possible role of this residue in relation to the substrate specificity of GLUT7. Comparing the previously modeled GLUT1 (assigned as 15UK, Protein Data Bank) (15) with GLUT7 (1GY7), also obtained using the glycerol phosphate transporter GlpT (1PW4, Protein Data Bank) as a template, it was found that Val-290 and Ile-314 appear to face the aqueous pore (Fig. 1, B and C).

Two mutants of human GLUT7 cDNA encoding a point mutation in position 314 were constructed in which the native Ile was replaced with either Val or Ser. After injection of the cRNA into oocytes, the subsequent expression of the mutants in the oocyte plasma membrane was assessed by immunohistochemistry and semiquantitative Western blot analysis of isolated oocyte plasma membranes. Both methods employed a commercially prepared antibody raised against the 10 C-terminal amino acids of the wild type human GLUT7. In all cases, these conservative single amino acid substitutions did not affect expression of the salt (three times normal NaCl) PBS and then PBS. The slides were then incubated with 1 mg/ml biotinylated goat anti-rabbit secondary antibody (Chemicon) diluted 1:200 in PBS for 1 h at room temperature. The sections were washed in high salt PBS followed by normal PBS and then treated with streptavidin-conjugated fluorescein isothiocyanate (American Biosciences) for 30 min in the dark. After three 5-min washes with PBS in the dark, the slides were mounted and sealed with nail polish. They were then viewed and photographed under a confocal microscope (Zeiss LSM510).

Multiple Alignments of GLUT Sequences and Docking Analysis—The sequences of GLUTs 1–5 and 7 were analyzed using CLUSTALX (14) software, and the conserved residues of interest were identified. The comparison between the GLUTs 1 and 7 structures around the exofacial vestibule of the aqueous pore were made using the coordinates from the GLUT1 model assigned the name 1SUKE (15). This template structure was subject to a 10-ns molecular dynamics simulation in water/octane and ions, using the force field OPLS (16). This run was at a constant number of molecules, pressure, and temperature (Langenv velocities) with Berendsen coupling for temperature and pressure. All bonds were constrained using the LINCS (17) algorithm for the protein and SETTLE (18) for water in the GROMACS suite (19). The resulting structure was then utilized as template for models of GLUTs 1 and 7, built, and refined with Modeler 7 (20) for each structure including the template, following the approach used in reference (15). The resulting GLUT 1 and 7 structures were deposited in the Protein Data Bank, codes 1YG1 and 1YG7, respectively. Docking of $\beta$-d-glucose and $\beta$-d-fructose (Fig. 1) was explored using Autodock and Autodock tools (21) with the GLUTs 1 and 7 structures as the receptors and the hexose structures as predicted by the PRODRG server.

Results

Two polyclonal antibodies were raised against the unique 10-amino acid sequence of the C-terminal tail of GLUT7 (PTASPaketSF) by Alpha Diagnostic International, San Antonio, TX. Testing of the affinity-purified product in C-terminal tail of GLUT7 (PTASPaketSF) by Alpha Diagnostic Interbodies were raised against the unique 10-amino acid sequence of the national, San Antonio, TX. Testing of the affinity-purified product in

Kinetic Analysis—Hexose uptake into oocytes expressing GLUT7 or its mutants was measured over a range of concentrations from 0.05 to 6 mM using 30-min incubations, which had been determined to be within the linear scope of uptake. Uptake was corrected for nonspecific entry using water-injected eggs from the same batch in each experiment. ENZFIT (Elsevier, Cambridge, UK) software was used to determine the transport kinetics for the GLUT7- and mutant-mediated hexose uptake by non-linear regression analysis.

Western Blotting and Immunohistochemistry—Two polyclonal antibodies were raised against the unique 10-amino acid sequence of the C-terminal tail of GLUT7 (PTASPaketSF) by Alpha Diagnostic International, San Antonio, TX. Testing of the affinity-purified product in Western blots indicated that the antibody 7440 recognized the expression of GLUT7 protein in plasma membranes of cRNA-injected oocytes but was negative for water-injected oocytes.

Oocytes were incubated in OCT (10% polyvinyl alcohol, 4% polyethylene glycol) embedding medium (Shandon) and flash-frozen in liquid nitrogen. Ten-micrometer-thick sections were cut on a cryostat (Leica cryostat, Richmond Hill, Ontario, Canada), mounted on slides, and stored at $-20^\circ \text{C}$. On the day of use, sections were brought to room temperature and fixed with methanol for 90 s. Following a 5-min wash with PBS, the sections were treated with 1% SDS for 5 min to increase antigen exposure and then washed with PBS for 5 min three times. The sections were then treated with 10% goat serum (0.1% Tween 20) to decrease nonspecific binding of the secondary antibody followed by a rapid rinse with PBS and incubation with primary antibody. The primary anti-GLUT7 antibody was diluted 1:200 in a 25% w/v milk solution (25 g of powdered milk in 100 ml of PBS with 0.05% Tween 20) and incubated with the slides for 24 h at 4 °C followed by washes with high

4 15UK refers to a GLUT1 homology model deposited in the Protein Data Bank (Ref. 15).
proteins in the oocytes, and all of the mutants were present at levels similar to or even higher than the wild type protein (Fig. 2).

To identify any alterations of substrate specificity for mutant proteins, we performed a comparison between the uptake of a series of hexoses by wild type GLUT7 and I314V mutant. There was a striking difference between the wild type GLUT7 and the I314V mutant functional activity, with the mutant being effectively unable to transport fructose, whereas glucose uptake was normal (see Fig. 4). Also, wild type GLUT7 does not transport D-galactose or 2-deoxy-D-glucose, and in this regard, the I314V mutant was no different (Fig. 3).

Next, we investigated whether the loss of fructose transport represented an effect on the binding of the substrates, or blocked translocation in some other way, by comparing the kinetic characteristics of the glucose uptake in both proteins. We reported previously that hGLUT7 has a high affinity for glucose and fructose (12). This was based upon the kinetic analysis of glucose uptake \( k_{m} \approx 300 \mu M \) and the ability of fructose to competitively inhibit glucose transport \( IC_{50} 60.3 \pm 25.8 \mu M \). Therefore, to complete this analysis, we measured the kinetics of fructose uptake by wild type GLUT7 and determined the \( IC_{50} \) for glucose inhibition of fructose uptake. Both sets of experiments confirmed...
that hGLUT7 has a high affinity for these two hexoses. The $K_v$ for fructose uptake was $113.9 \pm 40.1 \mu M$, $n=3$ (Fig. 4), and the IC$_{50}$ for glucose inhibition of 100 $\mu M$ fructose transport was found to be $121.8 \pm 56.0 \mu M$.

Kinetic analysis of glucose transport in the GLUT7 I314V mutant confirmed the initial uptake measurements, using a single glucose concentration, that this point mutation had no effect on glucose transport (Fig. 5). Uptake of glucose over the concentration range 0.05–3.0 mM was curvilinear and, when corrected for uptake into water-injected oocytes, exhibited Michaelis-Menten type kinetics with a $K_v$ of $32.7 \pm 26.5 \mu M$. Interestingly, glucose (100 $\mu M$) transport by this mutant was completely unaffected by the presence of excess cold fructose (10 mM) (Fig. 6), suggesting that fructose either was unable to access the hexose binding site or was no longer recognized.

To determine whether the role of Ile-314 was unique for GLUT7 or was a key residue for other fructose-transporting GLUTs, we then compared the effect of similar mutations in GLUTs 2 and 5 (I322V and I296V, respectively). Substitution of valine for isoleucine in both proteins resulted in a loss of fructose transport (Figs. 7 and 8), and in GLUT2, just as for GLUT7, had no effect on 100 $\mu M$ glucose uptake (Fig. 7). It could be argued that although the substitution of Ile with Val is very conservative, it could produce a very significant alteration to the entrance binding site in the protein. Therefore, the effect of a substitution of isoleucine with serine at position 314 in GLUT7 was also tested. This was found to have no effect on the specificity of the transporter, with glucose and fructose transport both being retained (Fig. 9).

**DISCUSSION**

Our simulation of the GLUT7 structure appeared to confirm the overall structural organization of the TMs in this protein as proposed previously for GLUT1, with TM7 forming part of the lining of the aqueous pore (15) (Fig. 1). (Note: This recent model has made obsolete all prior GLUT1 models advanced by the same group (22, 23).) Comparison of the sequence alignment of GLUTs 1–7 in the TM7 region showed that, in the position equivalent to Val-290 of GLUT1, GLUTs 2, 5, and 7 have an Ile residue, whereas, in contrast, the other class I GLUTs (1, 3, and 4), which transport glucose but not fructose, have a Val at this position. This indicated a possible role for the residue at this position in the permeation process and led us to hypothesize that the replacement of Ile with Val would alter the transport properties of GLUTs 7 and 5 and GLUT2.
Substitution of Val for Ile in GLUT7 dramatically altered the substrate specificity of the protein, almost completely eliminating the ability to transport fructose, whereas having no effect on the recognition or capacity to move glucose. The other transport characteristics were also identical to the wild type behavior, GLUT7 I314V showing no affinity for 2-deoxyglucose or galactose. Expression of the mutant in the oocyte membrane was also unaffected by this single amino acid substitution, as determined by immunohistochemistry and Western blotting. These results indicated that the recognition, binding, and translocation of the glucose through GLUT7 do not depend on the presence of the Ile at the 314 position.

Wild type GLUT7 has the same affinity for glucose and fructose, and these substrates can competitively inhibit each other’s transport equally effectively. The simplest interpretation of these observations would be that there is a single binding site that recognizes both hexoses. However, we now have to add another feature that appears to play a role in substrate access and that is the hydrophobic residue found at position 314. Clearly, it cannot form a hydrogen bond with the substrate, and yet it appears to be critical for the transport of fructose, but not glucose. The uptake of glucose by the I314V mutant was unaffected by the presence of 10 mM fructose, suggesting that in some way, this point mutation prevents fructose from accessing the binding site. At the same time, this indicated that this substitution cannot have any major effect on the overall structure or function of the protein. The differences in the size for Val and Ile are minor, with molecular volumes of 140 and 166.7 Å³; consequently, the global protein energy of GLUT7 is expected to vary very little when Val is substituted by Ile, about 10 k_BT (the Boltzmann constant times temperature).

Using molecular dynamics and energy minimization algorithms in a system already used to model GLUT1 (15), we have produced a putative three-dimensional structure for GLUT7. The model was created using fixed coordinates of the already crystallized E. coli protein GlpT and adjusted using homology modeling from the template after 10 ns of simulations. The position of isoleucine 314 is shown (Fig. 1C) as facing the aqueous pore and within the outer-facing vestibule. There also appears to be a tryptophan residues (C) on the other side of the vestibule, contributed by TM2 (Trp-89), which could form a hydrophobic interaction with Ile-314, producing a narrowing of the pore (Fig. 1C). The docking model further suggested that for the hexose substrates to move forward into the channel and reach the binding site, they first have to pass through this narrow passage. This hypothesis would indicate that a very specific orientation of fructose or glucose is required to allow them to access the binding site(s) within the channel. Interestingly, when we compare the GLUT7 structure with that of GLUT1, in which valine (Val-290) can also interact with the same conserved tryptophan residue, we see an even narrower passage (4.4 Å versus 5.2 Å, Fig. 1, B and C). Finally, the docking simulation also predicts a quite different orientation for fructose (Fig. 1C) when compared with glucose (Fig. 1B) as they bind below the vestibule, suggesting that fructose needs a wider opening to be able to enter the pore and complete the transport cycle.

To further test this hypothesis, we replaced Ile-314 with Ser, which has a smaller size and length of the side chain (residual volume 89 and surface area of 115) and is a hydrophilic residue. Our data showed an increase in both glucose and fructose transport in the GLUT7 I314S mutant.
mutant. Ser is shorter than Ile and will not be able interact hydrophobically with Trp-89, allowing TMs 7 and 2 to "relax." Thus, the vestibule would be opened up, allowing easier entry of both substrates.

We also extended our study to GLUTs 2 and 5, which are also known to be able to transport fructose (24, 25) and which have Ile at the position equivalent to 314. Both proteins lost the ability to recognize fructose when Val was substituted for Ile. In GLUT2, whereas the ability of GLUT2 to transport glucose was again unaffected by this substitution. These observations confirmed that Ile-314 is critical for fructose transport by the GLUTs tested to date.

There are many examples of significant effects on the functionality of SLC2A proteins when hydrophobic residues are mutated (4, 26, 27). One of the most frequent missense mutations encountered in De Vivo syndrome (a GLUT1 deficiency syndrome hypoglycorrhachia, and reduced erythrocyte glucose transport) is where the hydrophilic residue Thr-310 is replaced by an Ile (28). Thr-310 is conserved in human GLUTs 1–9 and also across many other species (29), suggesting that this residue may play an important role in glucose permeation. Also, when an experimental substitution of V197I was made in GLUT2, a severe reduction in glucose transport resulted (7).

From these examples, it is clear that the role of the hydrophobic residues in the biologically active conformation of the SLC2A membrane proteins is more complex than just creating a hydrophobic well inside the pore, but their structural importance is not yet fully understood. It could involve subtle changes in helix packing, changing the shape of the pore, and/or provide more direct interactions of the amino acid side chain with the substrate giving rise to steric hindrance. Our data indicated that isoleucine 314 in GLUT7 may form part of a substrate selectivity filter within the exofacial vestibule proximal to the binding site(s) and prior to translocation.

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