QLS Motif in Transmembrane Helix VII of the Glucose Transporter Family Interacts with the C-1 Position of D-Glucose and Is Involved in Substrate Selection at the Exofacial Binding Site†

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ABSTRACT: The liver-type (GLUT2) and brain-type (GLUT3) human facilitative glucose transporters exhibit distinct kinetics (K_m values for deoxyglucose transport of ∼11 mM and ∼1.5 mM, respectively) and patterns of substrate transport (GLUT2 is capable of D-fructose transport, while GLUT3 is not). Using a range of chimeric glucose transporters comprised of regions of GLUT2 and GLUT3 studied by expression in *Xenopus* oocytes after microinjection of cRNA, we have proposed that the seventh putative transmembrane helix is intimately involved in the selection of transported substrate and that this region plays an important role in determining the K_m for 2-deoxyglucose [Arbuckle, M. I., Kane, S., Porter, L. M., Seatter, M. J., and Gould, G. W. (1996) *Biochemistry 35*, 16519−16527]. Inspection of the predicted amino acid sequence of this region reveals that GLUTs 1, 3, and 4 (high-affinity glucose transporters) contain a conserved QLS motif in this helix (residues 277−279 in human GLUT3). In the glucose/fructose transporter (GLUT2) this motif is replaced by HVA. To study the role of the QLS motif in substrate selection, we have engineered substitutions in this region between GLUT2 and GLUT3. GLUT3 (QLS > HVA) exhibits a K_m for deoxyglucose transport identical to that of native GLUT3 but increased sensitivity for inhibition of deoxyglucose transport by D-fructose. However, unlike native GLUT3, this species is capable of transporting D-fructose. Compared to wild-type GLUT2, GLUT2 (HVA > QLS) exhibits a lower K_m for deoxyglucose transport (∼3 mM vs ∼11 mM), the ability to transport D-fructose is reduced, and D-fructose is a less efficient inhibitor of deoxyglucose transport. Analysis of the ability of a range of glucose epimers and analogues to inhibit transport by these species suggests that the QLS motif interacts with the incoming D-glucose at the C-1 position; this may be a key interaction in the high-affinity recognition of the transported substrate. We further argue that this interaction acts as a molecular filter that is involved in the selection of the transported substrate.

The transport of glucose across the plasma membrane of animal cells is mediated by a family of transporters of the facilitative diffusion type. These transporters are expressed in a tissue-specific fashion resulting in the highly coordinated control of blood glucose (for review see refs *1* and *2*). The heterologous expression of different members of the transporter family in a variety of cells has allowed the detailed analysis of the kinetic properties and substrate selectivities of these proteins (*3*−*7*).

The application of molecular biology to address issues of structure−function relationships within this important class of membrane transporters has begun to reveal details of the molecular basis of transporter function (*8*−*12*). For example, glucose transporters have been proposed to exist in either of two conformations, with the substrate binding site exposed to either the extracellular side of the membrane or the intracellular space. The structural separation of these exofacial and endofacial sugar binding sites, originally proposed on the basis of both kinetics and inhibitor selectivity (*13*−*18*), has now been further defined by mutation studies. These studies have suggested that putative transmembrane helices VII, VIII, and IX are involved in the exofacial recognition of substrate (*10*), and putative transmembrane helices X and XI may constitute at least part of the endofacial site (*19*). Such studies have provided important insight into functionally important regions within GLUT1[1] (*13*).

We have previously shown that the liver-type glucose transporter, GLUT2, is unique among the GLUT family by virtue of its ability to transport both D-fructose and D-glucose (*4*, *5*). In contrast GLUT3 transports glucose and D-galactose with high affinity but is unable to transport D-fructose (*4*, *5*). This suggested to us a potential assay with which we could identify regions of the transporters involved in substrate selection using chimeric GLUT2/GLUT3 glucose transporters (*20*). On the basis of such experiments, we proposed that sequences within transmembrane helix VII are important for

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[1] Abbreviations: DeGlc, 2-deoxy-D-glucose; GLUT, glucose transporter.

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Table 1: Sequence Alignments of Putative Transmembrane Helix 7 in the Five Human GLUTs[a]

GLUT1	...RQPILIAVVL**QLS**QQLSGINA...
GLUT2	...RQPILVALMLHVAQQFSGING...
GLUT3	...RQPIIISIVL**QLS**QQLSGINA...
GLUT4	...RQPLIIAVVL**QLS**QQLSGINA...
GLUT5	...RWQLLSIIVLMGGQQLSGVNA...

[a] Shown are the predicted amino acid sequences of the predicted seventh transmembrane helix of the five human GLUT isoforms. The QLS motif highlighted is notably absent from the isoforms that are capable of mediating fructose transport (GLUTs 2 and 5). Sequences are written from the N-terminus to C-terminus, right to left.

determining which sugar substrate is transported (D-fructose or D-glucose) and also influence the observed K_m for deoxyglucose transport (*20*). Other studies have implicated helix VII as a crucial region for GLUT function (*21*). For example, Hashiramoto et al. (*10*) have identified Q-282 in this helix as a crucial residue in the binding of the exofacial ligand ATB-BMPA. Studies of other related transport proteins such as the *Chlorella* monosaccharide/H^+ cotransporter have also supported an important role for helix VII in transporter function (*22*, *23*). Further evidence for an important role for helix VII in transporter function can be postulated on the basis of the high degree of identity observed within this helix among all of the transporter family members (recently reviewed in ref *24*).

Inspection of the predicted amino acid sequences of helix VII reveals that the members of the GLUT family that transport deoxyglucose with moderate to high affinity (GLUTs 1, 3, and 4) all contain a QLS sequence in this helix (residues 279−279 in the human GLUT3 isoform; Table 1) (*20*). In contrast, the low-affinity glucose/fructose transporter (GLUT2) and the fructose-specific transporter (GLUT5) do not contain this motif. We therefore prepared two mutant species in which the QLS of GLUT3 was substituted with the corresponding HVA from GLUT2 and *vice versa*: GLUT3 (QLS > HVA) and GLUT2 (HVA > QLS). These species, together with native GLUT2 and GLUT3, were expressed in *Xenopus* oocytes and their functional properties were examined. Our results suggest that the QLS motif in helix VII acts as a molecular filter that dictates the ability of GLUTs 1, 3, and 4 to transport D-glucose but not D-fructose and suggest that this region of helix VII is intimately involved in substrate recognition at the exofacial substrate binding site by interaction with the C-1 position of the incoming sugar molecule.

MATERIALS AND METHODS

Materials. Wild-caught *Xenopus laevis* were purchased from the African Xenopus Facility (Noordhoek, Republic of South Africa). All isotopes were from DuPont/NEN (U.K.), and sugars were purchased from Sigma (Poole, U.K.). Reagents for *in vitro* transcription, DNA ligations/digestions, *Taq* polymerase, and dNTPs were from Promega (Southampton, U.K.). *Pfu* polymerase was from New England Biolabs. All other reagents were as described (*4*, *5*).

Isolation of Oocytes and Microinjection. Female *Xenopus laevis* were maintained at 18 °C on a 12 h light/dark cycle. Individual oocytes were dissected and stored in DNOM buffer [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, and 5 mM Hepes-NaOH, pH 7.6, supplemented with nonessential and essential amino acids and MEM vitamins at 1× the manufacturer's recommended concentrations, 1 mM oxaloacetate, and 1 mM pyruvate]. All subsequent procedures were performed in DNOM buffer except for the assays of hexose transport, which were performed in Barth's buffer alone (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, and 5 mM Hepes-NaOH, pH 7.6). Oocytes were injected with water or 50 nL of cRNA (usually ∼50 ng), prepared and purified as described in ref *4*, and incubated in DNOM buffer at 18 °C for 48−72 h prior to assay; the medium was replaced every 12 h.

Hexose Transport in Oocytes: (A) Transport of deGlc. Groups of eight oocytes were washed three times in Barth's buffer and incubated in 0.45 mL of Barth's buffer at pH 7.4 in 13.5 mL centrifuge tubes at room temperature. Transport measurements were initiated by the addition of a 50 μL aliquot of [2,6-^{3}H]deGlc to the concentration indicated. The reaction was stopped after the requisite time interval (30 min unless otherwise stated) by quickly aspirating the medium and washing the oocytes with 5 mL of ice-cold phosphate-buffered saline (PBS; 150 mM NaCl and 10 mM sodium phosphate, pH 7.4) containing 0.1 mM phloretin. Oocytes were washed twice and individually dispensed to scintillation vials. These three washes were completed within 30 s. One milliliter of 1% sodium dodecyl sulfate was added to each vial and incubated for 1 h with agitation, prior to the addition of scintillant and measurement of radioactivity. Under these conditions, we have demonstrated that transport of deGlc, and not its subsequent phosphorylation, is rate-limiting in oocytes (*4*, *5*).

(B) D-Fructose Transport. Transport of D-fructose was determined exactly as for deGlc. Transport was determined over a 30 min period unless otherwise stated, at a substrate concentration of either 50 or 100 μM, as stated.

Note that for both deGlc and fructose transport assays, parallel assays were undertaken using oocytes microinjected with water as a control. These transport rates were subtracted from those obtained in oocytes expressing a functional transporter/chimera, and here we present only the value of transport rates obtained after such subtraction. Such control assays were performed for every condition in every experiment. In general, the water-injected oocytes exhibit a deGlc transport rate of between 3% and 15% that measured in an identical oocyte population microinjected with a functional transporter.

Where K_m values are reported, these were calculated from measurements of transport rates over the range 0.1−10 K_m, and when native transporters were compared to mutant species, the values presented for the native transporter were determined in the same oocyte preparation at the same time (see refs *5*, *20*, and *25* for details).

Generation of Chimeric Transporter Species. Mutant transporter cDNAs were constructed using a PCR-based approach (*26*). Complementary oligonucleotide primers and PCR were used to generate two fragments having overlapping ends at defined junction points and containing the mutation of choice. These primary PCR fragments were combined in a subsequent secondary PCR reaction in which the overlapping ends anneal, allowing the 3′ overlap of each strand to serve as a primer for the extension of the

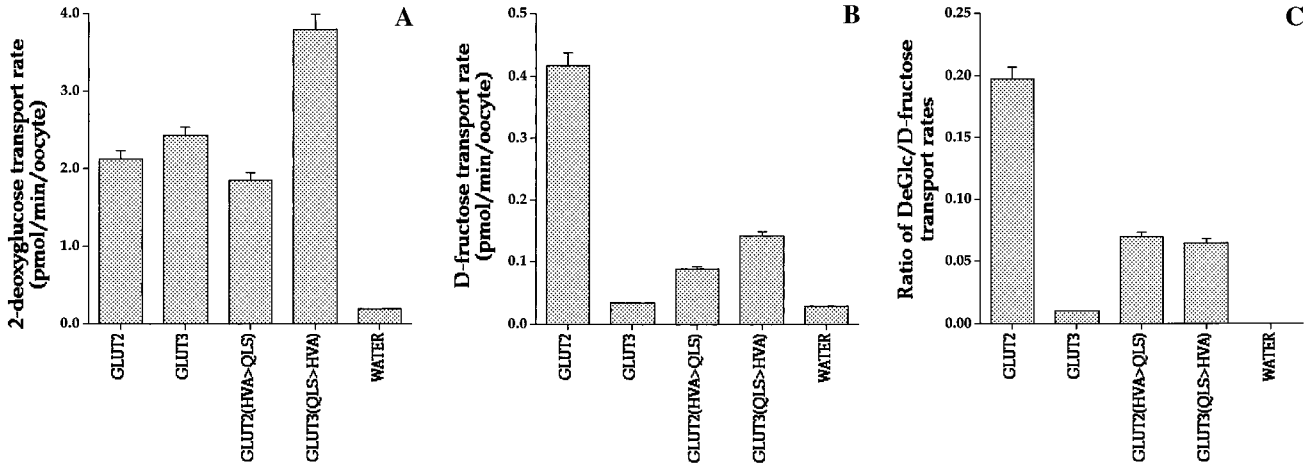

FIGURE 1: Deoxyglucose and D-fructose transport by wilD-type and mutant transporters. Deoxyglucose (panel A) or D-fructose (panel B) transport rates were measured in oocytes expressing GLUT2, GLUT3, GLUT2 (HVA > QLS), or GLUT3 (QLS > HVA) or in water-injected control oocytes. In these experiments, deGlc and D-fructose were used at a concentration of 50 μM. Shown are data from a single experiment performed in the same oocyte preparation; each point is the mean of 8 oocytes. This experiment was repeated 6 times with similar results. As the absolute transport rate measured varies between oocyte preparations, data from a single experiment are presented, rather than the averages of all experiments. In panel B, the transport rates measured for both GLUT2 (HVA > QLS) and GLUT3 (QLS > HVA) are significantly greater than that measured in water-injected oocytes ($p < 0.05$ for both), but D-fructose uptake by GLUT3 is not. Note the different *y*-axis scales between panels. In panel C, the ratio of D-fructose/deGlc transport rates measured in the same oocyte preparations in three independent experiments is shown as mean ±SD. GLUT2 (HVA > QLS) is significantly different from GLUT2 ($p \sim 0.01$) and GLUT3 (QLS > HVA) is significantly different from GLUT3 ($p < 0.05$).

complementary strand. PCR reactions were carried out using either *Taq* polymerase or *Pfu* polymerase. The PCR-generated primary products were purified by gel electrophoresis followed by passage through a DEAE-Sephacel column (EluTip, Schleicher and Schuell) before the subsequent overlap extension (secondary) PCR reaction. The sequences of the oligonucleotides used in the PCR constructions (written 5′ to 3′) were as follows: for GLUT2 (HVA > QLS), CTGATGCTGCAACTGTCTCAGCAA and TTGCTGAGACAGTTGCAGCATCAG (antisense and sense primers); and for GLUT3 (QLS > HVA), TTGTGCTCCATGTCGCTCAGCAGC and GCTGCTGAGCGACATGGAGCACAA (antisense and sense primers). Primary PCR reactions were performed using combinations of the antisense/sense primers and start/end oligos described in ref *20*. Secondary PCR reactions were then performed with start/end combinations. The start/end pairs of oligos for GLUT2 and GLUT3 were described previously (*20*, *25*) and were engineered to contain restriction sites allowing subcloning into plasmid pSP64T (*27*). All chimeras were fully sequenced on both strands at least twice prior to functional analysis. The GLUT2- and GLUT3-containing plasmids employed for both cRNA synthesis and PCR mutagenesis reactions were pHTL.217 and pSPGT3 (*27*).

RESULTS AND DISCUSSION

Functional Properties of the Mutant Transporters. We assayed the functional properties of the mutant transporters with a view to determining their substrate specificities. Figure 1 shows the result of a typical experiment in which GLUT2, GLUT3, GLUT2 (HVA > QLS), and GLUT3 (QLS > HVA) were expressed in the same oocyte preparation and their ability to transport deGlc and D-fructose was measured. As shown, all of the expressed proteins routinely gave high levels of functional expression as measured by deoxyglucose (deGlc) uptake (Figure 1A), indicating that the mutants generated were functional as glucose transporters. We also measured the ability of these proteins to transport D-fructose (Figure 1B). In agreement with previous studies, GLUT2 was capable of D-fructose transport but GLUT3 was not. If, as we speculate, the QLS motif is involved in substrate selection, then substitution of this motif between isoforms would be expected to modulate substrate selection. The data in Figure 1B show that GLUT3 (QLS > HVA) has acquired the ability to mediate fructose transport; similarly, GLUT2 (HVA > QLS) exhibits a significantly blunted rate of fructose transport compared to native GLUT2. It should be noted that the blunted ability of GLUT2 (HVA > QLS) to transport D-fructose is restricted to this sugar, as this mutant exhibits high levels of deGlc uptake when assayed in the same oocyte preparation (see Figure 1A).

Thus, replacement of the QLS motif in GLUT3 with the corresponding HVA motif from GLUT2 results in a species that has acquired the ability to transport D-fructose. In contrast, replacement of the HVA motif in GLUT2 with the QLS motif from GLUT3 significantly reduces the ability of this GLUT2 mutant to transport D-fructose. These differences were observed in proteins that exhibit comparable levels of deGlc transport, arguing that they were not a consequence of defective transporter function. Inspection of the ratio of D-fructose transport/deGlc transport from three independent experiments using three separate oocyte preparations (Figure 1C) clearly illustrates this point. Such results demonstrate that the presence of QLS is negatively correlated with the ability to transport D-fructose; we argue that one interpretation of such observations is that this motif acts to physically preclude D-fructose from the exofacial substrate binding site (see below).

Kinetic Parameters of Mutant Transporters. We measured the K_m of these transporters for deGlc and D-fructose (Table 2). The substitution of the QLS motif in GLUT3 with HVA did not significantly alter the K_m for deGlc compared to native GLUT3. In contrast, GLUT2 (HVA > QLS) exhibited a much reduced K_m compared to native GLUT2. The

Table 2: Kinetic Parameters of Mutant GLUTs Expressed in Oocytes[a]

transporter	K_m for deoxyglucose (mM)	K_m for D-fructose (mM)
GLUT2	10.9 ± 0.8	73 ± 6 ($n = 6$)
GLUT3	1.4 ± 0.02	
GLUT2 (HVA > QLS)	2.8 ± 0.8 ($n = 4$)	nd
GLUT3 (QLS > HVA)	1.3 ± 0.5 ($n = 11$)	173 ± 51 ($n = 3$)

[a] Kinetic parameters were determined as described in Materials and Methods. The values for the native GLUT2 and GLUT3 species were determined in parallel experiments, and the values presented above also include data from refs *4* and *20*. The values shown are mean ±SD (n = number of experimental determinations), employing at least three different batches of cRNA and three different oocyte preparations. nd = not determined, as rates were too low to accurately measure. Note that native GLUT3 does not transport D-fructose.

K_m for D-fructose transport by GLUT3 (QLS > HVA) was found to be significantly greater than native GLUT2; this is probably a reflection of the fact that other regions of the transporters also contribute to substrate selection, as well as the presence or absence of a QLS motif (see for example refs *20* and *28*). D-Fructose transport mediated by GLUT2 (HVA > QLS) was routinely too low to allow accurate measurement of this parameter (see Figure 1).

It is tempting to speculate that the introduction of a QLS motif into GLUT2 may have a more significant impact on function than the corresponding deletion of the QLS from GLUT3. Without definitive measurements of turnover numbers, however, it is difficult to precisely ascribe functional significance to these measurements. Comparisons between GLUT2 and GLUT3 in this regard are hindered by an inability to accurately determine the absolute levels of transporter expression for these isoforms (*20*, *25*). Nevertheless, these data show that the introduction of the QLS motif into GLUT2 results in the protein exhibiting an increased affinity for deGlc, implying that this motif is important in high-affinity substrate binding.

Substrate Interactions at the Exofacial Binding Site. Under the assay conditions employed in Figure 1, transport of deGlc is rate-limiting, and an inhibition of transport rate in response to a given hexose can be reasonably ascribed to inhibitory effects at the exofacial substrate binding site (*4*, *5*, *20*). We therefore examined the ability of other substrates to inhibit deGlc transport by GLUT2, GLUT3, and the two mutant species generated (Figure 2). Unlike GLUT2, deGlc transport by GLUT3 has been previously shown to be sensitive to D-galactose and D-xylose. In contrast, GLUT2 function is inhibited by D-fructose whereas GLUT3 function is not (*4*, *5*, *20*, *29*). We have measured the ability of a range of hexoses to inhibit deGlc transport in these mutants. D-Xylose (D-glucose without the C-6 group), D-galactose (C-4 epimer of D-glucose), and 3-deoxy-D-glucose were chosen in order to examine the interaction of the QLS motif with the transported substrate at the C-3, C-4, and C-6 positions. Figure 2 shows that substitution of the QLS motif between GLUT2 and GLUT3 isoforms does not modulate the inhibition by D-galactose, D-xylose (Figure 2), or 3-deoxyglucose (data not shown) compared to the corresponding native transporter. Such results therefore argue strongly that the QLS motif does not interact with either the C-6 moiety or either the C-3/C-4 position hydroxyls in the transported glucose molecule. The C-2 position hydroxyl has been shown previously not to play a role in substrate recognition by any of the GLUT isoforms (*21*, *29*, *30*). Therefore, we suggest that the QLS motif interacts with the C-1 position of the transported glucose moiety (see below).

The patterns of inhibition observed in response to D-fructose were found to be significantly different among the mutants compared to the native proteins (Figure 2). GLUT2 (HVA > QLS) exhibits a notably decreased inhibition by D-fructose compared to native GLUT2, further arguing that the presence of a QLS motif in this position is inhibitory on D-fructose recognition. In support of this, GLUT3 (QLS > HVA) was observed to acquire a degree of D-fructose inhibition of function. The stereochemistry of D-fructose (in both pyran and furan ring forms) is such that both the C-3 and C-4 position hydroxyls are oriented in space in an

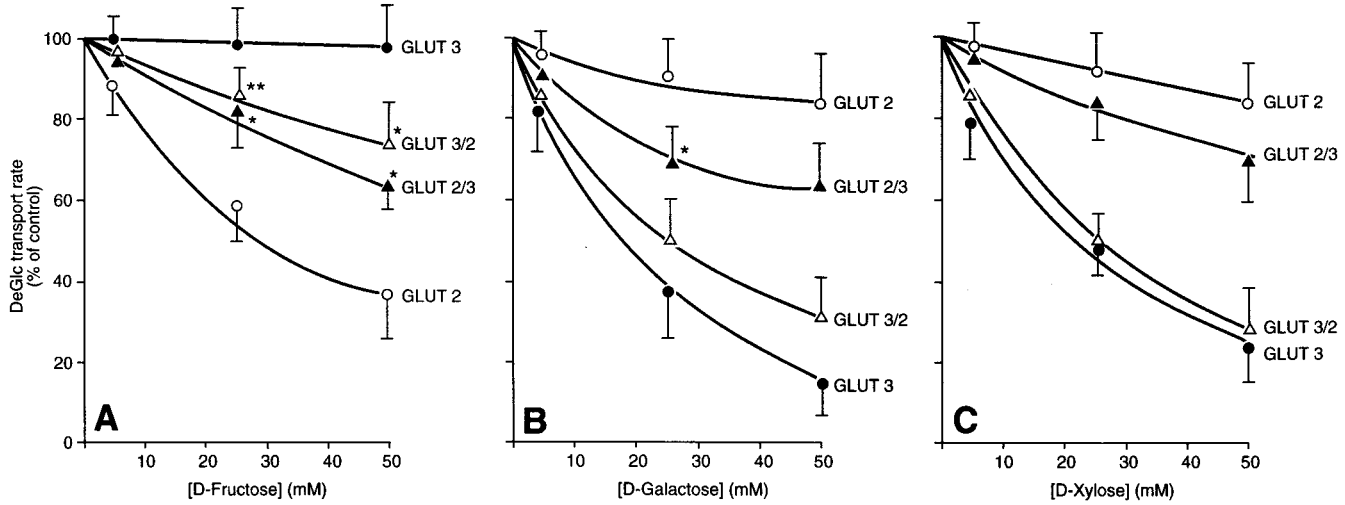

FIGURE 2: D-Fructose, D-galactose, and D-xylose inhibition of deGlc transport. Deoxyglucose transport was measured in oocytes expressing GLUT2, GLUT3, GLUT2 (HVA > QLS), or GLUT3 (QLS > HVA). In these experiments, hexoses added to the bathing medium at the indicated concentrations were rapidly mixed, then deGlc (50 μM, 0.5 μCi/mL) was added to initiate transport. Shown are data from a representative experiment in which each of the transporters was expressed in the same oocyte preparations. Statistically significant differences compared to the corresponding native transporter [i.e., GLUT2 compared to GLUT2 (HVA > QLS) and GLUT3 compared to GLUT3 (QLS > HVA)] are indicated. *, $p < 0.05$; **, $p \sim 0.05$.

identical fashion to that of D-glucose. The major difference between these sugars is observed at the C-1 position. Such data, together with our observation that the recognition of C-3, C-4, and C-6 positions is not altered in the mutants, provide a compelling argument that the QLS motif may recognize the C-1 position of the incoming D-glucose molecule. The absence of this motif in GLUT2 may explain this protein's reduced affinity for glucose and the ability to transport D-fructose with its distinct stereochemistry at this position.

It is important to note that our data clearly indicate that other regions of the transporters are important for both D-fructose transport and recognition. This is supported by several lines of evidence. For example, GLUT3 (QLS > HVA) transports D-fructose with a much higher K_m than that observed for GLUT2, and GLUT2 (HVA > QLS) still retains some ability to transport D-fructose, albeit much reduced (Figure 1). Nevertheless, the data presented here provide a compelling argument for an important role for the QLS motif in this regard. We suggest that this amino acid motif may function as a molecular filter that serves to prevent entry of D-fructose into the exofacial substrate binding site.

Summary. On the basis of several lines of evidence, we propose that the QLS motif within putative membrane-spanning helix VII of GLUTs 1, 3, and 4 is a crucial feature involved in high-affinity recognition of D-glucose by these transporters. We suggest that this motif is involved in recognition of the C-1 hydroxyl of the incoming D-glucose at the exofacial substrate binding site of these isoforms. Introduction of this QLS motif into the fructose/glucose transporter GLUT2 decreased the measured K_m for deGlc and significantly abrogated both D-fructose transport and D-fructose recognition at the exofacial substrate binding site. The corresponding substitution within GLUT3 resulted in this species acquiring some capacity to transport D-fructose and the ability to recognize D-fructose at the exofacial substrate binding site. We therefore propose that the QLS motif acts as a molecular filter that discriminates between hexoses at the exofacial binding site. This discrimination may arise via specific interaction with the C-1 hydroxyl of the D-glucose substrate, or alternatively may be a consequence of the bulky CH_2OH group present on fructose being sterically precluded from the exofacial binding site by the QLS motif.

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