Structure−Function Analysis of Liver-Type (GLUT2) and Brain-Type (GLUT3) Glucose Transporters: Expression of Chimeric Transporters in *Xenopus* Oocytes Suggests an Important Role for Putative Transmembrane Helix 7 in Determining Substrate Selectivity†

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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 11.2 ± 1.1 and 1.4 ± 0.06 mM, respectively) and patterns of substrate transport (GLUT2 is capable of D-fructose transport, GLUT3 is not) [Gould, G. W., Thomas, H. M., Jess, T. J., & Bell, G. I. (1991) *Biochemistry 30***,** 5139−5145]. We have generated a range of chimeric glucose transporters composed of regions of GLUT2 and GLUT3 with a view to identifying the regions of the transporter which are involved in substrate recognition and binding. The functional characteristics of these chimeras were determined by expression in *Xenopus* oocytes after microinjection of cRNA. Replacement of the region from the *start* of putative transmembrane helix 7 to the C-terminus of GLUT3 with the corresponding region from GLUT2 results in a chimera with the ability to transport fructose and exhibits a K_m for 2-deoxyglucose transport of close to that observed for wild-type GLUT2 (8.3 ± 0.3 mM compared to 11.2 ± 1.1 mM). Replacement of the region in GLUT3 from the *end* of helix 7 to the C-terminus with the corresponding region from GLUT2 resulted in a species which was unable to transport fructose and whose K_m for 2-deoxyglucose was indistinguishable from wild-type GLUT3. We have determined the affinity for 2-deoxyglucose, D-fructose, and D-galactose of these and other chimeras. In addition, the K_i for maltose, a competitive inhibitor of 2-deoxyglucose transport, which binds to the exofacial sugar binding site was determined for these chimeras. The results obtained support a model in which the seventh putative transmembrane-spanning helix is intimately involved in the selection of transported substrate and in which this region plays an important role in determining the K_m for 2-deoxyglucose. Additional data is presented which suggests that a region between the end of putative transmembrane helix 7 and the end of helix 10, together with sequences in the N-terminal half of the protein may also participate in substrate recognition and transport catalysis.

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 Bell et al. (1993) and Gould and Holman (1993)]. The heterologous expression of different members of the transporter family in a variety of cells has resulted in a detailed analysis of the kinetic properties and substrate selectivities of these proteins (Burant & Bell, 1992; Colville et al., 1993; Gould et al., 1991; Keller et al., 1989; Nishimura, 1993) and has allowed the application of molecular biology to address issues of structure−function relationships within this important class of membrane transporters (Hashiramoto et al., 1992; Hresko et al., 1994; Mori et al., 1994; Mueckler, 1994; Tamori et al., 1994).

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 (Baldwin, 1993). Structural separation of these exofacial and endofacial sugar binding sites has been proposed on the basis of both kinetics and inhibitor selectivity (Appleman & Lienhard, 1989; Baldwin, 1993; Baldwin et al., 1979; Bloch, 1973; Hebert & Carruthers, 1991, 1992). For instance, ATB-BMPA, a reagent proposed to interact only with the exofacial substrate binding site, has been shown to label GLUT1 in the region of helices 7 to 9 (Hashiramoto et al., 1992). In contrast, the endofacial binding site specific reagent cytochalasin B (CB) labels in the region of helices 10 and 11 (Inukai et al., 1994). Studies by Holman and colleagues using baculovirus expressed protein have shown that the C-terminal half of the erythrocyte-type (GLUT1) protein contains sufficient information to recognise and bind both CB and ATB-BMPA. Such studies suggest a key role for the C-terminal cluster of six putative transmembrane helices in the binding of glucose (Cope et al., 1994).

We previously observed that the liver-type glucose transporter, GLUT2, was unique among the GLUT family by

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virtue of its ability to transport both D-fructose and D-glucose (Colville et al., 1993; Gould et al., 1991). In contrast GLUT3, for example, transports glucose and D-galactose with high affinity but is unable to transport D-fructose (Colville et al., 1993; Gould et al., 1991). This suggested to us a potential assay with which we could identify regions of the transporters involved in substrate selection. We therefore prepared a series of chimeric glucose transporters in which regions of GLUT3 have been replaced with the corresponding regions from GLUT2, and *vice-versa*. Our choice of these two isoforms for this study was driven in part by the distinct substrate selectivities of these two isoforms and by the fact that GLUT2 exhibits a high K_m for deoxyglucose (deGlc) whereas in contrast the K_m of GLUT3 is much lower (11.2 $\pm$ 1.1 mM *vs* 1.4 $\pm$ 0.06 mM, respectively).

Here we describe the functional expression of a series of chimeras which provide evidence in favor of an important role for transmembrane helix 7 in substrate selectivity, and also in the affinity of 2-deoxyglucose transport. We further suggest that a region lying between the end of helix 7 and the end of helix 10 is also part of the exofacial substrate binding site, as evidenced by the ability of maltose to inhibit deGlc transport. Data is also presented which implicates sequences in the N-terminal half of the protein as being important in substrate recognition. The data is interpreted in terms of a working hypothesis for regions of the transporter that participate in substrate selection and recognition.

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 (Colville et al., 1993; Gould et al., 1991).

Isolation of Oocytes and Microinjection

Female *X. laevis* were maintained at 18 °C on a 12 h light/dark cycle. Individual oocytes were dissected and stored in DNOM buffer; this buffer is a modification of the more commonly used Barths buffer which we have used previously. DNOM buffer was prepared by supplementing Barths 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 $Ca(NO_3)_2$, 5 mM Hepes-NaOH, pH 7.6) with non-essential and essential amino acids (Gibco, Paisley, Scotland), MEM-vitamins (Gibco, Paisley, Scotland), all at 1× the manufacturers 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 Barths buffer alone. Oocytes were injected with water or 50 nL of cRNA (usually ~50 ng), prepared and purified as described in (Colville et al., 1993), 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

Transport of deGlc. Groups of 8 oocytes were washed three times in Barths buffer and incubated in 0.45 mL of Barths 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 in the figures and tables. The reaction was stopped after the requisite time interval (30 min unless otherwise stated) by quickly aspirating the media and washing the oocytes with 5 mL of ice-cold phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 7.4) containing 0.1 mM phloretin, a potent transport inhibitor. The oocytes were washed in this fashion a further two times and dispensed to scintillation vials, one oocyte per vial. These three washes were completed within 30 s. 1 mL of 1% sodium dodecyl sulfate was added to each scintillation vial and incubated at room temperature 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 (Colville et al., 1993; Gould et al., 1991).

D-Fructose and D-Galactose Transport. Transport of these sugars was determined exactly as for deGlc. Groups of oocytes (typically 8) were incubated in Barths buffer at pH 7.4 in 13.5 mL centrifuge tubes, and the transport rate measurement was initiated by the addition of the appropriate sugar/radiolabel to the media. Transport was determined over a 30 min period unless otherwise stated and was measured at room temperature. Transport was stopped by three washes in ice-cold PBS containing 0.1 mM phloretin, and the radiolabel in each oocyte was determined as for deGlc transport.

Note that for both deGlc and fructose/galactose 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% of that measured in an identical oocyte population microinjected with a functional transporter.

3-O-Methyl-D-glucose Equilibrium Exchange. Measurement of the equilibrium exchange K_m values for 3-*O*-methyl-D-glucose was performed exactly as described in Gould et al. (1991).

Generation of Chimeric Transporter Species

Recombinant chimeric transporter cDNAs were constructed using a PCR-based approach (Katagiri et al., 1992). Complementary oligonucleotide primers and PCR were used to generate two fragments having overlapping ends at defined junction points. 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 complementary strand. PCR reactions were carried out using either *Taq* polymerase or *Pfu* polymerase. The PCR generated primary and secondary products were purified by gel electrophoresis followed by passage through a DEAE-

Table 1: Oligonucleotide Sequences[a]

Oligonucleotides for GLUT2/GLUT3 Series	
external amplification oligos	
GLUT2 start	*gtcgacgtcgac*TTCCGCACAAGACCTGGAATTGACA
GLUT3 end	*gtcgacgtcgac*CGAGGGAGAGGTGGCTTTCCCATGCC
junction oligos	
1511	CAGCTCTTCACCAATTCCAGCTACCGACAGCCCATCATCATTTCCATTGTGCTCCAGCTC
1510	CAATTTTCCGGAATCAATGGCATTTTTTACTACTCAACAGGAATCTTCAAGGATGCAGGT
1509	ATTGGGCCAGGCCCCGATCCCCTGGTTCATGGTGGCCGAACTCTTCAGCCAGGGCCCCCGC
1504	CTCCTGGCCTTTACCCTGTTCACATTTTTTAAAGTCCCTGAGACCCGTGGCAGGACTTTT
1505	GAGAGCTGGAGCACAATGGAAATGATGATGGGCTGTCGGTAGCTGGAATTGGTGAAGAGC
1506	ACACCTGCATCCTTGAAGATTCCTGTTGAGTAGTAAAAAATGCCATTGATTCCGGAAAAT
1507	GGGCCGGGGGCCCTGGCTGAAGAGTTCGGCCACCATGAACCAGGGGATCGGGCCTGGCCCA
1508	TCAAAAGTCCTGCCACGGGGTCTCAGGGACTTTAAAAAATGTGAACAGGGTAAAGGCCAGG
Oligonucleotides for GLUT3/GLUT2 Series	
external amplification oligos	
GLUT3 start	*gtcgacgtcgac*TCACCCCTAGATCTTTCTTGAAGAC
GLUT2 end	*gtcgacgtcgac*CAGACGGTTCCCTTATTGTTTCTGT
junction oligos	
1577	GAGCTCTTTAGAGTGTCCAGCTACCGACAGCCTATTCTAGTGGCACTGATGCTGCATGTG
1583	CAGCTCTCTGGGATCAATGCTGTGTTCTATTACTCAACCAGCATTTTTCAGACGGCTGGT
1589	ATTGGACCAGGCCCCATTCCCTGGTTTATTGTGGCTGAGTTTTTCAGTCAAGGACCACGT
1587	CTCATTACCTTCTTGGCTTTTACCTTCTTCAAAGTTCCAGAAACCAAAGGAAAGTCTTTT
1588	GCCACATGCAGCATCAGTGCCACTAGAATAGGCTGTCGGTAGCTGGACACTCTAAAGAGC
1584	ATACCAGCCGTCTGAAAAATGCTGGTTGAGTAATAGAACACAGCATTGATCCCAGAGAGC
1585	GGACGTGGTCCTTGACTGAAAAACTCAGCCACAATAAACCAGGGAATGGGGCCTGGTCCA
1586	TCAAAAGACTTTCCTTTGGTTTCTGGAACTTTGAAGAAGGTAAAAGCCAAGAAGGTAATG

[a] Sequences corresponding to GLUT2 are underlined. Sequences which encode a *Sal*I restriction site used in subcloning of PCR fragments are written in lower case italics. All oligos are written 5′ to 3′.

Sephacel column (EluTip, Schliecher and Scheull). Primary products were purified in this fashion before the subsequent overlap extension PCR reaction. The sequences of the oligonucleotides used in the PCR constructions are given in Table 1, and details of the precise joining points and nomenclature adopted are presented in Figure 1.

The PCR fragments were engineered such that restriction sites at the 5′ and 3′ ends allowed subcloning into plasmid pSP64T (Kayano et al., 1990). Large-scale plasmid preparations of all positive clones were then made for subsequent analysis. DNA sequencing was performed using DyeTermination Reactions on an ABI automated DNA sequence analysis unit. All chimeras were fully sequenced on both strands at least twice prior to functional analysis.

RESULTS

The nomenclature and composition of the series of chimeric transporters prepared by recombinant PCR for this analysis is shown in Figure 1. Thus, G3(7St) is a chimera composed of GLUT3 from the N-terminus to the start of helix 7 and GLUT2 from this point to the C-terminus. G3-(10Ed) is a chimera composed of GLUT3 to the end of helix 10, and GLUT2 from this point to the C-terminus, etc. These species were used as templates for *in vitro* transcription reactions, and cRNA microinjected into oocytes. The oocytes were then assayed for their ability to transport deGlc or D-fructose.

Figure 2 shows the results of the GLUT3 series of chimeras (so-called because GLUT3 contributed at least the first six putative helices for all species). As well as mediating deGlc transport, all of the chimeras were capable of mediating the transport of D-galactose (data not shown, but see below), and one member of this series, G3(7St), mediated D-fructose transport. We performed a kinetic analysis of these chimeras, and determined the K_m for deGlc together with the K_i for maltose inhibition of deGlc transport. We have also determined the K_m for 3-*O*-methyl-D-glucose under equilibrium exchange conditions for some of these chimeras, and this data is also presented in Table 2. The results of this analysis are presented in Table 2, and data from a representative experiment with two of the chimeras are presented in Figure 3. Since these chimeras were constructed with the intention of determining which regions dictate substrate specificity, we have also measured the K_ms for D-fructose and D-galactose transport by these proteins. The results of this analysis are presented in Table 3.

We have also prepared and analysed a series of mirror-image chimeras, which, in contrast to those described above, contained at least the first six putative transmembrane helices of GLUT2 (GLUT2 series; Figure 1). These were expressed in oocytes and their properties determined (Figure 4). We consistently found that for three of these chimeras, the level of functional expression obtained was much lower than that of the GLUT3 series; potential reasons for this will be discussed below. Nevertheless, some kinetic analysis was possible, particularly for G2(7Ed) and G2(12Ed) which gave sufficient expression to allow us to determine K_m values for

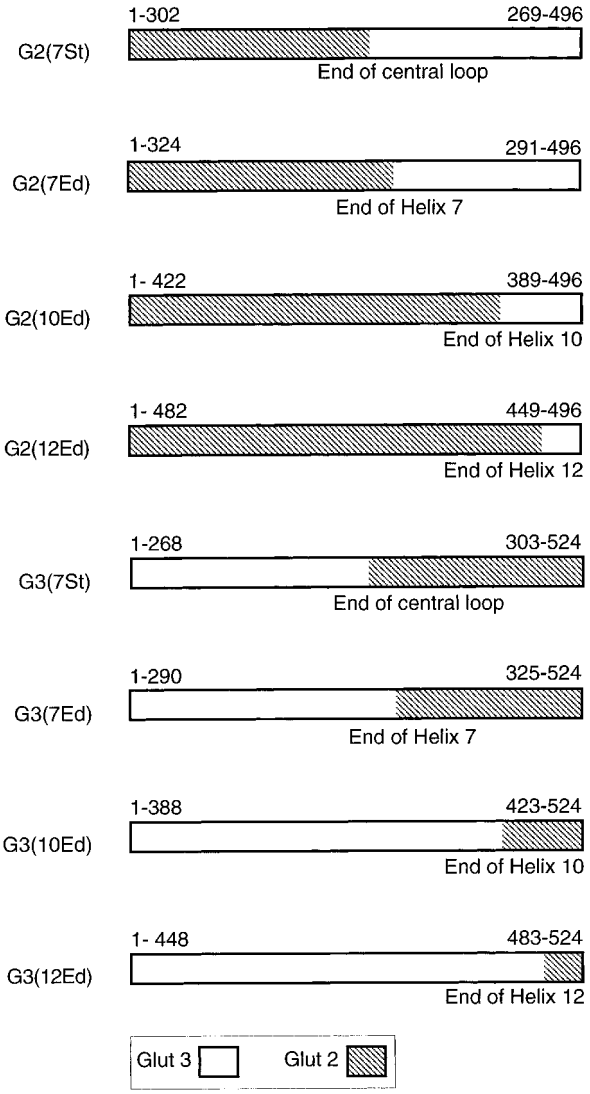

FIGURE 1: Chimeric transporters. Shown is a diagrammatic representation of the junction points of the chimeric species used in this study. These correspond to the start of helix 7, the end of helix 7, the start of helix 10, and the end of helix 12. The nomenclature used in this study is indicated, and the precise amino acids which constitute each chimera are indicated; amino acid numbering refers to the human isoforms, from which these chimeras were assembled.

deGlc and different substrates. The results of these experiments are presented in Table 4.

DISCUSSION

We have employed a chimeric transporter approach in order to identify regions within the liver-type glucose transporter, GLUT2, and the brain-type glucose transporter, GLUT3, which dictate their unique patterns of substrate specificity. Previous studies from this and other laboratories has shown that GLUT2 is capable of mediating the transport of deGlc, D-galactose, and D-fructose, but in contrast GLUT3 is not capable of transporting D-fructose (Burant & Bell, 1992; Colville et al., 1993. Gould et al., 1991). Using this information as the basis of an assay to identify important regions of the proteins responsible for substrate recognition, we have generated a range of chimeric transporters composed of reciprocal domain swaps between these two isoforms (Figure 1). The results of this analysis will be considered

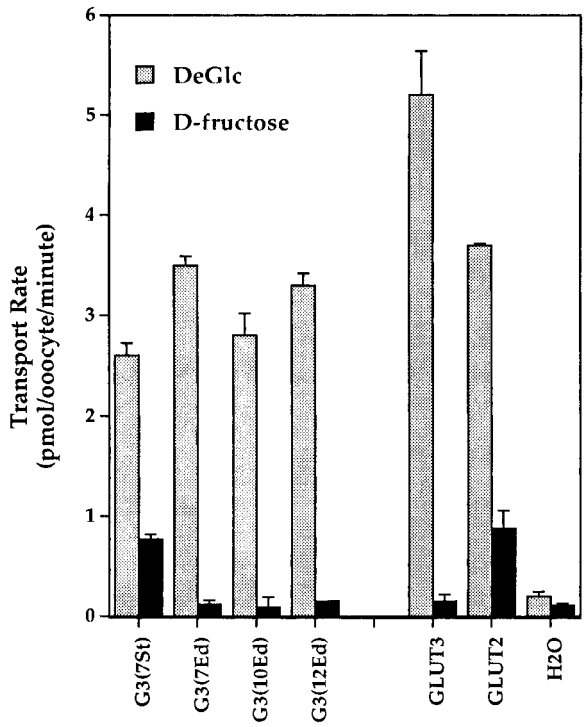

FIGURE 2: 2-Deoxyglucose and D-fructose transport by the GLUT3 series of chimeric transporters. Oocytes were microinjected with water, 50 ng of cRNA encoding wild-type GLUT2 or GLUT3, or ~50 ng of cRNA encoding the chimeric transporter species indicated. After 48 h, transport was determined by a 30 min incubation with either 50 μM deGlc or 100 μM D-fructose. The results of a typical experiment are presented, and are mean ± SD of eight oocytes (representative of seven separate experiments using oocytes from seven different animals and four different batches of cRNA). Only wild-type GLUT2 and G3(7St) exhibited fructose transport which was statistically increased above water-injected oocytes ($p < 0.001$ for both).

Table 2: Kinetic Parameters of the GLUT3 Series of Chimeric Transporters Expressed in Oocytes[a]

chimera	fructose transport?	K_m for deGlc (mM)	K_i for maltose (mM)	K_m^{ee} for 3-*O*-MG (mM)
G3(7St)	yes	8.3 ± 0.3	135 ± 15	38 ± 7
G3(7Ed)	no	1.4 ± 0.03	69 ± 7	12.4 ± 4
G3(10Ed)	no	1.05 ± 0.07	26 ± 4	nd
G3(12Ed)	no	1.89 ± 0.35	25 ± 6.1	11.3, 12.6
GLUT3	*no*	*1.4 ± 0.06*	*28 ± 2.5*	*42.3 ± 4.1*
GLUT2	*yes*	*11.2 ± 1.1*	*125 ± 24*	*10.6 ± 1.3*

[a] Kinetic constants were determined for each chimera as described. The data presented above are the mean ± SEM of at least three independent determinations for each chimera performed using different batches of cRNA and different oocyte populations. The values for wild-type GLUT2 and 3 (*italics*) are from Colville et al. (1993), but in some of the experiments these values were determined simultaneously with those for the chimeric transporters to insure consistency of results. nd, not determined.

in two groups, the GLUT3 series (the lower four chimeras in Figure 1), and the GLUT2 series (the upper four chimeras in Figure 1). Several models have been proposed for GLUT structure, based upon a combination of mutagenic studies, biophysical analysis, and computational modeling. Our data will be summarized in terms of both the "twelve membrane-spanning domains" model (Gould & Holman, 1993; Hresko et al., 1994; Mueckler, 1985; Zeng et al., 1996) and in terms of the rigid β-barrel model proposed by others (Fishbarg et al., 1994; Fishbarg & Vera, 1995).

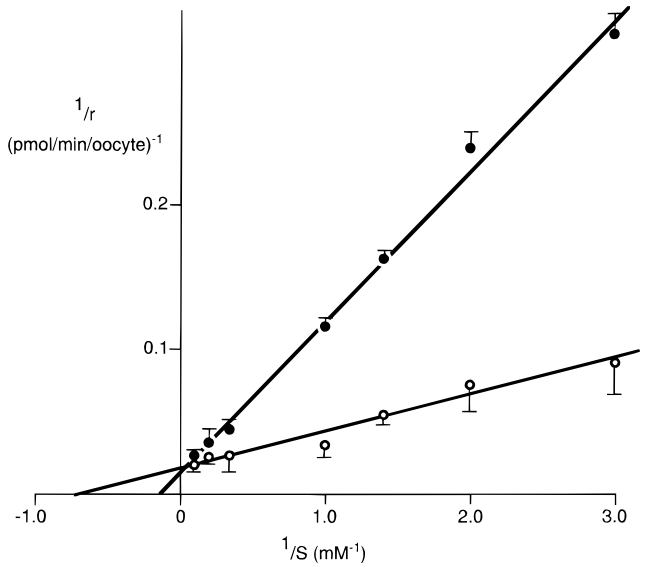

FIGURE 3: Lineweaver−Burk plot of deoxyglucose transport for chimeras G3(7St) and G3(7Ed). Shown are representative Lineweaver−Burk plots of deGlc transport mediated by two of the chimeric transporters analysed in this study, G3(7St) (●) and G3-(7Ed) (○). Each point represents the mean of 8 oocytes (± SD). DeGlc transport rates were determined by incubating oocytes with deGlc for 30 min at each concentration. Under these conditions, we have previously demonstrated that transport of deGlc and not its subsequent phosphorylation are rate limiting. A summary of all experiments of this type is presented in Tables 2 and 3. Note that for clarity of presentation, not all data points have been presented.

Table 3: Kinetic Parameters of D-Fructose and D-Galactose Transport by Chimeras

chimera	K_m for D-fructose (mM)	K_m for D-galactose (mM)
G3(7St)	365 ± 21	25.4 ± 2.1
G3(7Ed)	nd	2.55 ± 0.4
G3(10Ed)	nd	2.8 ± 0.9
G3(12Ed)	nd	4.8 ± 0.88
GLUT2	76 ± 11	92 ± 8.4
GLUT3	nd	6.2 ± 1.5

[a] Kinetic parameters were determined for the transport of D-fructose and D-galactose as described. Measurements of the K_m values of wild-type GLUT2 and GLUT3 were performed in parallel for some of these experiments, and the mean value presented is the sum of the data from this study and our previous analysis (Colville et al., 1993). nd, not determined, as these species do not transport D-fructose (see Figure 2).

Functional Properties of the GLUT3 Series of Chimeras

Analysis of the transport of deGlc, D-fructose, and D-galactose by these proteins was performed and the results of a typical experiment (Figure 2) show that all chimeras retain the ability to transport deGlc and D-galactose but that fructose transport was mediated only by G3(7St). This chimera is composed of GLUT3 from the amino terminus to the approximate start of helix 7, followed by GLUT2 to the carboxy-terminus. The next chimera in this series, G3-(7Ed), composed of GLUT3 to the end of helix 7 and then GLUT2 to the carboxy-terminus, was not capable of mediating D-fructose transport. The high rate of deGlc transport observed for this and the other chimeras in this series precludes this inability to mediate D-fructose transport being a consequence of lack of functional expression of this chimera at the cell surface. Rather, this result strongly suggests that helix 7 is crucial for the ability of GLUT2 to transport D-fructose, since identical chimeras in which helix 7 is contributed from either GLUT2 or GLUT3 exhibit quite distinct functional characteristics (Figure 2).

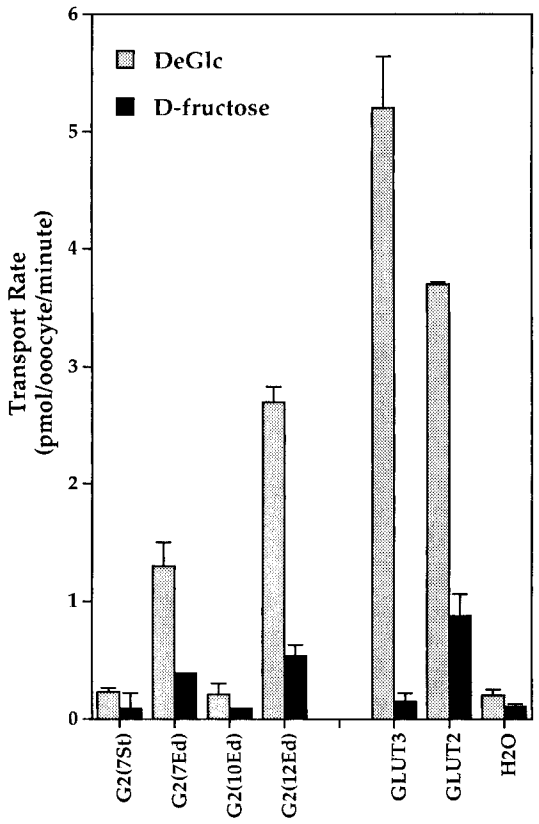

FIGURE 4: 2-Deoxyglucose and D-fructose transport by the GLUT3 series of chimeric transporters. Oocytes were microinjected with water, ~50 ng of cRNA encoding wild-type GLUT2 or GLUT3, or ~50 ng of cRNA encoding the chimeric transporter species indicated. After 48 h, transport was determined by a 30 min incubation with either 50 μM deGlc or 100 μM D-fructose. The results of a representative experiment are presented, and are mean ± SD of eight oocytes.

Table 4: Kinetic Parameters of the GLUT2 Series of Chimeric Transporters Expressed in Oocytes

chimera	fructose transport?	K_m for deGlc (mM)	K_i for maltose (mM)	K_m for D-fructose (mM)	K_m for D-galactose (mM)
G2(7St)	–	–	–	–	–
G2(7Ed)	yes	8.5 ± 2.1	nd	166, 195	nd
G2(10Ed)	–	–	–	–	–
G2(12Ed)	yes	9.5 ± 3.4	133 ± 21	139 ± 21	nd
GLUT3	*no*	*1.4 ± 0.06*	*28 ± 2.5*	*nd*	*6.2 ± 1.5*
GLUT2	*yes*	*11.2 ± 1.1*	*125 ± 24*	*76 ± 11*	*92 ± 8.4*

[a] Kinetic constants were determined for each chimera as described. The data presented above as mean ± SEM are results of at least three independent determinations, using different batches of cRNA and different oocyte populations. Only two values of the K_m for G2(7Ed) have been determined, and both are presented. The values for wild type GLUT2 and 3 (*italics*) are from Colville et al. (1993), but in some of the experiments, these values were determined simultaneously with chimeric transporters to insure consistency of results. nd, not determined.

Kinetic Analysis of the GLUT3 Series of Chimeras

GLUT2 and GLUT3 exhibit distinct K_m values for deGlc transport in oocytes. GLUT3 is a high-affinity transporter ($K_m \approx 1$ mM for deGlc), whereas GLUT2 has a much lower affinity ($K_m \approx 10$ mM) (Colville et al., 1993). A similar distinction between these two transporters is manifest in the

Table 5: Sequence Alignments of Putative Transmembrane Helix 7 in the Five Human GLUTs

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 which are capable of mediating fructose transport (GLUTs 2 and 5).

K_i for maltose inhibition of deGlc transport by these proteins, with the K_i value of maltose inhibition being much lower for GLUT3 than GLUT2 (28 mM *vs* 125 mM, respectively) (Colville et al., 1993). We therefore performed an analysis of the kinetic properties of the GLUT3 series chimeras, and the data is presented in Table 2. The results of this analysis are striking in that the K_m for deGlc transport for G3(7St) is markedly "GLUT2-like" (i.e., ~10 mM), whereas all of the other chimeras in this series are more "GLUT3-like" (i.e., exhibit K_m values of ~1 mM). A similarly large difference was observed between the K_m for 3-*O*-methyl-D-glucose measured under equilibrium exchange conditions for G3-(7St) and G3(7Ed) (Table 2). The fact that large differences in K_ms between G3(7St) and G3(7Ed) are also observed under equilibrium exchange conditions indicates that the endofacial to exofacial conformational change in the presence or absence of substrate is unlikely to contribute to this difference, and further argues that the main role of putative helix 7 is in substrate selection (see below).

Taken together, these results suggest that putative transmembrane helix 7 plays an important role in determining the kinetic behavior of the transporter. Consistent with this suggestion, Hashiramoto et al. (1992) have proposed an important role for helix 7 in recognition of the exofacial ligand ATB-BMPA.

Of course it should be pointed out that the K_m values obtained here are a function not only of substrate binding to the exofacial binding site, but also of the reorientation of the transporter substrate binding site from exofacial to endofacial, and back again. Hence, it cannot be stated unequivocally that the differences in K_m values between these chimeras are solely a function of affinity of the protein for deGlc. Ideally, measurements of turnover number for these chimeras would be obtained. Unfortunately, in our hands, procedures for the preparation of pure plasma membrane fractions from oocytes frequently result in poor yields, and are highly irreproducible. This has precluded an accurate determination of plasma membrane associated transporters. Another handicap in this regard is the inability to accurately quantitate GLUT2 levels by cytochalasin B binding due to the low affinity of this isoform for this inhibitor (Axelrod & Pilch, 1983; Jordan & Holman, 1992). Hence we cannot, for example, compare the turnover numbers for G3(7Ed) with wild-type GLUT3, as the chimera has a GLUT2 carboxy-terminus.

Therefore, we have not made any direct measurements of turnover number for these species. However, in some experiments, estimation of total cellular levels of chimera expression were performed by immunoblot analysis in parallel with a kinetic study. Making the (major) assumption that a similar proportion of each chimera is at the plasma membrane, the *estimated* relative V_{max} values are all within a factor of 2 for the GLUT3 series. Given the nature of the assumptions and the inability to directly compare species with GLUT2 or GLUT3 carboxy-termini, this avenue of investigation was not pursued. This caveat apart, the changes in K_m for deGlc transport among the chimeras reported in Table 2 clearly indicate an important role for putative transmembrane helix 7.

Further evidence in favor of this conclusion comes from analysis of the inhibition of deGlc transport in these chimeras by maltose. Maltose is not transported by members of the GLUT family as it is a di-saccharide. Values of K_i for this sugar therefore represent an effect mediated at the exofacial binding site and are independent of changes in turnover number between chimeras. The K_i for maltose inhibition of deGlc transport mediated by GLUT2 is much higher than the value obtained for GLUT3 (see Table 2). Inspection of the data in Table 2 clearly shows that two regions dictate the ability of maltose to inhibit deGlc transport. The first region is composed of helix 7, and the second region lies between the end of helix 7 and the end of helix 10. G3(10Ed) is kinetically indistinguishable from GLUT3 both in terms of deGlc transport K_m and the K_i for maltose. Hence we propose that the information contained within helices 11 and 12 does not contribute to the recognition of substrate by the exofacial substrate binding site. The difference in maltose K_i values between G3(7St) and G3(7Ed) provides further evidence for an important role for helix seven in substrate interaction at the exofacial substrate binding site, as has been proposed based upon changes in K_m values (see above). Differences between G3(7Ed) and G3(10Ed) further indicate that residues within this region are also important in this regard, a result in agreement with point mutation studies of GLUT1 (Hashiramoto et al., 1992; Mori et al., 1994), antibody binding data (Davies et al., 1990), and studies on expressed domains of GLUT1 (Cope et al., 1994).

Finally, further support for an important role for helix 7 is provided by inspection of the K_m values for D-galactose transport in this series (Table 3). Again, a profound change in K_m is observed upon substitution of helix 7 in these chimeras (see below).

Since we have observed D-fructose transport by G3(7St), we measured the apparent K_m for D-fructose transport by this species. The value obtained was significantly greater than that obtained for wild-type GLUT2 measured in parallel. This significant (~5-fold) increase in K_m value may be accounted for in several ways. For example, it is possible that the amino-terminal cluster of six putative transmembrane helices and the intracellular loop between helices 6 and 7, which are contributed by GLUT3 in this chimera, contain information important in the constitution of the actual substrate binding site. This would be consistent with data from GLUT1, which have implicated a residue in transmembrane helix 5 as playing an important role in sugar interaction with the transporter (Mueckler et al., 1994), and is also consistent with photolabeling data, which have suggested that helices within the amino-terminal half of GLUT1 are directly involved in the formation of the "channel" through which glucose is transported (Lachaal et al., 1996). Alternatively, the amino-terminal domain (contributed from GLUT3 in this chimera) may influence the packing of the amino- and carboxy-terminal halves of the protein, with concomitant alterations in function. This would be consistent with data

from Holman's laboratory where researchers demonstrated an important role for the amino terminal half of GLUT1 in maintaining the ligand binding capacity of the carboxy-terminal region of the protein (Cope et al., 1994). We will return to this point below.

Functional Properties of the GLUT2 Series of Chimeras

We have attempted to express the GLUT2 series of chimeras shown in Figure 1. We have been unable to obtain functional expression of two of these species, G2(7St) and G2(10Ed) whatsoever (Figure 4). Furthermore, expression of G2(7Ed) was found to be much lower than G2(12Ed), which in turn exhibited levels of functional expression approaching that obtained for wild-type GLUT2. [Again, we are unable to directly compare the levels of expression of G2(7Ed) or G2(12Ed) with wild-type GLUT2 as the chimeras contain the GLUT3 carboxy-terminus.]

Expression of chimeric transporters which contain regions of GLUT2 at the amino terminus would appear to be difficult to achieve. Buchs et al have expressed in oocytes chimeras of GLUT4/GLUT2, with GLUT2 at the *carboxy-terminus*, but were unable to express any chimeras with GLUT2 at the *amino-terminus* (Buchs et al., 1995). Similarly, others have reported problems expressing chimeras containing the GLUT2 amino-terminus in other cells (Piper, 1993). Interestingly, Buchs et al. (1995) reported the ability to express immunoreactive GLUT2/GLUT4 chimeras in oocytes but were unable to demonstrate any functional activity of these expressed proteins.

Despite the low level of expression of G2(7Ed), we were able to determine some kinetic parameters for this protein. These are presented in Table 4; the relatively low levels of functional expression have precluded all but a basic analysis of this protein. Data for G2(12Ed) are also shown, a chimera which in our hands expressed well. The K_m for deGlc of these two chimeras was observed to be similar to wild-type GLUT2. This again argues for an important role of helix 7 in determining the kinetic parameters of the transporter, as the observed K_m of G2(7Ed) is close to that observed for wild-type GLUT2, despite the fact that this chimera contains transmembrane helices 8 to 12 and the carboxy-terminal region of GLUT3.

We have found that both G2(7Ed) and G2(12Ed) retain the ability to transport D-fructose. In the case of G2(7Ed), this represents an important result in support of the data outlined above which argues for an important role for transmembrane helix 7 in substrate selection. The K_m value for D-fructose transport for this chimera was found to be ~2-fold higher than native GLUT2, suggesting that regions within helices 8 to 12 contain information important in conferring the kinetic parameters of transport for this sugar. This is further emphasized by G2(12Ed), which exhibits a K_m for D-fructose transport which is similar to wild-type GLUT2. These data may be interpreted to imply that regions within the amino-terminal half of the protein are also important in dictating the ability to transport D-fructose as G3(7St), a chimera containing the entire carboxy-terminal half of GLUT2 from the start of helix 7, exhibits a higher K_m for D-fructose than either G2(7Ed) or G2(12Ed). However, an analysis of the V_{max} of these chimeras would be required to definitively prove this point.

Working Model for Transporter Substrate Selection

On the basis of the results presented above, together with data from other laboratories, we propose that putative transmembrane helix 7 of the human GLUT family plays an important role in both substrate selection and in defining kinetic behavior of the transporters. Our data has shown that sufficient information is retained within transmembrane helix 7 to the carboxy-terminus of GLUT2 to enable a chimeric protein containing this region to transport D-fructose, and to exhibit a K_m for deGlc approximating that of wild-type GLUT2. In marked contrast, a chimera containing helices 8 to the carboxy-terminus of GLUT2 does *not* transport D-fructose and exhibits K_m values for both deGlc and D-galactose that resemble GLUT3. Such results strongly suggest that transmembrane helix 7 plays an important role in substrate selection and also determines the kinetic parameters of deGlc transport. This is consistent with the data of Hashiramoto et al. (1992), who have provided good evidence that helix 7 constitutes part of the binding site for the exofacial-specific ligand, ATB-BMPA. Inspection of the sequence of putative helix 7 from the five cloned human GLUT isoforms reveals some interesting clues regarding which region of this helix may be important in substrate selection. GLUTs 1, 3, and 4 all contain a QLS motif within this helix. This motif is notably absent from GLUT2 and GLUT5, both of which are capable of transporting D-fructose [see Gould and Holman (1993)]. Future studies aimed at identifying the role of this QLS motif are in progress.

Evidence that other regions within the carboxy-terminal half of the protein are involved in the formation of the exofacial binding site is provided by inspection of the K_i values for maltose inhibition of deGlc transport (Table 2). These data indicate that regions within helix 7 and within helices 8 to 10 are involved in recognition of substrate, as chimeras containing these regions exhibit modulated K_i values for maltose inhibition.

Our data further suggest that regions within the amino-terminal half of the protein are also involved in substrate selection. This is based in part on the observation that G3-(7St) is capable of transporting D-fructose with a much reduced affinity than that observed for wild-type GLUT2, despite this chimera containing the entire carboxy-terminal half of GLUT2. This is in contrast to both G2(7Ed) and G2(12Ed), which exhibit K_m values closer to the wild-type GLUT2. Similar results are observed for D-galactose transport in the GLUT3 series. G3(7St) exhibits a K_m for D-galactose which, although higher than any of the other chimeras in this series is still significantly less than that observed for wild-type GLUT2. Chimeric transporters in which helices within the amino-terminal half of these proteins are swapped will provide useful further information on this point.

The data presented here is in broad agreement with the data of Buchs et al. (1995), who propose that regions within the carboxy-terminal half of GLUT2 are responsible for the unique kinetic parameters of this protein. These authors ascribed an important role for the carboxy-terminal 30 amino acids in dictating both the K_m and V_{max} for deGlc but also found that a region located between the start of transmembrane helix 7 and the end of transmembrane 12 was at least equally as important. An important role for the carboxy-terminal 30 amino acids in determining both K_m and V_{max}

for deoxyglucose transport was also suggested by Katagiri et al. (1992) on the basis of a single GLUT1/GLUT2 chimera expressed in CHO cells. The extreme carboxy-terminal region has also been implicated in catalytic turnover of transporters on the basis of studies which have shown that deletion of the carboxy-terminus "locks" the transporter in an endofacial conformation (Oka et al., 1990). Such studies have proposed that the cytoplasmic carboxy-terminal region of the transporters is important in determining the catalytic turnover of the protein (Oka et al., 1990; Katagiri et al., 1992; Lachaal et al., 1996).

In our hands, the extreme carboxy terminus would not seem to play a significant role in determining the K_m for deGlc transport by GLUT2/GLUT3 chimeras. Comparison of the G3(7St) and G3(7Ed) chimeras, which are identical except for helix 7, clearly shows that these chimeras exhibit pronounced differences in the K_m for deGlc. These K_m values are a function not only of substrate binding to the exofacial binding site, but also of the re-orientation of the transporters from the exofacial to endofacial conformation and back again. Hence, it cannot be stated unequivocally that the changes in K_m between such chimeras are solely a function of affinity of the protein for deGlc. However, we would point out that G3(7St) and G3(7Ed) are identical in all respects except for transmembrane helix 7; hence, if, as has been suggested, the extreme carboxy-terminus is crucial for determining V_{max} (Oka et al., 1990; Katagiri et al., 1992), then the behavior of these two chimeras in that regard would be expected to be similar. Whatever the explanation, it is clear that substitution of helix 7 profoundly alters the kinetic properties of the expressed proteins. Further support for this argument comes indirectly from the distinct substrate specificity of G3(7St) and G3(7Ed) and from the demonstration that the K_i for maltose is notably different between these species.

This difference in the relative importance of the carboxy terminus may be accounted for by several potential reasons. These include the different chimeras prepared (for example, Buchs et al. (1995) used GLUT4/GLUT2 chimeras compared to GLUT3/GLUT2 chimeras employed here), and may also be complicated by a failing of all analyses of chimeric transporters reported to date (this study included) to accurately determine transporter turnover numbers (discussed above). We would suggest that another potential reason for the identification of helix seven as being of central importance is that here we have generated a systematic series of substitutions throughout the carboxy-terminal half of the transporters; in contrast, other studies have examined a smaller number of chimeric species. Further studies using such a chimeric transporter approach will undoubtedly provide much more detail to the present models of transporter function.

Finally, some discussion of recent transporter models in which the putative twelve transmembrane helix model has been questioned would seem appropriate. Fishbarg and Vera have recently proposed a model for GLUT structure which proposes that these proteins exist in a relative rigid β-barrel structure (Fishbarg & Vera, 1995). These authors suggest that a relatively rigid translocation structure (or "channel") of diameter ~20 Å exists within the transporter, which is gated shut by mobile loops at both ends. It is proposed that these gates open in response to aromatic interactions with the transported sugar (Fishbarg & Vera, 1995). In this model, the region referred to here as putative transmembrane helix 7 is predicted to form two β-strands, separated by a mobile exofacial loop (s9 in the model of Fishbarg and Vera). Thus, our results when interpreted within the context of the β-barrel model would argue that this region is key for the selection of transported substrate. This region has been proposed by Fishbarg and Vera to be crucial for exofacial substrate binding.

Clearly, these (Fishbarg & Vera, 1995) and other (Zeng et al., 1996) models will only be distinguished upon solution of the structure of the protein. Nevertheless, our data argue strongly suggest that the chimeric transporter approach can provide important information on the functional role of defined regions of this important class of membrane transporters.

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