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Identification of a key residue determining substrate affinity in the human glucose transporter GLUT1

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ABSTRACT

Asn^{331} in transmembrane segment 7 of the yeast *Saccharomyces cerevisiae* transporter Hxt2 has been identified as a single key residue for high-affinity glucose transport by comprehensive chimera approach. The glucose transporter GLUT1 of mammals belongs to the same major facilitator superfamily as Hxt2 and may therefore show a similar mechanism of substrate recognition. The functional role of Ile^{287} in human GLUT1, which corresponds to Asn^{331} in Hxt2, was studied by its replacement with each of the other 19 amino acids. The mutant transporters were individually expressed in a recently developed yeast expression system for GLUT1. Replacement of Ile^{287} generated transporters with various affinities for glucose that correlated well with those of the corresponding mutants of the yeast transporter. Residues exhibiting high affinity for glucose were medium-sized, non-aromatic, uncharged and irrelevant to hydrogen-bond capability, suggesting an important role of van der Waals interaction. Sensitivity to phloretin, a specific inhibitor for the presumed exofacial glucose binding site, was decreased in two mutants, whereas that to cytochalasin B, a specific inhibitor for the presumed endofacial glucose binding site, was unchanged in the mutants. These results suggest that Ile^{287} is a key residue for maintaining high glucose affinity in GLUT1 as revealed in Hxt2 and is located at or near the exofacial glucose binding site.

1. Introduction

Glucose is an important nutrient for most living cells. Facilitated diffusion of glucose and related sugars across biological membranes is mediated by monosaccharide transporters. One group of such transporters, referred to as GLUT transporters, belongs to the major facilitator superfamily (MFS) [1]. Human GLUT transporters are classified in the SLC2A family, with GLUT1 (SLC2A1) being the prototype of this family. GLUT1 is expressed in many tissues and cell types including blood–tissue barriers and erythrocytes [2]. It remains to date the only glucose transporter to be purified and functionally reconstituted into liposomes [3,4].

Studies of glucose transport, which have focused on human erythrocytes, have resulted in the identification of several specific inhibitors. Phloretin is a biphenolic compound and inhibits the glucose transport activity of GLUT1 [5]. Kinetic analysis has indicated that this compound binds competitively to a presumed exofacial glucose binding site [6]. Cytochalasin B inhibits GLUT1 with kinetic analysis indicating that it binds to a presumed endofacial glucose binding site [6].

Various mutational studies have been extensively performed on the molecular mechanism of sugar transport by GLUT1 and other GLUT transporters [2,7,8]. Above all, many amino acid residues in TM7 were implicated for the substrate binding [9–13].

Detailed structural insight into bacterial MFS transporters was provided by the low resolution crystal of OxlT [14] and then the high resolution crystals of GlpT [15], LacY [16] and EmrD [17]. The configurations of the transmembrane segments (TMs) in these transporters are highly similar. These findings suggest that MFS transporters share similar conformations regardless of the substantial differences in their amino acid sequences and substrate specificities.

The yeast *Saccharomyces cerevisiae* expresses 17 hexose transporters (Hxt1 to Hxt11, Hxt13 to Hxt17, and Gal2) [18,19] that belong to the MFS and are homologs of the mammalian GLUT transporters. In contrast to mammalian GLUT transporters, however, no specific inhibitors, with the exception of sulfhydryl inhibitors, have been identified for the yeast hexose transporters. We have previously studied the yeast high-affinity glucose transporter Hxt2 by generating a comprehensive series of chimeric transporters. We found that Asn^{331} in TM7 is a key residue responsible for the high-affinity glucose transport activity of Hxt2 [20]. The replacement of this residue with each of the other 19 amino acids yielded transporters with K_m values

Abbreviations: ATB-BMPA, 2-*N*-4- (1-azi-2,2,2-trifluoroethyl) benzoyl-1,3-bis (D-mannos-4-yloxy) -2-propylamine; MFS, major facilitator superfamily; TM, transmembrane segment

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ranging from 0.87 to 54 mM, compared with a K_m of 3.3 mM for the wild-type transporter.

We have now examined the possibility that a similar mechanism of substrate recognition might operate in the mammalian glucose transporter GLUT1, although the amino acid identity between Hxt2 and GLUT1 is only 27% even within TM regions [21]. We thus investigated the role of Ile^{287} in human GLUT1, which corresponds to Asn^{331} of yeast Hxt2 [21], with the use of a recently developed heterologous yeast expression system based on a glucose transport-null strain of *S. cerevisiae* with an additional mutation to allow expression of GLUT1 in the plasma membrane in a fully functional form [22]. Our results indicate that Ile^{287} is indeed a crucial residue for high affinity of GLUT1 for glucose, suggesting that MFS transporters may also share a common mechanism for substrate recognition.

2. Materials and methods

2.1. Construction of vectors

The plasmid GLUT1-pVT, which comprises human GLUT1 cDNA under the control of the *ADH1* promoter in the multicopy vector pVT102-U (YEp *URA3 bla*), was constructed as described previously [23]. The initiation codon of the open reading frame of *GLUT1* is replaced in this plasmid with a 45-nucleotide sequence: GGATCC (BamHI site) –TACACA (Kozak sequence) –ATGCACCATCACCACCATCAC (initiation codon followed by six histidine codons) –ATCGAAGGTCGT (codons for Ile-Glu-Gly-Arg as an Xa substrate). The open reading frame of *GLUT1* was also modified in this plasmid to generate (i) a ClaI site (underline) immediately downstream of the termination codon (by changing TGA to TAATCGAT), (ii) a SacII site (by changing CCGCGC to CCGCGG, corresponding to nucleotides 631 to 636 of the open reading frame), and (iii) a SalI site (by changing GCCGGC to GTCGAC, corresponding to nucleotides 995 to 1000), all without changing the deduced amino acid sequence.

2.2. Mutagenesis

Replacement of Ile^{287} in GLUT1 with each of the other 19 amino acids was performed with the use of a polymerase chain reaction-based approach in which the target codon (ATC) was replaced with a specific codon for each of the other 19 residues, as described previously [20]. The amplification products were digested with SacII and SalI and substituted for the corresponding region of GLUT1 cDNA in GLUT1-pVT. Plasmids were introduced into *S. cerevisiae* S7 (*MAT***a** *Δhxt1–17 Δgal2 Δagt1 Δmph2 Δmph3 Δstl1 leu2–3,112 ura3–52 trp1–289 his3-Δ1 MAL2–8^c SUC2 fgy1–1*) [22]. The DNA sequences were confirmed for each of the mutated transporters with the use of a DNA sequencer (model 310, Applied Biosystems).

2.3. Transport assay

Cells harboring plasmids were grown to early stationary phase (optical density at 650 nm, 1.0 to 1.3) at 30 °C in synthetic liquid medium supplemented with adenine, amino acids, and maltose (20 mg/ml) but not with uracil (S2Mal) [24]. The initial rate of glucose uptake by the cells was measured at 30 °C for 5 s in the assay buffer of pH 6.0 as described [25,26]. Transport activities measured at a D-[^{14}C]glucose concentration of 0.1 or 20 mM were expressed as picomoles of glucose per 1×10^7 cells per 5 s and were corrected for the background activity determined with 0.1 or 20 mM L-[^{14}C]glucose as substrate. Kinetic parameters were measured under the zero-trans entry condition and were determined by nonlinear regression analysis. For examination of the effects of inhibitors, cells were exposed to the inhibitor for at least 5 min at 30 °C before measurement of transport activity. We confirmed that the inhibitory effect of phloretin was dependent on pH as described by LeFebre and Marshall [27] and our assay condition at pH 6.0 was found appropriate (Supplemental Fig. S1). In some experiments, transport activity was calculated as a percentage of that observed with cells expressing wild-type GLUT1. Kinetic data for the yeast chimeric transporter C1578 were obtained previously [20].

2.4. Other assays

Cell homogenates were prepared as described [25] and were subjected to immunoblot analysis with rabbit polyclonal antibodies generated against human erythrocyte GLUT1 and with ^{125}I-labeled protein A (GE Healthcare) [28]. The intensity of bands corresponding to immune complexes was measured with imaging plates (BAS 1800II, Fuji Film) within the range proportional to the amount of protein. Cell number was determined with a particle counter (Z2, Beckman Coulter). Protein concentration was measured with bicinchoninic acid (Pierce).

3. Results and discussion

3.1. Expression of 287X mutants of GLUT1

We made use of a yeast multicopy expression plasmid containing human GLUT1 cDNA (GLUT1-pVT) to study the role of Ile^{287} in the putative TM7 of GLUT1, which corresponds to Asn^{331} in yeast Hxt2 and in our yeast chimeric transporter C1578, studied previously [20, see Supplemental Fig. S2]. Isoleucine-287 was replaced with each of the other 19 amino acids to generate the 287X series of mutants, and each modified GLUT1 was expressed in *S. cerevisiae* strain S7, in which the genes for all known endogenous hexose transporters have been deleted (*Δhxt1–17 Δgal2*) and in which the heterologous GLUT1 was targeted to the plasma membrane in a fully active form as a result of an additionally introduced mutation (*fgy1-1*) [22]. Expression of each mutant protein was confirmed by immunoblot analysis of cell homogenates with antibodies to GLUT1 (Fig. 1A). All the mutants showed a predominant immunoreactive band at 40 kDa corresponding to the position of wild-type GLUT1. The extent of expression of each mutant protein was 60 to 107% of that of wild-type GLUT1, with the exception of 287H (51%) and 287K (56%) (Fig. 1B).

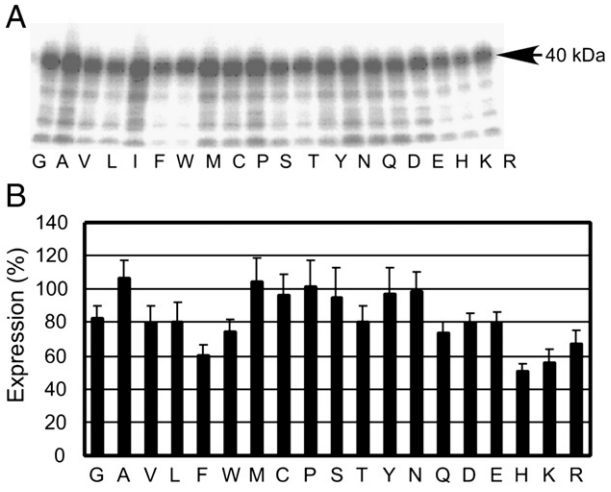

Fig. 1. Expression of 287X mutants. (A) S7 cells harboring plasmids encoding each of the 287X mutants of GLUT1 were cultured to early stationary phase at 30 °C in S2Mal synthetic medium, after which cell homogenates were prepared. A portion of each homogenate (5 μg of protein) was subjected to immunoblot analysis with antibodies to GLUT1 and ^{125}I-labeled protein A. (B) The band intensity for each mutant protein in immunoblots similar to that shown in A was measured and expressed as a percentage of that for GLUT1 (287I). Data are means ± SE from five or six experiments.

Table 1
Characterization of the 287X series of GLUT1 mutants

Mutant	Glucose transport (0.1 mM)		Glucose transport (20 mM)		K_m (mM)	V_{max} (pmol/5 s per 10^7 cells)	Relative V_{max} Normalized by IB
	pmol/5 s per 10^7 cells	Normalized by IB	pmol/5 s per 10^7 cells	Normalized by IB			
287G	1.4 ± 0.2	0.07	172 ± 21	0.74	ND	ND	
287A	2.6 ± 0.2	0.10	187 ± 11	0.63	13 ± 2	300 ± 10	1.04
287V	12.7 ± 3.4	0.67	185 ± 32	0.83	1.6 ± 0.4	160 ± 20	0.75
287L	8.4 ± 0.4	0.43	230 ± 26	1.02	3.3 ± 0.6	230 ± 30	1.06
287F	0.4 ± 0.0	0.03	58 ± 18	0.35	ND	ND	
287W	0.2 ± 0.1	0.01	58 ± 29	0.28	ND	ND	
287M	8.7 ± 0.7	0.35	329 ± 58	1.11	5.3 ± 1.5	410 ± 70	1.45
287C	6.7 ± 0.6	0.29	255 ± 45	0.94	4.4 ± 0.6	270 ± 50	1.03
287P	4.6 ± 0.7	0.19	320 ± 38	1.12	12 ± 0 5	20 ± 60	1.89
287S	3.3 ± 0.3	0.15	310 ± 48	1.16	16 ± 3	530 ± 130	2.07
287T	15.5 ± 1.0	0.81	556 ± 37	2.46	4.5 ± 0.5	630 ± 30	2.90
287Y	0.2 ± 0.1	0.01	74 ± 7	0.27	ND	ND	
287N	2.2 ± 0.2	0.09	203 ± 25	0.73	15 ± 1	270 ± 40	1.01
287Q	1.2 ± 0.1	0.07	188 ± 41	0.91	ND	ND	
287D	1.0 ± 0.0	0.05	77 ± 4	0.34	14 ± 1	120 ± 10	0.56
287E	2.5 ± 0.3	0.14	170 ± 6	0.77	13 ± 3	270 ± 30	1.26
287H	0.4 ± 0.1	0.04	114 ± 0	0.78	ND	ND	
287K	0.2 ± 0.1	0.02	58 ± 10	0.38	ND	ND	
287R	0.4 ± 0.1	0.03	65 ± 7	0.34	ND	ND	
GLUT1 (287I)	23.8 ± 2.5	1.00	281 ± 29	1.00	1.1 ± 0.0	270 ± 30	1.00

The 287X mutants were generated by replacing Ile^{287} of GLUT1 with each of the other 19 amino acids and were expressed in S7 cells. The cells were grown to early stationary phase at 30 °C in S2Mal synthetic medium, after which glucose transport activity was measured for 5 s at 30 °C with 0.1 or 20 mM D-glucose as substrate. Transport activities were normalized either by cell number or by the mean level of protein expression as determined by quantitative immunoblot (IB) analysis (Fig. 1B); the latter values are expressed relative to the normalized value for GLUT1. The K_m, V_{max}, and relative V_{max} (normalized by IB) were determined with 0.1 to 80 mM D-glucose as substrate as described [20]. ND, not determined. Transport activities and kinetic parameters are means ± SE from at least three experiments.

3.2. Characterization of 287X mutants of GLUT1

Wild-type GLUT1 exhibited a K_m for glucose of 1.1 mM (Table 1). As a first step to evaluate changes in the kinetics of glucose transport mediated by the 287X series of mutants, we measured transport activities at D-glucose concentrations of 0.1 and 20 mM and normalized the measured activities by the expression level of each mutant as determined by immunoblot analysis (Table 1). The K_m and V_{max} values of the GLUT1 mutants were determined under the zero-trans entry condition with 0.1 to 80 mM D-glucose as substrate. The mutants could be divided into three groups on the basis of their K_m values: (i) a group of high-affinity transporters (K_m = 1 to 6 mM) consisting of 287V, 287L, 287M, 287C, and 287 T; (ii) a group of lower-affinity transporters (K_m = 12 to 16 mM) consisting of 287A, 287P, 287S, 287N, 287D, and 287E; and (iii) a group of transporters whose activities were too low for assessment of kinetic parameters, consisting of 287G, 287F, 287W, 287Y, 287Q, 287H, 287K, and 287R. The K_m values of the 287X series of GLUT1 mutants correlated well with those of the 331X series of C1578 mutants (Fig. 2). The rank order of affinities for the GLUT1 mutants was 287I (wild type) > 287V > 287L > 287C, whereas that for the C1578 mutants was 331I > 331V > 331C. All of these high-ranking residues are medium-sized, non-aromatic and uncharged amino acids with a low or no hydrogen bonding capability. Given the predicted location of Asn^{331} of C1578 in the middle of TM7 where the residue is surrounded by three other residues in different TMs within van der Waals distances [20], the size and shape of the residue at this position are likely important for it to fit well. The similar effects of residue replacement at this position in both GLUT1 and C1578 suggest the existence of a common structure around this site in both transporters. Minor differences were observed between the rank order of affinities for replacement of Asn^{331} of C1578 and that for replacement of the corresponding residue of Hxt2 [20].

We examined the substrate specificities of the five GLUT1 mutants that exhibited high-affinity glucose transport activity: 287V, 287L, 287M, 287C, and 287T. No marked differences in substrate specificity were apparent between these mutants and wild-type GLUT1 (Fig. 3).

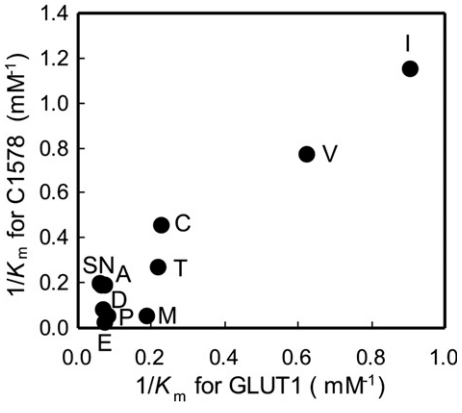

Fig. 2. Correlation of glucose affinities for 287X mutants of GLUT1 and those for 331X mutants of C1578. Kinetic parameters of glucose transport for the indicated 287X mutants of GLUT1 were measured for 5 s at 30 °C as described in Materials and methods and Table 1. Similar measurements were previously performed with KY73 cells expressing 331X mutants of C1578 [20]. Since the activity of 331L of C1578 was too low for kinetic study, data for Leu is not shown.

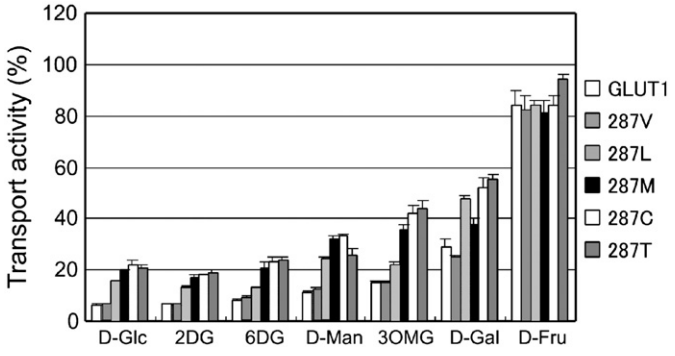

Fig. 3. Substrate specificity of 287X mutants of GLUT1 exhibiting high-affinity glucose transport. The transport activities of the 287V, 287L, 287M, 287C, and 287T mutants as well as of wild-type GLUT1 were measured at 30 °C for 5 s with 0.1 mM D-[^{14}C]glucose as substrate in the presence of 20 mM nonradioactive sugar indicated. Glucose transport activity for each mutant was expressed as a percentage of that determined in the presence of 20 mM L-glucose. Data are means ± SE from four experiments. D-Glc, D-glucose; 2DG, 2-deoxy-D-glucose; 6DG, 6-deoxy-D-glucose; D-Man, D-mannose; 3OMG, 3-*O*-methyl-D-glucose; D-Gal, D-galactose; D-Fru, D-fructose.

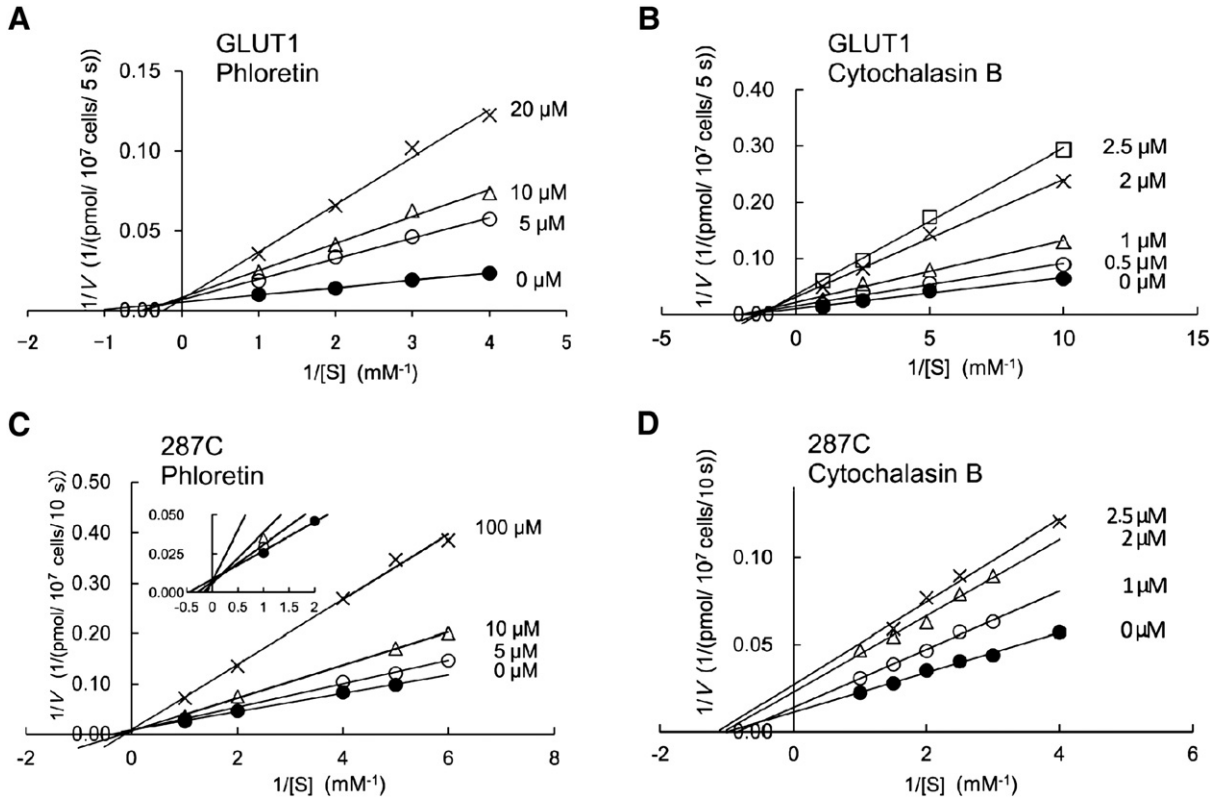

Fig. 4. Lineweaver–Burk plots of glucose transport activity of GLUT1 and the 287C mutant in the presence of phloretin or cytochalasin B. The transport activities of GLUT1 (A, B) and the 287C mutant of GLUT1 (C, D) were measured at 30 °C for 5 s (GLUT1) or for 10 s (287C) with 0.1 to 1.0 mM D-[^{14}C]glucose as substrate and in the presence of the indicated concentrations of phloretin (A, C) or cytochalasin B (B, D). The inset in C shows the portion of the graph close to the origin.

3.3. Kinetic analysis with inhibitors

We next investigated the effects of two potent inhibitors thought to bind asymmetrically to GLUT1. Kinetic analysis has shown that phloretin and cytochalasin B bind to exofacial and endofacial glucose binding sites, respectively [6]. Under the zero-trans entry condition, phloretin competitively inhibits glucose transport whereas cytochalasin B acts as a noncompetitive inhibitor. We confirmed that phloretin and cytochalasin B acted as competitive and noncompetitive inhibitors, respectively, of glucose entry in yeast cells expressing GLUT1 (Fig. 4A, B). The same patterns of inhibition were observed with the GLUT1 mutant 287C (Fig. 4C, D). Table 2 shows K_i values for seven mutants: five high-affinity mutants (287V, 287L, 287M, 287C, and 287T) and two lower-affinity mutants (287P and 287S). The sensitivity to phloretin was markedly reduced for 287C and 287S compared with that for GLUT1, whereas that to cytochalasin B did not differ substantially between the examined mutants and GLUT1. The reason why only the phloretin K_i values of 287C and 287S were increased is not clear, but our results are consistent with the notion that Ile^{287} of GLUT1 is located close to or at the exofacial glucose binding site and is a key residue for maintaining glucose affinity. Given that, with the exception of 287V (0.75) and 287D (0.56), the relative V_{max} values of 287X mutants, regardless of their affinity for glucose, were approximately equal to or greater than that of the wild-type protein (Table 1), it is not likely that the replacement of Ile with most other residues alters the basic high-affinity structure of the transporter or impairs its targeting to the plasma membrane, although these possibilities cannot be excluded.

Table 2
Inhibition constant (K_i) values of 287X mutants for phloretin and cytochalasin B

Mutant	K_i for phloretin (μM)	K_i for cytochalasin B (μM)
287V	3.3 ± 0.0	1.2 ± 0.3
287L	3.3 ± 0.5	2.1 ± 0.3
287M	4.5 ± 0.1	2.2 ± 0.3
287C	22 ± 4	1.5 ± 0.3
287P	5.3 ± 0.3	1.7 ± 0.2
287S	42 ± 4	5.0 ± 1.5
287T	6.0 ± 1.7	4.8 ± 2.3
GLUT1 (287I)	3.0 ± 0.5	2.0 ± 0.3

S7 cells expressing the indicated 287X mutants of GLUT1 were grown to early stationary phase at 30 °C in S2Mal synthetic medium. Glucose transport activity was measured for 5 s at 30 °C with 0.1 mM D-glucose as substrate and in the absence or presence of phloretin (1 to 100 μM) or cytochalasin B (0.5 to 10 μM). The K_i values for phloretin and cytochalasin B were calculated based on competitive and noncompetitive inhibition, respectively, and are means ± SE from three or four experiments.

3.4. Important role of TM7

The role of TM7 in transport function has been previously studied in members of the mammalian GLUT family. For GLUT1, the substitution of Leu for Gln^{282}, which is situated about one helix turn below Ile^{287} (Fig. 5), and the use of inhibitors (4,6-*O*-ethylidene D-glucose and ATB-BMPA) specific for the exofacial glucose binding site suggested that this residue is important for transport and that it faces the hydrophilic side of the permeation pathway either at or close to the exofacial site [9]. The replacement of Ile^{287} with Cys in a cysteine-less form of GLUT1 and examination of the effects of a hydrophilic sulfhydryl inhibitor (*p*-chloromercuribenzenesulfonate) and of *N*-ethyl maleimide indicated that this residue is also important for transport and faces the hydrophilic side [11,12]. Studies with GLUT2 and GLUT3 suggested that the QLS motif present in the middle of TM7 (corresponds to Q^{279}, L^{280} and S^{281} of GLUT1) interacts with the C-1 position of D-glucose [10]. In GLUT7, a fructose and glucose transporter, Ile^{314}, which corresponds to Val^{290} of GLUT1 and is situated at the outer edge of TM7, was shown to be required for fructose transport [13]. In addition, analysis of a plant monosaccharide transporter, *Chlorella* HUP1, showed that

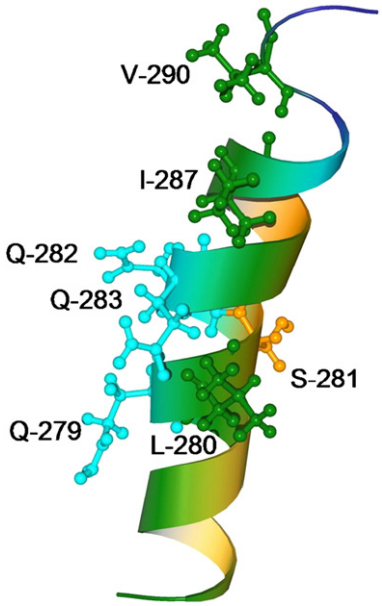

Fig. 5. Amino acid residues in TM7 implicated for functional roles. TM7 is shown as α-helix. Amino acid residues pointed out as functionally important in previous studies and Ile^{287} are shown in ball and stick representation.

substitution of Asn for Ile^{303}, which corresponds to Ile^{287} of GLUT1, increased the K_m for glucose from 1.5×10^{-5} to 6×10^{-3} M without affecting V_{max} [29]. Such an increase in K_m was also observed for 287N in the present study. Studies of bacterial MFS transporters have also shown the importance of TM7. TM7 thus contributes to the substrate permeation pathway in the crystal structures of GlpT and LacY [15,16], and one of the putative substrate binding sites (Arg^{269}) in TM7 of GlpT corresponds to Gln^{283} of GLUT1. These various observations thus indicate that TM7 plays a key role in substrate recognition by MFS transporters.

4. Conclusion

We observed that in GLUT1, medium-sized, non-aromatic and uncharged residues are well accommodated at position of 287. Among 17 homologs of *Saccharomyces cerevisiae*, we recognized Asp and Glu also accommodated in this position (Supplemental Fig. S2). Thus, it appears that "medium-size" is a general feature of the residue at this position, suggesting that hydrogen bonding at this position does not contribute to high-affinity glucose transport. We propose that Ile^{287} interacts with surrounding residues within van der Waals distance that directly communicate with the substrate and thereby contributes to fine-tuning of the binding reaction at the presumed exofacial site. Alternative possibilities are that this residue contributes to steric hindrance of the passing substrate or that it may serve for the dynamic movement of the transporter caused by substrate binding.

Our present data have shown that Ile^{287} of the human glucose transporter GLUT1, like the corresponding residue (Asn^{331}) of the yeast glucose transporter Hxt2, is a key residue for determining high glucose affinity in GLUT1. In addition, the effects of phloretin and cytochalasin B support the notion that Ile^{287} of GLUT1 is located at or close to the presumed exofacial binding site for glucose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2009.01.014.

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