Identification of an Amino Acid Residue That Lies between the Exofacial Vestibule and Exofacial Substrate-binding Site of the Glut1 Sugar Permeation Pathway*

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A valine-to-isoleucine mutation at amino acid residue 197 of Glut2 or the equivalent residue 165 of Glut1 has been shown to impair glucose transport activity. This mutation was originally discovered in the Glut2 gene of a patient with type 2 diabetes. We investigated the mechanism of the effect of this mutation on transport activity via the analysis of Glut1 mutants expressed in Xenopus oocytes combined with cysteine substitution mutagenesis and the use of cysteine-reactive chemical probes. Aliphatic side chain substitutions at position 165 that were bulkier than the native valine residue inhibited glucose transport activity, whereas substitutions of less bulky side chains had little effect on transport, suggesting a role for steric hindrance. A cysteine residue was introduced at position 165 of a functional, cysteine-less Glut1 construct, and this mutant was then tested for inhibition of transport activity by a membrane-impermeant sulfhydryl-specific reagent (p-chloromercuribenzenesulfonate). p-Chloromercuribenzenesulfonate inhibited activity of the Cys165 mutant when it was added to the external buffer but not when it was injected directly into oocytes, indicating that this residue is accessible from the external solvent but not from the cytoplasm. Competition experiments indicated that Cys165 lies near the exofacial substrate-binding site or directly in the sugar permeation pathway. These data provide evidence that the side chain of Val165, which resides in the middle of transmembrane helix 5, juts into the aqueous permeation pathway of Glut1, probably between the exofacial substrate-binding site and the outer vestibule of the pathway.

Glut1 is the prototype member of the Glut family of membrane glycoproteins, which comprises four glucose transporter isoforms (Glut proteins 1–4) and a fructose transporter (Glut5) (1, 2). Glut1 is perhaps the most extensively studied of all of the facilitated diffusion-type membrane transport systems. Its kinetic properties have been studied for over four decades in the human erythrocyte membrane (3, 4), and more recently in a patient with type 2 diabetes. We investigated the mechanism of the effect of this mutation on transport activity via the analysis of Glut1 mutants expressed in Xenopus oocytes combined with sulfhydryl-reactive chemical probes. Khorana and colleagues (18, 19) were the first to use this approach to explore the structure and function of a

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This paper is dedicated to the memory of Julio V. Santiago and Wesley R. Mueckler.

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membrane transporter, and the approach has subsequently been exploited in various permutations to investigate the structure-function relationships of a variety of membrane transporters (18–24) and ion channels (25–28). Our data suggest that Val165 lies within the aqueous sugar translocation pathway of Glut1, in close proximity to both the exofacial substrate-bind- ing site and the outer vestibule of the transporter. These observations lend additional support to our model concerning the importance of helix 5 in transporter catalytic activity (9).

**EXPERIMENTAL PROCEDURES**

Procedures for the site-directed mutagenesis and sequencing of human Glut1 cDNA and the in vitro transfection and purification of Glut1 mRNAs (29), isolation, microinjection, and incubation of Xenopus oocytes (30), preparation of total oocyte membranes and laser confocal immunofluorescence microscopy of sectioned oocytes (31), SDS-polyacrylamide gel electrophoresis and immunoblotting with Glut1 C-terminal antibody (16), and 2-deoxyglucose uptake measurements (5) have been described in detail previously.

Stage 5 Xenopus oocytes were injected with 50 ng of wild-type or mutant Glut1 mRNA. Three days after injection, groups of ~30 oocytes were incubated for 15 min in the presence or absence of the indicated concentrations of p-chloromercuribenzoate (pCMB), p-chloromercuri- risulfonate (pCMBS), iodomethane, or iodoacetate in Barth’s saline at 22 °C. The 100-fold concentrated reagent stocks were prepared in 50% dimethyl sulfoxide, and control oocytes were treated with the appropriate concentration of vehicle alone. In some experiments, sugars (2-deoxyglucose, ethylidene glucose, D-glucose) were included in the incubation solution at 200 mM to test for their ability to delay the sulfhydryl reaction. After the 15-min incubation, the oocytes were washed three times in Barth’s saline and then used for the determination of [1H2]deoxyglucose uptake (50 μM, 30 min at 22 °C).

**RESULTS**

We reported previously that the highly conservative substitution of an isoleucine for Val165 of Glut1, a residue that lies in the middle of transmembrane helix 5, dramatically reduces transport activity (17). Val165 lies one helical turn distant from Gln161, a residue that appears to be involved in forming the exofacial substrate-binding site of Glut1 (16) (see Fig. 1). We reasoned that the isoleucine side chain, which bears an extra methylene group compared with valine, might sterically inhibit interaction between glucose and the amide side chain of Gln161 as a result of its greater bulk. We tested this hypothesis by examining the effect of other amino acid substitutions at position 165 on transport activity. If the hypothesis is correct, bulkier aliphatic side chain substitutions at position 165 should inhibit activity, whereas less bulky side chains should have little if any effect on transport function.

The aliphatic series of amino acid substitutions at position 165 of human Glut1 that were generated and analyzed are listed in Table I. The mutant CDNs were transcribed into mRNAs that were then injected into Xenopus oocytes. Transporter expression and function were analyzed 3 days after injection. Western blot analyses of total oocyte membranes demonstrated that the relative level of protein expression varied somewhat among the mutants and wild-type Glut1 (Fig. 2A) and also varied from one experiment to another, as is often the case with the oocyte expression system. However, laser confocal immunofluorescence microscopy of oocyte sections demonstrated that all of the mutants were consistently expressed in the plasma membrane as efficiently as wild-type Glut1 (Fig. 2B). 2-Deoxyglucose uptake measurements showed that amino acid side chain substitutions at position 165 that were bulkier than valine (leucine, isoleucine) inhibited activity of Glut1 by 76–82%, whereas side chain substitutions less bulky than valine (glycine, alanine) had little or no effect on transport activity (Fig. 3). Although there was a tendency for the alanine substitution mutant to display a lower activity than wild-type Glut1, the difference was not statistically significant (p = 0.0543).

The above observations, coupled with the fact that Val165 lies approximately one helical turn distant from a residue that appears to interact directly with glucose during its passage through the membrane, suggest that Val165 may extend into the aqueous pathway by which glucose traverses the lipid bilayer, and that bulky substitutions at this position either inhibit substrate interaction with Gln161 or interfere with transport activity in some other way. To determine more directly whether Val165 lies exposed in the aqueous transmembrane pathway formed by Glut1, we first created a Glut1 molecule devoid of cysteine residues by employing site-directed mutagenesis to change all six native cysteine residues to either

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1 The abbreviations used are: pCMB, p-chloromercuribenzoate; pCMBS, p-chloromercuribenzenesulfonate; C-less, Glut1 molecule in which all six native cysteine residues were changed to either glycine or serine.
**TABLE I**

Mutations introduced into wild-type or C-less Glut1

<table>
<thead>
<tr>
<th>Residue no.</th>
<th>Amino acid change</th>
<th>Codon change</th>
</tr>
</thead>
<tbody>
<tr>
<td>133</td>
<td>Cys → Ser</td>
<td>TGC → AGC</td>
</tr>
<tr>
<td>201</td>
<td>Cys → Gly</td>
<td>TGC → GGC</td>
</tr>
<tr>
<td>207</td>
<td>Cys → Ser</td>
<td>TGC → AGC</td>
</tr>
<tr>
<td>347</td>
<td>Cys → Ser</td>
<td>TGC → AGT</td>
</tr>
<tr>
<td>421</td>
<td>Cys → Gly</td>
<td>TGC → GGC</td>
</tr>
<tr>
<td>429</td>
<td>Cys → Ser</td>
<td>TGT → TCT</td>
</tr>
</tbody>
</table>

165 in C-less Glut1

- Val → Cys: GTC → TGC
- Val → Gly: GTC → GGA
- Val → Leu: GTC → GCT
- Val → Ala: GTC → GCC
- Val → Ile: GCT → ATC

Sugar Translocation Pathway of the Glut1 Glucose Transporter

If Cys165 lies in close proximity to the exofacial substrate-binding site or directly in the sugar translocation pathway, then sugar substrates might compete with and delay the covalent reaction of pCMBS with Cys165. A complete inhibition of the reaction would not be expected, because sugar ligand binding is a transient, non-covalent interaction. Fig. 6 demonstrates that sugar substrates can indeed compete with and delay the interaction of pCMBS with Cys165.
used as an osmotic control that lacks affinity for Glut1-binding sites. The relative efficacy of the different sugars at competing out the pCMBS reaction parallels their relative affinity (1/Km) for Glut1, i.e. 2-deoxyglucose > ethyliene glycol ≈ D-glucose (8). Additionally, the observation that the non-transported Glut1 exofacial ligand, ethyliene glycol, inhibited the reaction of pCMBS with Cys165 indicates that interaction with the exofacial substrate-binding site is sufficient to cause inhibition, and that inhibition was not the result of transport into the cytoplasm or binding to the inward-facing substrate-binding site.

pCMBS and pCMB were used as sulphydryl reactive reagents in the above experiments, in part because these molecules bear some resemblance to hexose molecules in overall size and shape (see Fig. 4). To determine whether there are structural constraints on molecules that have access to Cys165, we examined the ability of two other sulphydryl-reactive reagents, both smaller in size than either pCMB or pCMBS, to interact with Cys165. Fig. 7A demonstrates that neither methyliodide nor iodoacetate inhibited transport activity of the Cys165 C-less mutant. Because this lack of inhibition could be due to the inability of the reagents to gain access to Cys165, or to the inability of the methylated or carboxymethylated cysteine residue to inhibit transport activity, we performed the experiment shown in Fig. 7B. In this experiment, oocytes expressing the Cys165 C-less mutant were first incubated with either methyl iodide or iodoacetate, rinsed with buffer to remove the reagents, and then incubated with pCMBS under conditions that normally elicit inhibition of transport activity. If the iodo reagent reacts with Cys165 but the reactions do not inhibit transport, then subsequent incubation with pCMBS should not result in transport inhibition of the Cys165 C-less mutant, because the sulphydryl group would already have formed adducts with the iodo reagents. The data in Fig. 7B show that subsequent incubation with pCMBS did result in inhibition of transport activity, indicating that the iodo reagents did not have access to Cys165 within the permeation pathway of Glut1.

DISCUSSION

The experiments described herein identify an amino acid residue that appears to lie within the sugar translocation pathway of Glut1. This residue was originally identified because of its alteration in the GLUT2 gene of a patient with type II diabetes (34). The valine-to-isoleucine mutation at position 197 of Glut2 and at the equivalent position 165 of Glut1 dramatically reduced transport function of the respective isoforms (17). The unexpected effect of this mutation on transport activity motivated the present studies to determine the role of this residue in transporter function. Although Val165 is unlikely to be directly involved in the binding of sugar substrates or in transporter conformational changes given its unreactive aliphatic side chain and in light of the results described here, it does appear to lie within the sugar translocation pathway in the vicinity of the exofacial sugar-binding site. Previous studies have demonstrated that Glu161, which lies about one helical turn distant from Val165 within transmembrane helix 5, may be involved in both exofacial substrate binding and in transporter conformational changes (16). Thus, larger aliphatic side chains at position 165 may either inhibit interaction of substrates with the amide side chain of Gln161 or sterically inhibit transporter...
and iodoacetate do not have access to this residue. If one structurally exclusive environment, given that iodomethane in exofacial substrate binding, and also appears to lie in a turn distant from a residue that appears to be directly involved

and 200 mM of the indicated sugar in Barth’s saline for either 1, 2, or 5 min at 22 °C. After washing three times in Barth’s saline, the oocytes were subjected to 2-deoxyglucose uptake measurements as described in the legend to Fig. 3. l-Glucose served as an osmotic control with no affinity for Glu1. Values represent mean ± S.E. The data shown represent one of four independent experiments.

conformational changes.

Yan and Maloney (21), using a cysteine substitution experimental approach, have defined three distinct regions within putative transmembrane helix 7 of the bacterial glucose 6-phosphate antiporter, UhpT. They discovered that amino acid residues were either accessible to pCMBS only in intact cells, accessible only in inverted membrane vesicles, or accessible in both circumstances, depending on their relative location within the transmembrane helix. Residues close to the exoplasmic membrane face fell into the first category, residues close to the cytoplasmic membrane face fell into the second category, and residues near the middle of the helix fell into the third category. They interpreted their observations in terms of the classic single-site membrane carrier model (3, 4), where one would expect amino acid residues involved directly in substrate binding (in the middle of helix 7 in the case of UhpT) that become exposed to both aqueous compartments during the course of the transport cycle. Residues that lie on the exofacial side of this region may only be exposed to the exoplasmic compartment, and residues that lie endofacial to this region may only be exposed to the cytoplasmic compartment. Their data provide compelling physical evidence for a general model of facilitative membrane transport derived solely on the basis of kinetic transport data.

The observation that Cys165 in C-less Glut1 was accessible to pCMBS from the external medium but not from the cytoplasm suggests that this residue lies in the outer vestibule of Glut1 that does not become exposed to the cytoplasm during transporter conformational changes accompanying the transport cycle. The negative result obtained after injecting the reagent into oocytes must be interpreted with caution, however. It is possible that the intracellular pCMBS did not react with the cysteine residue at position 165 for a reason other than inaccessibility of the side chain. For example, there may be a large surplus of intracellular sulfhydryl groups that affectively compete for reaction with the pCMBS. Cys165 lies only one helical turn distant from a residue that appears to be directly involved in exofacial substrate binding, and also appears to lie in a structurally exclusive environment, given that iodomethane and iodoacetate do not have access to this residue. If one assumes that the Cys165 side chain is inaccessible to intracellular substrate, as suggested by the pCMBS injection experiment, then Val165 most likely lies at an intermediate position between the outer vestibule of Glut1 and the region of helix 5 involved directly in binding exofacially disposed glucose.

Regardless of the exact mechanism of the inhibition at Val165, the results reported here lend additional support to our original simplified structural model for Glut1 in which helix 5, along with other amphipathic helices, participates in forming an aqueous transmembrane channel, only accessible from one membrane face at any instant, and containing one or more substrate-binding sites (35). Interestingly, helix 5 of the Lac permease of *E. coli* has been proposed to play a direct role in the transport of disaccharides. The Lac permease belongs to the 12 transmembrane helix transporter superfamily along with the mammalian Glut proteins, but much more is known about the structure/function relationships of the Lac permease compared with Glu1. Based on a variety of data, Kaback (36) has proposed that lactose is transported across the permease through a pocket or channel formed by helices 5, 7, and 8, and that Cys148 and Met154 within helix 5 are in direct contact with lactose during transport. It is tempting to speculate that overall helix bundling is conserved in all members of the 12 transmembrane helix superfamily, and that these same helices compose the sugar translocation pathway in Glut1. This would be
consistent with existing data that Gln\textsuperscript{282} in helix 7 and Gln\textsuperscript{161} in helix 5 participate in exofacial substrate binding.

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REFERENCES
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