Transmembrane Segment 5 of the Glut1 Glucose Transporter Is an Amphipathic Helix That Forms Part of the Sugar Permeation Pathway

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Facilitative transport of glucose into mammalian cells is mediated by members of the Glut family of membrane glycoproteins (reviewed in Refs. 1–3). Glut1, the prototype member of this family, may be the most extensively studied of all mammalian membrane transporters. Kinetic studies on human red blood cell Glut1 are mostly compatible with a simple alternating conformation mechanism for sugar transport (4), although anomalies have been observed that appear to contradict this hypothesis (5). The human Glut1 polypeptide exhibits a molecular mass of 54,117 and contains a single N-linked oligosaccharide (6). Analysis of the amino acid sequence obtained by translation of the cDNA sequence suggested the presence of 12 transmembrane segments (6), and this prediction has been experimentally verified using glycosylation-scanning mutagenesis (7).

Five of the 12 putative transmembrane segments (segments 3, 5, 7, 8, and 11) are capable of forming amphipathic α-helices, which led to the hypothesis that these amphipathic helices cluster together in the membrane to form the walls of a water-filled pathway through which glucose traverses the fatty acyl core of the lipid bilayer (6). It was further suggested that hydroxyl- and amide-containing amino acid side chains within these helices form the glucose binding pocket within Glut1 via the formation of hydrogen bonds with sugar hydroxyl groups.

Several pieces of evidence are consistent with this model for the structure of Glut1. First, glutamine-161 within helix 5 (8) and glutamine-282 within helix 7 (9) both appear to participate in forming the exofacial substrate binding site. Second, valine-165, which lies near the center of helix 5 one helical turn distant from glutamine-161, is accessible to aqueous sulfhydryl reagents and appears to be near the exofacial substrate binding site based on mutagenesis and inhibitor studies (10). Third, tryptophan-412 within helix 11 is essential for transport activity (11). Fourth, hydrogen exchange studies indicate that 30% of peptide hydrogen atoms are freely exposed to water in purified reconstituted Glut1, consistent with the existence of a water-accessible permeation pathway (12).

In this study we used cysteine-scanning mutagenesis in conjunction with a sulfhydryl-specific chemical reagent to more directly address the role of transmembrane segment 5 in forming the Glut1 sugar permeation pathway. Our results suggest that transmembrane segment 5 is an amphipathic α-helix with a polar water-accessible face that lines the exofacial portion of the sugar permeation pathway.

EXPERIMENTAL PROCEDURES

Procedures for the site-directed mutagenesis and sequencing of human Glut1 cDNA and the in vitro transcription and purification of Glut1 mRNAs (13), isolation, microinjection, and incubation of Xenopus oocytes (14), preparation of total oocyte membranes (11), SDS-polyacrylamide gel electrophoresis and immunoblotting with Glut1 C-terminal antibody (8), and 2-deoxyglucose uptake measurements (15) have been described in detail previously.

Treatment with p-Chloromercuribenzenesulfonate (pCMBS)‡—Stage 5 Xenopus oocytes were injected with 50 ng of wild-type or mutant Glut1 mRNA. Two days after injection, groups of ~20 oocytes were incubated for 15 min in the presence or absence of the indicated concentrations of

‡ The abbreviations used are: pCMBS, p-chloromercuribenzenesulfonate; C-less, Glut1 molecule in which all 6 native cysteine residues were changed to either glycine or serine.
TABLE I
Cysteine scanning mutagenesis of helix 5

| Residue | Amino Acid Change | Codon Change |
|---------|------------------|--------------|
| 156     | Leu → Cys        | CTG → TGC    |
| 157     | Gly → Cys        | GGC → TGC    |
| 158     | Thr → Cys        | ACC → TGC    |
| 159     | Leu → Cys        | CTG → TGC    |
| 160     | His → Cys        | CAC → TGC    |
| 161     | Glu → Cys        | CAG → TGC    |
| 162     | Leu → Cys        | CTG → TGC    |
| 163     | Gly → Cys        | GGC → TGC    |
| 164     | Ile → Cys        | ATC → TGC    |
| 165     | Val → Cys        | GTC → TGC    |
| 166     | Val → Cys        | GTC → TGC    |
| 167     | Gly → Cys        | GGC → TGC    |
| 168     | Ile → Cys        | ATC → TGC    |
| 169     | Leu → Cys        | CTC → TGC    |
| 170     | Ile → Cys        | ATC → TGC    |
| 171     | Ala → Cys        | GGC → TGC    |
| 172     | Glu → Cys        | CAG → TGC    |
| 173     | Val → Cys        | CTG → TGC    |
| 174     | Phe → Cys        | TTC → TGC    |
| 175     | Gly → Cys        | GGC → TGC    |
| 176     | Leu → Cys        | CTG → TGC    |

pCMBS in Barth’s saline at 22 °C. The 100× concentrated reagent stock was prepared in 100% dimethyl sulfoxide, and control oocytes were treated with the appropriate concentration of vehicle alone. After a 15 min incubation period, the oocytes were washed 4 times in Barth’s saline and then used for the determination of [3H]2-deoxyglucose uptake (50 μM, 30 min at 22 °C).

RESULTS

We previously constructed a mutant human Glut1 cDNA encoding cysteine-less (C-less) Glut1 polypeptide in which all 6 native cysteine residues were changed to either serine or glycine residues (10). The C-less transporter expressed in Xenopus oocytes exhibits transport activity nearly indistinguishable from wild-type Glut1. We used C-less Glut1 cDNA to construct cysteine-scanning mutants for transmembrane segment 5, which is predicted to form an amphipathic α-helix that lines the sugar permeation pathway (6). Each of the 21 residues within transmembrane segment 5 was individually changed to a cysteine residue using oligonucleotide-mediated site-directed mutagenesis, producing a series of 21 mutant Glut1 molecules, each containing only a single cysteine residue (see Table 1).

Mutant mRNAs were injected into Xenopus oocytes and the expression and function of the corresponding mutant transporters were evaluated by immunoblotting oocyte membrane fractions and by conducting 2-deoxyglucose uptake assays. As demonstrated previously (13), the immunoblots revealed the presence of two glycosylated forms of Glut1 in oocyte membranes, a faster-migrating, core-glycosylated, high mannose form, and a slower migrating, fully processed, complex N-glycosylated form (see Fig. 1). Roughly half of the cysteine-scanning mutants were expressed in oocyte membranes at levels similar to the parental C-less construct, whereas the other half were expressed at lower levels. V166C, V167C, and A171C were expressed at particularly low levels relative to the parental C-less transporter.

Transport activity was detectable for all 21 mutants as determined by uptake of [3H]2-deoxyglucose (Fig. 2). Consistent with our previously published results (8), cysteine substitution at glutamine-161 greatly reduced transport activity, even when normalized to the amount of steady-state protein. This residue has been shown to be involved in exofacial substrate binding and in a conformational change during transporter cycling (8). Cysteine substitutions at valine-166, glycine-167, alanine-171, and glutamine-172 also significantly inhibited transport activity, most likely because of the reduced steady-state levels of the mutant proteins relative to the parental C-less transporter (see Fig. 1). All of the other mutants exhibited transport activities that were at least 50% of the activity of the parental C-less transporter. Cysteine substitution at leucine-164 enhanced transport activity by ~46%, perhaps because of the increased steady-state level of the mature glycosylated form of this mutant relative to that of the parental C-less construct (see Fig. 1). We previously demonstrated that only the fully glycosylated form of Glut1 is present in the plasma membrane (13).
Three days after injection of mRNAs, groups of 15–20 oocytes were incubated in the presence or absence of 0.5 mM pCMBS in Barth’s saline and then subjected to 2-deoxyglucose uptake measurements under the conditions described in the legend to Fig. 2. Results are expressed as mean ± S.E. of four to twelve independent experiments, each experiment using 15–20 oocytes per experimental group. Each uptake value was normalized to the uptake observed in the presence of vehicle alone (see Fig. 3). The activity of 7 cysteine-substituted amino acid side chains reacted with the membrane-impermeant sulfhydryl-specific reagent, pCMBS, and compared with the activities measured in the presence of vehicle alone (see Fig. 3). The activity of 7 cysteine-substituted amino acid side chains reacted with the pCMBS and therefore must be accessible from the external aqueous solvent. Our results with Glut1 are reminiscent of those reported for cysteine-scanning mutagenesis experiments conducted on the Escherichia coli lac permease (reviewed in Ref. 16) and the glucose-6-P antiporter (17), both of which belong to the same 12 transmembrane helix superfamily of membrane transporters as Glut1. Helix 7 of the glucose-6-P antiporter and several helices within the lac permease have been shown to possess solvent-accessible faces that appear to line a portion of their respective substrate permeation pathways. Our results extend these findings to a mammalian facilitative transporter, and the results when taken together imply that a similar structural paradigm may exist for cotransporters, antiporters, and facilitative transporters in organisms ranging from E. coli to the human.

Yan and Maloney (17) demonstrated that residues within helix 7 of the glucose-6-P antiporter that are accessible to the external solvent are clustered along the water-accessible face proximal to the exoplasmic surface of the plasma membrane, and that the 6 residues along the water-accessible face most proximal to the cytoplasmic surface of the membrane displayed sensitivity to pCMBS in the external solvent. These results are consistent with a simple alternating conformation or carrier model for the sugar permeation pathway (8). Our results with Glut1 are hypothesized to be exposed within the aqueous sugar permeation pathway based on two separate series of experiments (8, 10). The opposite face of helix 5, which is presumably in contact with the fatty acyl core of the lipid bilayer, is composed primarily of highly hydrophobic amino acid side chains. These data comprise the first experimental evidence for the existence of an amphipathic transmembrane helix in a glucose transporter and also support our original hypothesis that transmembrane segment 5 comprises part of the sugar permeation pathway (6).

To determine which transmembrane residues are accessible to the external aqueous solvent and therefore may comprise part of the sugar permeation pathway, transport activity was measured for each of the 21 mutants after incubation in the presence of the membrane-impermeant sulphydryl-specific reagent, pCMBS, and compared with the activities measured in the presence of vehicle alone (see Fig. 3). The activity of 7 cysteine-scanning mutants was inhibited by at least 40% after incubation with pCMBS, indicating that the corresponding 7 cysteine-substituted amino acid side chains reacted with the pCMBS and therefore must be accessible from the external aqueous solvent. Interestingly, none of the 5 side chains predicted to lie closest to the cytoplasmic surface of the membrane appeared to be accessible to the external reagent. Note that the reduction in relative transport activity after pCMBS treatment did not reach statistical significance for the glutamine-161 mutant because of the extremely low absolute transport activity for this particular mutant (see Fig. 2). However, we have previously demonstrated an important role for glutamine-161 in transport activity consistent with this residue being exposed to the sugar permeation pathway (8).
mechanism of transport whereby the outer binding site is largely inaccessible to cytoplasmic substrate and the inner binding site is largely inaccessible to substrate in the external medium. At least in the case of the glucose-6-P antiporter, there also appears to exist a central transmembrane region that is alternately accessible from either aqueous domain as the transporter moves between its two basic configurations. As pointed out by Yan and Maloney (17), this general model for membrane transport was proposed decades ago based purely on kinetic analyses, and it is only now that molecular biological approaches are providing more direct experimental support for this fundamental hypothesis.

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