Transmembrane segment 12 of the Glut1 glucose transporter is an outer helix and is not directly involved in the transport mechanism*

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Running Title: Transmembrane Segment 12 of the Glut1 Glucose Transporter

A model has been proposed for the exofacial configuration of the Glut1 glucose transporter in which 8 transmembrane domains form an inner helical bundle stabilized by 4 outer helices. The role of transmembrane segment 12, predicted to be an outer helix in this hypothetical model, was examined by cysteine-scanning mutagenesis and the substituted cysteine accessibility method using the membrane-impermeant, sulfhydryl-specific reagent, p-chloromercuribenzenesulfonate (pCMBS). A previously characterized, functional, cysteine-less Glut1 molecule was used to produce 21 Glut1 point mutants by changing each residue along helix 12 to a cysteine residue. These mutants were then expressed in Xenopus oocytes and their protein levels, functional activities, and sensitivities to pCMBS were determined. Strikingly, in contrast to all 9 other predicted Glut1 transmembrane helices that have been previously examined by this method, none of the 21 helix 12 single-cysteine mutants exhibited significant inhibition of specific transport activity. Also unlike most other Glut1 transmembrane domains whose solvent-accessible residues lie along a single face of the helix, mutations in 5 consecutive residues predicted to lie close to the exofacial face of the membrane resulted in sensitivity to pCMBS-induced transport inhibition. These results suggest that helix 12 plays a passive stabilizing role in the structure of Glut1 and is not directly involved in the transport mechanism. Additionally, the pCMBS data indicate that the predicted exoplasmic end of helix 12 is completely exposed to the external solvent when the transporter is in its exofacial configuration.

The transport of glucose across mammalian cell membranes is mediated by proteins produced by the Glut gene family [reviewed in (1-3)], part of the Major Facilitator Superfamily (MFS) that contains several thousand members present in virtually every organism examined thus far (4). MFS proteins are involved in the transport of a diverse collection of low molecular weight molecules across membranes (5).

Glut1 is expressed at its highest known levels in the human erythrocyte and is the only member of the Glut protein family to have been purified (6,7). It remains one of the most extensively studied and thoroughly characterized members of the MFS (8). Based on analysis of the protein sequence deduced from a human cDNA clone, Glut1 was the first MFS protein predicted to possess 12 transmembrane helices (9), a major feature of most if not all members of this superfamily. A number of experimental observations, most notably a comprehensive glycosylation-scanning mutagenesis study (10), are consistent with this structural prediction. Several of the 12 predicted transmembrane helices are amphipathic, an observation that led to the suggestion that these helices form an exposed cavity in the membrane-buried portion of the protein that participates in the binding and translocation of glucose across the lipid bilayer (9). Hydrogen bond-forming amino acid side chains within these helices were predicted to form the sugar-binding site(s) of Glut1 via interaction with glucose hydroxyl groups (9), an hypothesis supported by earlier biochemical observations (11).

The existing experimental evidence generally supports this crude model for the exofacial configuration of Glut1 and implicates transmembrane segments 1 (12), 2 (13), 5 (14), 7 (13,15), 8 (16), 10 (17), and 11 (18) in the formation of an inner helical bundle that comprises a water-accessible cavity within the membrane. Data suggest that transmembrane segment 3 is an outer helix (19). This model is also largely in agreement with homology modeling of Glut1 based on the high resolution structures recently reported for the lac permease (20) and glycerol-

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3-P antiporter (21), two members of the Major Facilitator Superfamily from E. coli. Homology modeling of the endofacial configuration of Glut1 based on these structures suggests that helices 1, 2, 4, 5, 7, 8, 10, and 11 comprise an inner bundle of transmembrane helices that form a substrate-binding cavity near the center of the bilayer, whereas, helices 3, 6, 9, and 12 are predicted to encircle and stabilize this inner helical bundle. Interestingly, experimental data suggest that the exofacial half of helix 4 may not be exposed to the external aqueous environment in the exofacial conformation (22), even though it comprises an inner helix in the endofacial conformations of the bacterial transporters. It is thus possible that the endofacial and exofacial helical bundle orientations of these transporters may not be identical.

In the current study, cysteine-scanning mutagenesis was used along with a membrane impermeant, sulfhydryl-specific chemical reagent to examine the role of transmembrane segment 12 in the predicted exofacial structure of Glut1. Our results demonstrate that the exofacial end of helix 12 lies in direct contact with the external solvent in the outward-facing configuration of Glut1. Additionally, none of the helix 12 side chains appears to be essential for transport activity. These data suggest that helix 12 does not participate directly in the transport mechanism but likely plays a role in stabilizing Glut1 structure.

EXPERIMENTAL PROCEDURES

Materials—Imported female African Xenopus laevis frogs were purchased from Xenopus Express (Homesassa, FL), \(^{3}H\)-2-deoxyglucose and Diguanosine triphosphate were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL), Megascript™ RNA synthesis kits were purchased from Ambion Inc (Austin, TX), and Transformer™ Site-Directed mutagenesis kits were obtained from Clontech (Paolo Alto, CA).

General Procedures—Procedures for the site-directed mutagenesis and sequencing of human Glut1 cDNA and the in vitro transcription and purification of Glut1 mRNAs (23), isolation, microinjection, and incubation of Xenopus oocytes (24), preparation of purified oocyte plasma membranes and indirect immunofluorescence laser confocal microscopy (25), SDS polyacrylamide gel electrophoresis and immunoblotting with Glut1 C-terminal antibody (26), and \(^{2}H\)-deoxyglucose uptake measurements (27), have been described in detail previously.

Treatment with 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 p-chloromercuribenzenesulfonate (pCMBS\(^{1}\)), in Barth’s saline at 22°C. The 100x concentrated reagent stock was prepared in 100% dimethylsulfoxide and control oocytes were treated with the appropriate concentration of vehicle alone. After a 15 min incubation period, the oocytes were washed 4x in Barth’s saline and then used for the determination of \(^{3}H\)-2-deoxyglucose uptake (50 µM, 30 min at 22°C).

Specific Activity Determinations—Membranes were prepared 3 days following injection of 50 ng of mutant RNA per oocyte. Western blot analysis of each of the mutant transporters was performed on ~1 µg of total membrane protein and the intensity of the Glut1 band was quantified by scanning densitometry using a Molecular Dynamics Phosphorimagier SI. Analysis was performed using the ImageQuant NT program (Version 4.0). \(^{3}H\)-2-DOG uptake (pmol/oocyte/30 minutes) of each mutant was concomitantly determined in each set of experiments. Specific activity is expressed as the 2-deoxyglucose uptake per ng of mutant Glut1 protein expressed per µg of total oocyte membrane protein, and the data were then normalized by assigning the uptake activity of the parental C-less protein a value of 1.0. Purified human erythrocyte membranes were loaded on the same gels as the oocyte membrane samples for use as quantitative standards.

Statistical Analysis—Uptake data were analyzed for statistical significance using the two-tailed, unpaired Student T-test.
RESULTS

Mutation of all 6 native cysteine residues of human Glut1 to either serine or glycine residues (C-less Glut1) has no discernable effect on transport activity (28,29). C-less Glut1 is robustly expressed at the plasma membrane, and thus none of the native Glut1 cysteine residues is required for correct subcellular targeting, structural integrity, or transport activity when expressed in Xenopus oocytes (29). C-less Glut1 was used to produce 21 mutant transporters (single-C mutants) in which each amino acid residue within predicted transmembrane helix 12 was individually changed to a cysteine residue (see Table 1). All of the mutated cDNAs were sequenced to confirm the presence of the desired mutation as well as the absence of spurious mutations within the coding region.

Stage 5 Xenopus laevis oocytes were injected with mRNAs encoding each of the 21 single C-mutants. In order to confirm that the mutants were expressed in the plasma membrane, frozen sections of the injected oocytes were subjected to indirect immunofluorescence laser confocal microscopy using a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the C-terminal 15 residues of Glut1 (Figure 1a). All 21 of the single-C mutants were clearly expressed in the oocyte plasma membrane with efficiencies similar to that of the parental C-less protein. Expression levels for all 21 mutants were then quantitated by immunoblot analysis of purified oocyte membranes (Figure 1b). In Xenopus oocytes, Glut1 appears as two broad bands by SDS-PAGE, the lower band being an incompletely glycosylated form of the protein present in early Golgi compartments, and the upper band representing the fully glycosylated form of the protein that has largely reached the plasma membrane (23). As we have previously shown for other Glut1 transmembrane helices, the expression levels of the individual helix 12 single-C mutants varied somewhat, necessitating that transport data be normalized to the membrane content of mutant protein in order to directly compare the catalytic activities of the mutants to that of their C-less parent. Only the upper bands corresponding to the fully glycosylated mutants were quantitated for this purpose.

All 21 of the single-C mutants exhibited transport activities similar to that of the C-less parent as determined by the uptake of [3H]-2-deoxyglucose. The raw uptake data are shown in Figure 2a and the specific transport activities, normalized to the membrane content of each mutant, are presented in Figure 2b. Strikingly, the helix 12 residues were insensitive to cysteine substitution with regard to inhibition of transport activity compared to the residues comprising all 9 of the other Glut1 helices examined thus far. None of the 21 single-C mutants exhibited transport activity that was statistically less than that of C-less. Cysteine substitution at Thr448 resulted in a ~50% stimulation of transport activity.

Transport activity was then measured after incubation in the presence of the sulfhydrol-specific, membrane-impermeant reagent, p-chloromercuribenzenesulfonate (pCMBS) (Figure 3), in order to determine if any of the residues are exposed to the external solvent. We previously showed that pCMBS has very close access to the exofacial sugar-binding site (29). The V165C mutant represents a positive control for reaction with pCMBS (14,29). Figure 3 shows the transport activities observed in the presence of pCMBS normalized for each single-C mutant to the activity measured in the absence of the reagent, i.e., a value of 1 indicates no effect of pCMBS, values greater than 1 indicate stimulation by pCMBS, and values of less than 1 indicate inhibition by pCMBS. Surprisingly, 5 consecutive residues near the predicted exofacial end of the helix demonstrated significant sensitivity to pCMBS and thus must be exposed to the external solvent (see Figure 4).

DISCUSSION

Helix 12 is by far the most hydrophobic of all 12 Glut1 transmembrane helices, which is consistent with its proposed role as an outer stabilizing helix that is largely in contact with lipid as opposed to lining an exofacial aqueous cavity. Our observation that none of the helix 12 amino acid side chains is critical for transport activity is consistent with the hypothesis that this helix is not directly involved in substrate binding or transporter conformational changes. In contrast, all 8 proposed inner helices possess multiple residues that are sensitive to cysteine substitu-
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...with regard to transport activity, including helices 1 (12), 2 (13), 4, (22) S (14), 7 (13,15), 8 (16), 10 (17), and 11 (18). One mutant, T448C, exhibited a 50% increase in specific transport activity relative to the C-less parent. This residue lies near the predicted cytoplasmic face of helix 12. The reason for this increase is not apparent based on our current knowledge of Glut1 structure, but a similar increase has been observed for several other single-C mutants in other transmembrane helices that have been analyzed by scanning mutagenesis.

Surprisingly, however, the predicted exofacial end of helix 12 appears to be completely immersed in the external solvent (see Figure 4). There are at least 3 possible explanations for this observation. First, these residues may not reside in the transmembrane helical bundle per se, but may instead comprise part of the last exofacial loop of Glut1 in the exofacial conformation, or the end of this helix may extend into the exoplasm in the exofacial conformation. A second possibility is that helix 12 is in fact an “inner” helix in the exofacial configuration of the transporter. This possibility seems unlikely given the complete lack of effect of cysteine substitution on transport activity for residues within this helix. A third possibility is that the central region and cytoplasmic end of helix 12 lie outside the inner helical bundle, but that the helix is kinked in such a way that the exoplasmic end twists into the aqueous cavity.

If this latter possibility is correct, the exoplasmic end of helix 12 likely lies in the vestibule to the aqueous cavity and likely plays no role in substrate binding or conformation changes, given the lack of effect of the cysteine mutations.

An updated model for the exofacial binding site of Glut1 that summarizes all of the scanning mutagenesis and SCAM data to date is shown in Figure 5. Note that the orientations of helices 4 and 12 are arbitrary because of the lack of periodicity in their pCMBS sensitivities. Even though helix 4 is shown as an “inner” helix in this simple diagram, it is possible that it lies primarily outside of the inner bundle in the exofacial orientation (22). One caveat that should be considered concerning these studies is that it is uncertain as to whether the biophysical properties of Glut proteins are identical in native mammalian cells versus Xenopus oocytes. Molecular modeling of the endofacial conformation of Glut1 based on homology and energy minimization (30) suggests that this mammalian transporter shares much structural similarity to the Lac permease (20) and glycerol-3-P antiporter (21). However, most of the experimental data for Glut1 pertain to the exofacial conformation, and it will be most interesting to see high resolution structures for one or more members of the MFS to see how well the experimental data match the crystal structure.
Table 1

**Cysteine Scanning Mutagenesis of Helix 12**
cDNA encoding cysteine-less human Glut1 was subjected to oligonucleotide-mediated, site-directed mutagenesis, creating a series of 21 mutant cDNAs in which each of the 21 residues within transmembrane helix 12 was individually changed to cysteine. Residue # refers to the amino acid numbering for human Glut1 given in reference (9). Amino acids are designated by the three-letter code.

| Residue # | Amino Acid Change | Codon Change |
|-----------|-------------------|--------------|
| 430       | Gly → Cys         | GGT → TGT    |
| 431       | Pro → Cys         | CCC → TGC    |
| 432       | Tyr → Cys         | TAC → TGC    |
| 433       | Val → Cys         | GTC → TGC    |
| 434       | Phe → Cys         | TTC → TGC    |
| 435       | Ile → Cys         | ATC → TGC    |
| 436       | Ile → Cys         | ATC → TGC    |
| 437       | Phe → Cys         | TTC → TGC    |
| 438       | Thr → Cys         | ACT → TGT    |
| 439       | Val → Cys         | GTG → TGC    |
| 440       | Leu → Cys         | CTC → TGC    |
| 441       | Leu → Cys         | CTG → TGC    |
| 442       | Val → Cys         | GTT → TGT    |
| 443       | Leu → Cys         | CTG → TGC    |
| 444       | Phe → Cys         | TTC → TGC    |
| 445       | Phe → Cys         | TTC → TGC    |
| 446       | Ile → Cys         | ATC → TGC    |
| 447       | Phe → Cys         | TTC → TGC    |
| 448       | Thr → Cys         | ACC → TGC    |
| 449       | Tyr → Cys         | TAC → TGC    |
| 450       | Phe → Cys         | TTC → TGC    |

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FOOTNOTES

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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, single-C, a Glut1 mutant constructed using the C-less parent in which a single cysteine mutation was introduced in place of one the transmembrane residues.

FIGURE LEGENDS

FIG. 1. Expression of helix 12 single-C mutant transporters in Xenopus oocytes. Stage 5 Xenopus oocytes were injected with 50 ng of wild-type, C-less, or mutant C-less mRNAs and two days later frozen.
sections were prepared and analyzed by indirect immunofluorescence laser confocal microscopy or oocytes were used to prepare purified membrane fractions for immunoblot analysis. a) Confocal Micrographs of oocytes expressing each of the 21 single-C mutants; b) Immunoblot: 10 µg of total oocyte membrane protein were loaded per lane. Rabbit antiserum A674 raised against the C-terminal 15 residues of human Glut1 was used at 1:500 dilution. Numbers above the lanes on the right represent the quantity of human erythrocyte Glut1 loaded in each lane as quantitative standards.

FIG. 2. 2-Deoxyglucose uptake activity of helix 12 single-C mutants. [3H]-2-deoxyglucose uptake (50 µM, 30 min at 22 °C) and the plasma membrane content of each single-C mutant were quantitated 2 days after injection of mRNAs. Results represent the mean ± SE of 6-8 independent experiments, each experiment using 15-20 oocytes per experimental group. a) raw uptake data; b) the data are normalized per ng of each mutant protein expressed per 10 µg of total oocyte membrane. Background values observed in sham-injected oocytes were subtracted prior to normalization. *, p < 0.01 for single-C mutants compared to parental C-less Glut1.

FIG. 3. Effect of pCMBS on transport activity of helix 12 single-C mutants. 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 at 22 °C for 15 min. Oocytes were washed 4x in Barth’s saline and then subjected to 2-deoxyglucose uptake measurements under the conditions described in the legend to Fig. 2. Results represent the mean ± SE of 4-14 independent experiments, each experiment using 15-20 oocytes per experimental group. Data are expressed as relative uptake activity, i.e., uptake observed in the presence of pCMBS divided by the uptake observed in the absence of pCMBS. C-less represents the parental cysteine-less Glut1 construct. V165C is a well-characterized positive control whose activity is inhibited by pCMBS (29).

FIG. 4. Helical wheel representation of helix 12. Transmembrane helix 12 of Glut1 as viewed from the exoplasmic surface of the plasma membrane. Amino acids are represented by the single letter code. The black arrows point to the 5 consecutive residues near the predicted exofacial end of the helix that are sensitive to inhibition by pCMBS.

FIG. 5. 2-Dimensional model for the arrangement of the 12 transmembrane helices of Glut1 and the exofacial sugar-binding site. Proposed low-resolution model of the exofacial glucose-binding site as viewed from the exoplasmic face of the membrane. For simplicity all transmembrane segments are drawn as perfect helices perpendicular to the plane of the membrane. The model is consistent with numerous experimental observations (see text and reference (16) for discussion). Glucose is not drawn to scale. The dotted lines represent possible hydrogen bonds formed between glucose hydroxyl groups and various side chains on Glut1. Numbered residues are accessible to pCMBS from the external solvent. The helices shown in gray have not yet been analyzed by scanning mutagenesis. The relative orientations of helices 4 and 12 are arbitrary because of the lack of periodicity in sensitivity to pCMBS.
Transmembrane Segment 12 of the Glut1 Glucose Transporter

Figure 1a

Sham  C-Less  G430C  P431C  Y432C
V433C  F434C  I435C  I436C  F437C
T438C  V439C  L440C  L441C  V442C
L443C  F444C  F445C  I446C  F447C
T448C  Y449C  F450C
Figure 1b
Figure 2a
Transmembrane Segment 12 of the Glut1 Glucose Transporter

Figure 2b
Figure 3

Transmembrane Segment 12 of the Glut1 Glucose Transporter

pCMBS Sensitivity Relative to Untreated Control
Figure 4
Figure 5
Transmembrane segment 12 of the Glut1 glucose transporter is an outer helix and is not directly involved in the transport mechanism

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