Cysteine-Scanning Mutagenesis of Transmembrane Segment 11 of the GLUT1 Facilitative Glucose Transporter†

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ABSTRACT: The glucose permeation pathway within the GLUT1 facilitative glucose transporter is hypothesized to be formed by the juxtaposition of the hydrophilic faces of several transmembrane α-helices. The role of transmembrane segment 11 in forming a portion of this central aqueous channel was investigated using cysteine-scanning mutagenesis in conjunction with sulfhydryl-directed chemical modification. Each of the amino acid residues within transmembrane segment 11 were individually mutated to cysteine in an engineered GLUT1 molecule devoid of all native cysteines (C-less). Measurement of 2-deoxyglucose uptake in a *Xenopus* oocyte expression system revealed that all of these mutants retain measurable transport activity. Four of the cysteine mutants (N411, W412, N415, and F422) had significantly reduced specific activity relative to the C-less protein. Specific activity was increased in five of the mutants (A402, A405, V406, F416, and M420). The solvent accessibility and relative orientation of the residues to the glucose permeation pathway were investigated by determining the sensitivity of the mutant transporters to inhibition by the sulfhydryl-directed reagent *p*-chloromercuribenzenesulfonate (pCMBS). Cysteine replacement at five positions (I404, G408, F416, G419, and M420) produced transporters that were inhibited by incubation with extracellular pCMBS. All of these residues cluster along a single face of the α-helix within the regions showing altered specific activities. These data demonstrate that the exofacial portion of transmembrane segment 11 is accessible to the external solvent and provide evidence for the positioning of this α-helix within or near the glucose permeation pathway.

The facilitative transport of glucose across mammalian cell membranes is mediated by a family of at least four highly homologous 50−60 kDa transmembrane glycoproteins (*1*, *2*). Within the GLUT protein family, GLUT1 has been most extensively studied because of its relative abundance within the erythrocyte cell membrane. It remains the only glucose transporter to have been purified and reconstituted into lipid vesicles in a functional form (*3*). Extensive kinetic analysis of GLUT1-mediated transport in human erythrocytes has largely supported a simple alternating conformational model for facilitative transport (*4*), although alternate mechanistic models have been proposed (*1*, *5*). Several amino acid residues that likely play crucial roles in glucose binding and/or transport have been identified from affinity labeling studies and limited site-directed mutagenesis (*6*−*8*). The tertiary structure of the GLUT proteins, however, remains poorly characterized. This is due in part to the inherent difficulties in obtaining diffraction-quality crystals of transmembrane proteins.

A 12 transmembrane-spanning α-helical model for the structure of the GLUT proteins was first proposed by Mueckler et al. (*9*) in 1985 based upon hydropathy plot analysis of the GLUT1 primary sequence. This membrane topology has been confirmed by enzymatic (*10*), immunologic (*11*), and glycosylation-scanning mutagenesis analysis (*12*). Recognition that five of the putative transmembrane helices (numbers 3, 5, 7, 8, and 11) are capable of forming amphipathic helices has led to the hypothesis that these helices are arranged together to form an aqueous pathway that the glucose molecule traverses through the cell membrane (*9*). This is supported by hydrogen exchange experiments, which revealed that 80% of the polypeptide backbone is solvent-accessible (*13*).

Support for the participation of TM[1] segments 5 and 7 in forming part of this aqueous channel has recently been obtained using cysteine-scanning mutagenesis in conjunction with sulfhydryl-directed chemical modification (*14*, *15*). Molecular modeling of GLUT1 has suggested that at least five transmembrane α-helices would be required to form an aqueous channel of sufficient size to accommodate the glucose moiety (*16*). The identity of the remaining TM segments participating in the formation of this channel has yet to be determined. Within TM segment 11, mutations of W412 have been previously shown to markedly disrupt

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[1] Abbreviations: pCMBS, *p*-chloromercuribenzenesulfonate; C-less, GLUT1 in which all six native cysteine residues were changed to either glycine or serine; 2-DOG, 2-deoxyglucose; TM, transmembrane.

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Table 1: Cysteine-Scanning Mutagenesis of TM Segment 11[a]

residue	amino acid change change	mutagenic oligonucleotide
402	Ala→Cys	GGTCCACGTCCA**TGT**GCCATTGCCGTTGCA
403	Ala→Cys	CCACGTCCAGCT**TGC**ATTGCCGTTGCAGGC
404	Ile→Cys	CGTCCAGCTGCC**TGT**GCCGTTGCAGGGTTC
405	Ala→Cys	CCAGCTGCCATT**TGC**GTTGCAGGCTTCTCC
406	Val→Cys	GCTGCCATTGCC**TGT**GCAGGCTTCTCCAAC
407	Ala→Cys	GCCATTGCCGTT**TGT**GGCTTCTCCAACTGG
408	Gly→Cys	ATTGCCGTTGCA**TGC**TTCTCCAACTGGACC
409	Phe→Cys	GCCGTTGCAGGC**TGC**TCCAACTGGACCTCA
410	Ser→Cys	GTTGCAGGCTTC**TGC**AACTGGACCTCAAAT
411	Asn→Cys	GCAGGCTTCTCC**TGC**TGGACCTCAAATTTC
412	Trp→Cys	GGCTTCTCCAAC**TGC**ACCTCAAATTTCATT
413	Thr→Cys	TTCTCCAACTGG**TGC**TCAAATTTCATTGTG
414	Ser→Cys	TCCAACTGGACC**TGT**AATTTCATTGTGGGC
415	Asn→Cys	AACTGGACCTCA**TGT**TTCATTGTGGGCATG
416	Phe→Cys	TGGACCTCAAAT**TGC**ATTGTGGGCATGGGC
417	Ile→Cys	ACCTCAAATTTC**TGT**GTGGGCATGGGCTTC
418	Val→Cys	TCAAATTTCATT**TGC**GGCATGGGCTTCCAG
419	Gly→Cys	AATTTCATTGTG**TGC**ATGGGCTTCCAGTAT
420	Met→Cys	TTCATTGTGGGC**TGC**GGCTTCCAGTATGTG
421	Gly→Cys	ATTGTGGGCATG**TGC**TTCCAGTATGTGGAG
422	Phe→Cys	GTCGGCATGGGC**TGC**CAGTATGTGGAGCAA

[a] Oligonucleotide-mediated site-directed mutagenesis was performed on cDNA encoding cysteine-less human GLUT1 to introduce a single cysteine at each position within putative transmembrane helix 11. Residue refers to the amino acid numbering for human GLUT1 given in ref *9*. Amino acid residues are designated by their three-letter code. Mutated codons within each oligonucleotide are shown in boldface type with the mutated bases underlined.

transport activity (*8, 17*). Furthermore, this TM segment is capable of forming an α-helix with moderate amphipathicity. This suggested that TM segment 11 may also contribute to the formation of the glucose permeation pathway. To investigate the functional importance, solvent accessibility, and relative orientation of TM segment 11 with respect to this aqueous glucose channel, we performed cysteine-scanning mutagenesis and measured the sensitivity of each of these mutants to modification by the sulfhydryl-directed reagent pCMBS. The presented data support the participation of TM segment 11 in forming a portion of the aqueous glucose permeation pathway in GLUT1.

MATERIALS AND METHODS

Materials. *Xenopus laevis* imported African frogs were purchased from Xenopus Express (Homosassa, FL), [^{3}H]-2-deoxyglucose was obtained from Sigma (St. Louis, MO), diguanosine triphosphate (mRNA cap) was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL), Megascript RNA synthesis kit was purchased from Ambion Inc. (Austin, TX), and Transformer Site-Directed mutagenesis kit was obtained from Clontech (Palo Alto, CA).

General Procedures. Mutant transporters were constructed using the Transformer Site-Directed mutagenesis kit as previously described (*18*). Procedures for the synthesis and purification of mutant RNA (*19*); isolation, microinjection, and incubation of *Xenopus* oocytes (*20*); preparation of total oocyte membranes and laser confocal microscopy (*8*); SDS−polyacrylamide gel electrophoresis and immunoblotting with GLUT1 C-terminal antibody (*7*); and 2-deoxyglucose uptake measurements (*20*) have all been previously described in detail.

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 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**.** The cysteine mutant of V165, which has previously been shown to be sensitive to pCMBS

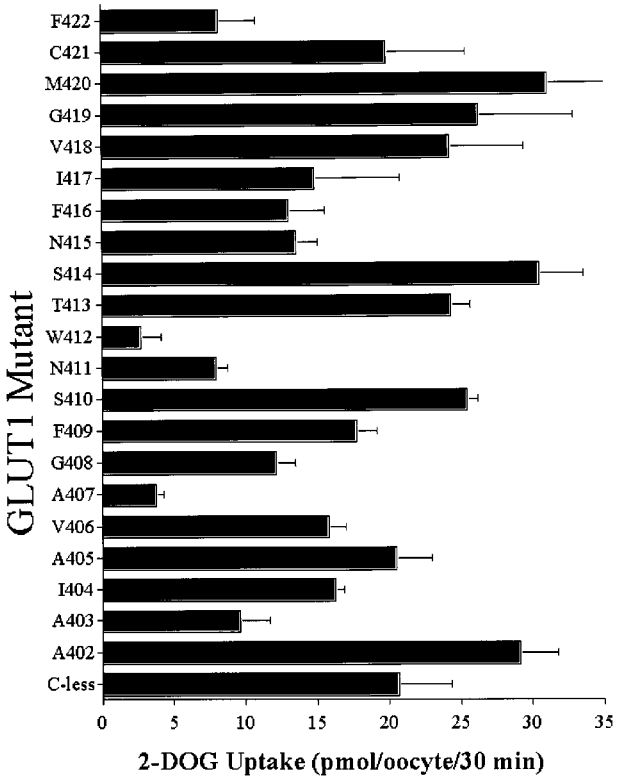

FIGURE 1: 2-Deoxyglucose uptake activity of GLUT1 cysteine mutants. [^{3}H]-2-Deoxyglucose uptake (50 μM, 30 min at 22 °C) was measured 2 days after injection of 50 ng of C-less or mutant mRNA into stage 5 *Xenopus* oocytes. Results represent the mean of 3−5 independent experiments, each using 10−20 oocytes per experimental group. C-less mRNA and sham water injected oocytes served as positive and negative controls in each experiment.

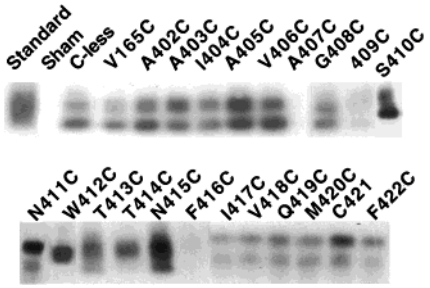

FIGURE 2: Expression of mutant GLUT 1 transporter proteins in *Xenopus* oocytes. Stage 5 *Xenopus* oocytes were injected with 50 ng of C-less or mutant C-less mRNAs. After incubation of the transfected oocytes in Barth's saline at 18 °C for 2 days, total membrane fractions were prepared for immunoblot analysis. Ten micrograms of total oocyte membrane protein was loaded per lane. Rabbit antiserum A674 raised against the C-terminal 15 residues of human GLUT1 was used at 1:500 dilution. The standard represents ~10 ng of GLUT1 protein from human erythrocyte ghosts.

treatment (*21*), served as a positive control. After a 15 min incubation period, the oocytes were washed 4 times 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. Total membranes were prepared 3 days following injection on 50 ng of mutant RNA per oocyte. Western blot analysis of each of the mutant transporters was performed on 10 μg of total membrane protein, and the intensity of the upper fully glycosylated GLUT1 band was quantified by scanning densitometry using a Molecular Dynamics Phosphorimager SI. Analysis was performed using the ImageQuant NT program (Version 4.0). Protein levels were normalized to the C-less GLUT1 control. [^{3}H]-2-DOG uptake (pmol/oocyte/30 min) of each mutant was concomitantly determined in each set of experiments and also normalized to C-less 2-DOG uptake. Specific activity was calculated by dividing the relative 2-deoxyglucose uptake by the relative amount of mature GLUT1 protein (compared to the C-less transporter).

RESULTS

Construction of Cysteine Mutants and Expression in Xenopus Oocytes. Using a previously constructed GLUT1 molecule devoid of all native cysteines (C-less) (*21*), we individually replaced each of the amino acid residues within putative transmembrane helix 11 of human GLUT1 with cysteine. The specific nucleotide substitutions made and the resulting amino acid changes are shown in Table 1. The C-less transporter has been previously shown to facilitate 2-deoxyglucose (2-DOG) uptake at levels comparable to the wild-type protein (*22*), demonstrating that none of the native cysteines are required for transport activity. As shown in Figure 1, measurable 2-DOG uptake activity in *Xenopus* oocytes was observed for each of the 21 individual cysteine mutants. The rate of 2-DOG uptake, however, varied considerably between mutants. In agreement with the previously reported sensitivity of W412 to mutagenesis, the

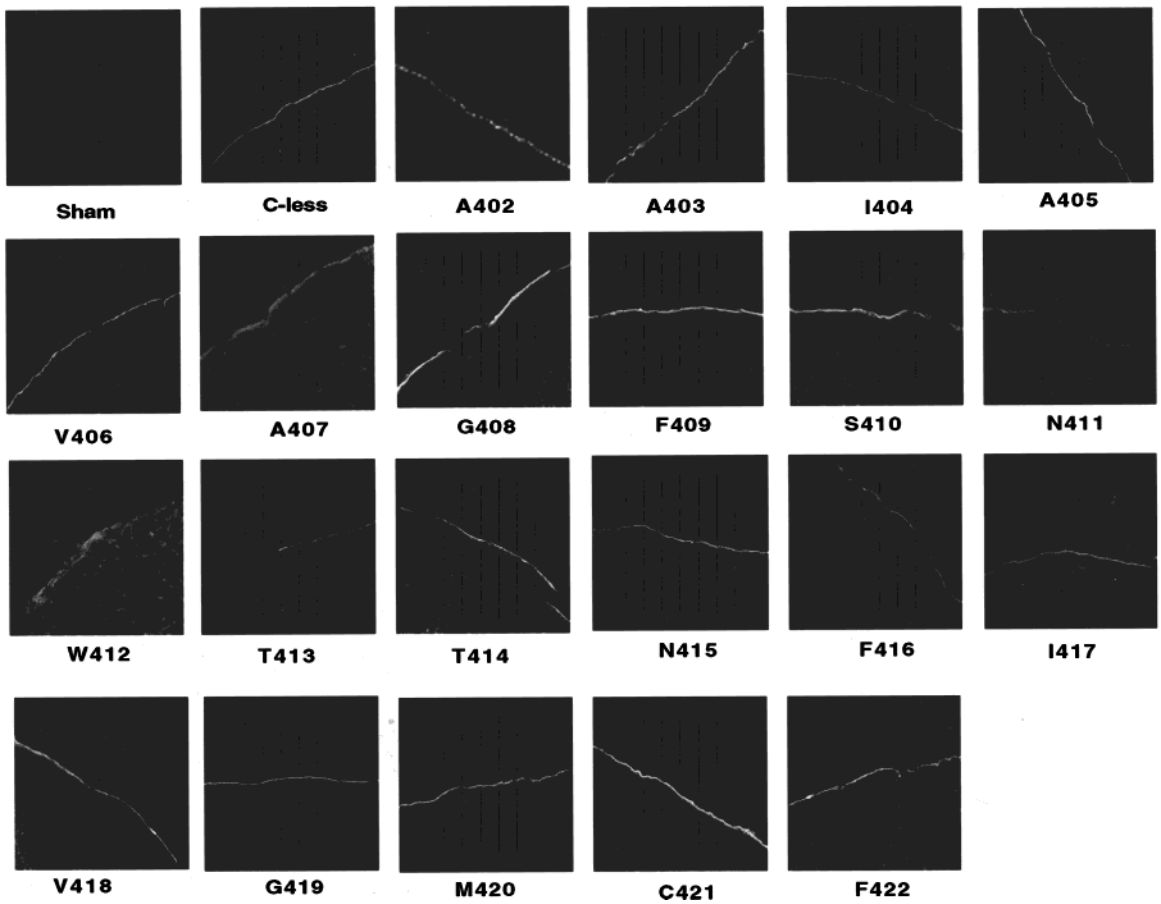

FIGURE 3: Localization of mutant GLUT1 transporter proteins in *Xenopus* oocytes. Two days after injection of stage 5 *Xenopus* oocytes with 50 ng of C-less or mutant C-less mRNAs, oocytes were frozen, sectioned, and subjected to indirect immunofluorescence laser confocal microscopy using rabbit antiserum A674 at a dilution of 1:250. Background staining in water-injected oocytes is shown in the sham-labeled micrograph.

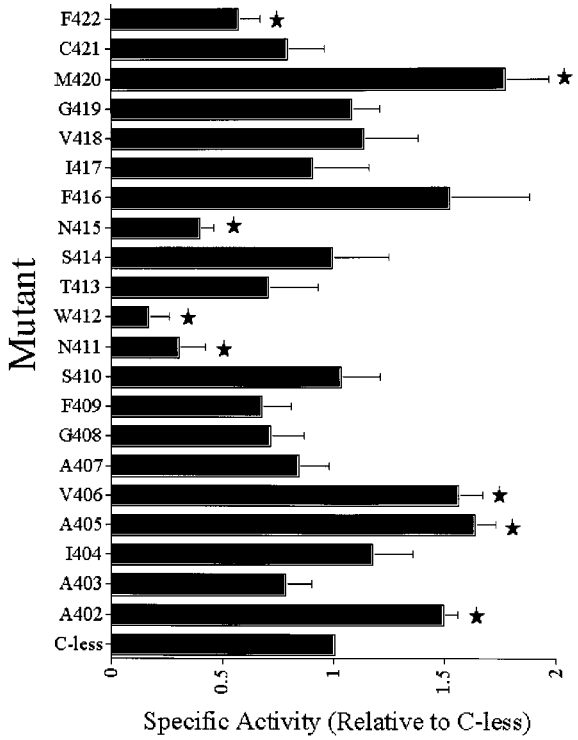

FIGURE 4: Relative specific activities of GLUT1 cysteine mutants. The upper GLUT1 band from total membrane western blots of each of the cysteine mutants was quantified by scanning densitometry as described under Materials and Methods. Results were normalized to the intensity of the C-less protein band. 2-DOG uptake was measured using the same batch of ooctyes used for the western blots, and activity was again normalized to C-less 2-DOG uptake activity. "Relative specific activity" is the relative activity divided by relative GLUT1 protein concentration. Each data point represents the mean ± SE of 3−5 independent experiments; ★, $p < 0.05$ for mutant vs C-less transporters.

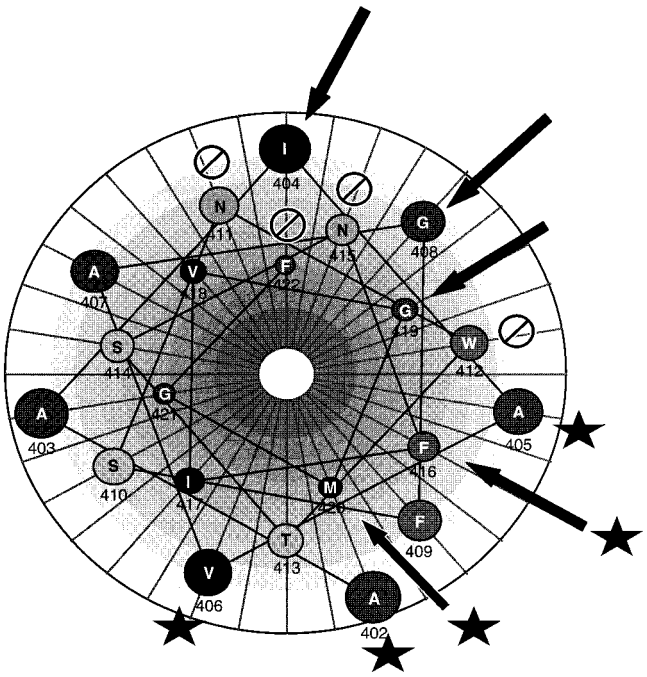

FIGURE 5: Helical wheel representation of transmembrane helix 11. Transmembrane helix 11 of GLUT1 as viewed from the cytoplasmic surface of the plasma membrane. Amino acids are represented by the single-letter code. Arrows point to residues that are accessible to pCMBS. ★ and ∅ designate significantly increased and diminished specific activity relative to C-less GLUT1, respectively.

W412C mutant exhibited less than 5% of C-less transporter 2-DOG activity. The A407 mutant also showed a greater than 95% reduction in the ability to facilitate 2-DOG uptake compared to the C-less transporter. In contrast, nearly a third of the mutants showed an increase in 2-DOG transport over C-less GLUT1.

Estimation of Mutant Specific Activities. To determine whether the observed differences in transport activity were due to changes in the intrinsic activity of the mutant proteins or simply a reflection of total protein expression, we first measured the amount of GLUT1 protein detected by western blot analysis of total membrane preparations (Figure 2). In the majority of mutants, diminished 2-DOG uptake activity relative to the C-less protein correlated with decreased steady-state protein expression. Since the introduced mutations could affect assembly and/or transport of these proteins to the cell membrane in addition to their effect on steady-state protein levels, we investigated the subcellular localization of the mutants by laser confocal microscopy (Figure 3). Immunofluorescence staining at the PM was clearly demonstrated in each of the cysteine mutants. As with the 2-DOG uptake measurements, the intensity of staining varied considerably between mutants. The two mutants with markedly diminished 2-DOG uptake activity (A407 and W412) also showed a significant amount of intracellular staining, suggesting that these mutations may affect the intracellular processing and transport of the transporters to the PM. Attempts at quantifying the amounts of GLUT1 at the PM by confocal microscopy were hindered by the significant variability in fluorescence intensity both between and within individual preparations of sectioned oocytes. Specific activities were therefore calculated using the upper protein band seen on western blot analysis as a reflection of the relative amount of functional GLUT1. This methodology has been previously shown to be a reliable estimate of the fully processed complex N-glycosylated transporter within the PM (*15*).

As shown in Figure 4, changes in relative specific activity were seen in nearly half of the cysteine mutants in TM segment 11. The specific activity of four of the mutants (N411, W412, N415, and F422) was significantly reduced ($p < 0.05$) relative to the C-less protein. All of these residues cluster along a single face of a putative α-helix (Figure 5). A significant increase in relative specific activity was seen in four mutants (A402, A405, V406, and M420). The F416 mutant also showed increased relative specific activity, although the difference from parental C-less activity was not statistically significant. The residues with increased specific activity were also found along a single portion of the α-helix, adjacent to the area with reduced specific activity.

pCMBS Modification. The solvent accessibility of the cysteine residues was investigated by measuring the effect of extracellular incubation with the sulfhydryl-directed reagent *p*-chloromercuribenzene sulfonate (pCMBS). This compound is membrane-impermeant and roughly of the same size as glucose, making it a suitable molecule to study the topology of the glucose permeation pathway (*21*). This reagent in conjunction with cysteine-scanning mutagenesis has been used previously to probe the structure of several transmembrane proteins including the lactose permease, which is a member of the facilitative transport protein

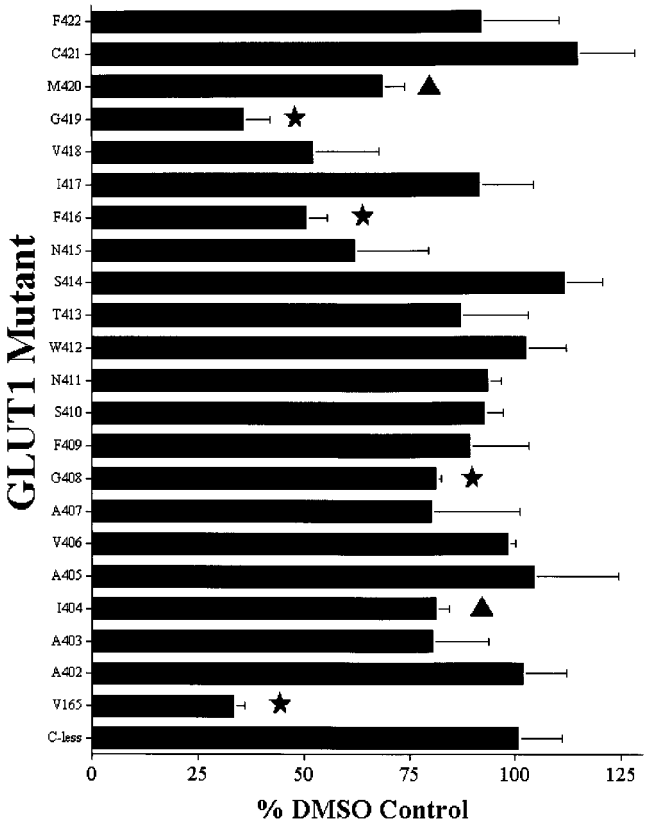

FIGURE 6: Effect of extracellular pCMBS on 2-deoxyglucose transport activity. Groups of 10−20 oocytes were incubated in Barth's saline at 22 °C in the presence or absence of 0.5 mM *p*-chloromercuribenzene sulfonate (dissolved in 100% dimethyl sulfoxide) for 15 min. Oocytes were washed 4× in Barth's saline and then subjected to [^{3}H]-2-deoxyglucose uptake measurements under the same conditions described in the legend to Figure 1. Results represent a percentage of the pCMBS-treated oocytes compared to vehicle alone within the same batch of oocytes. Each data point represents the mean ± SE of 3−5 independent experiments; ▲, $p < 0.05$; and ★, $p \leq 0.01$ for mutant vs C-less control. The V165 mutant served as a positive control in each experiment.

superfamily (*23*). Mutant transporters that are sensitive to incubation with extracellular pCMBS must be exposed to the aqueous environment. Furthermore, pCMBS sensitivity implies that the cysteine is either positioned sufficiently close to the glucose permeation pathway to inhibit glucose binding and/or transport or within a critical area of the protein such that modification results in a significant conformational change in the protein. Measurement of 2-deoxyglucose uptake in pCMBS-treated oocytes (Figure 6) revealed five residues that were pCMBS-sensitive (I404, G408, F416, G419, and M420). The three mutants with the greatest effect on 2-DOG uptake following pCMBS modification were all located within the exofacial portion of the TM segment. Although the decrease in 2-DOG uptake activity following pCMBS treatment did achieve statistical significance in the I404 and G408 mutants, the absolute decrease in 2-DOG uptake was minimal. All of the pCMBS-sensitive residues are positioned along a single face of a helical wheel plot, within the regions of the helix showing altered specific activity (Figure 5).

DISCUSSION

The identification of solvent-accessible residues along a single face of a putative α-helix within TM segment 11

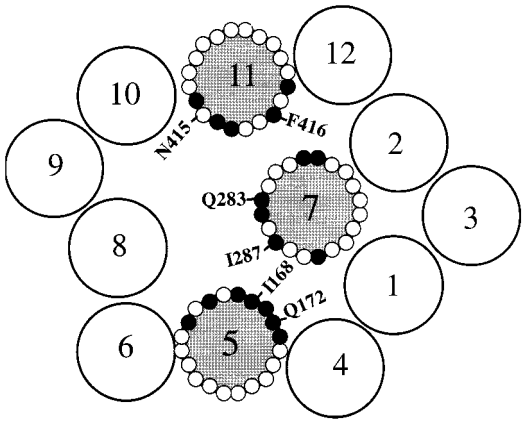

FIGURE 7: Proposed model for GLUT1 helix packing. Schematic representation of a potential arrangement of the 12 transmembrane α-helices in GLUT1 as viewed from the exofacial surface of the plasma membrane. The proposed positions of helices known to have solvent-accessible residues are shaded. ● represents cysteine residues sensitive to pCMBS modification as they appear on a helical wheel diagram. Adapted from Kaback's model for helix packing within the *E. coli* lactose permease (*23*).

supports our current model for GLUT1 tertiary structure as shown in Figure 7. The proposed arrangement of the 12 TM segments in GLUT1 is similar to the helix packing predicted for the *E. coli* lactose permease, one of the most extensively characterized of the facilitative transport proteins. The helical packing in the lactose permease has been supported by a number of experimental approaches including electron spin resonance labeling, fluorescence labeling, cysteine-scanning mutagenesis, and chemical cross-linking (*23*). Although the lactose permease shares little sequence homology with GLUT1 and facilitates the transport of the disaccharide lactose as opposed to the monosaccharide glucose, both proteins have very similar membrane topologies. A common helix packing arrangement between the lactose permease and GLUT1 would not be unreasonable. Although direct experimental evidence for the positioning of the TM segments in GLUT1 is not yet available, the results of cysteine-scanning mutagenesis on three separate TM segments provide indirect evidence regarding the orientation of these α-helices. Although TM segments 5, 7, and 11 all likely participate in the formation of the aqueous glucose permeation pathway, differences in the patterns of pCMBS sensitivity provide some clues to their positioning relative to the aqueous channel.

The relatively small number of pCMBS-sensitive residues in TM segment 11 compared to TM segments 5 and 7 suggests that a larger portion of TM segment 11 is inaccessible to solvent from the exofacial side of the membrane. Alternatively, several solvent-accessible residues in TM segment 11 may not be positioned sufficiently close to the glucose permeation pathway to inhibit transport activity following pCMBS modification. Both of these explanations would be consistent with a more peripheral positioning of this TM segment as shown in Figure 7.

Although specific activities were not determined in the cysteine-scanning mutagenesis study of TM segment 5 (*14*), residues throughout the circumference of helix 7 showed diminished specific activity following mutation to cysteine (*15*). This suggested a more central positioning of helix 7 within the aqueous channel. Our current study of TM

segment 11 reveals a pattern of specific activity changes following cysteine substitution that further supports a more peripheral positioning of this region relative to the permeation pathway (Figure 7). The location of the pCMBS-sensitive residues within the regions of the α-helix with alterations in specific activity is consistent with the positioning of this region along the border of the aqueous glucose permeation pathway. The pattern of both increased and decreased specific activities along different portions of the α-helix suggests that mutations within these regions of the protein may alter activity by causing movement of this helix relative to the aqueous channel. This may in part be due to interactions with the yet to be identified TM segments that are adjacent to helix 11 within the cell membrane.

Similar to the results seen in TM helices 5 and 7, the residues with the greatest sensitivity to pCMBS are found within the exofacial portion of the TM segment. This is entirely consistent with the alternating conformational model for facilitative glucose transport (*4*). This model predicts that the exofacial and cytoplasmic glucose binding sites are not simultaneously accessible. Extracellular pCMBS may only be capable of accessing the exofacial portion of the glucose channel. The small but statistically significant decrease in 2-DOG uptake activity in two residues within the cytoplasmic portion of the TM segment following pCMBS treatment suggests that a small amount of pCMBS may gain access to this cytoplasmic binding site. The current methodology, however, is not able to establish whether additional residues within the cytoplasmic portion of TM segment 11 participate in forming part of the glucose permeation pathway.

It has been previously suggested that W412 is involved in substrate binding (*8*). The inability of extracellular pCMBS to significantly affect the activity of the W412C mutant does not rule out this possibility. As noted above, W412 may participate in the cytoplasmic binding site and thus would be largely inaccessible to extracellular pCMBS. Alternatively, the introduction of the aromatic residue of pCMBS at this site may be able to partially substitute for the aromatic ring of typtophan. If this is the case, either the efficiency of modification at this site or steric effects of the pCMBS molecule prevent full restoration to C-less uptake activity.

Despite the absence of crystallographic data, an elementary understanding of the tertiary structure of the monosaccharide transport proteins is now beginning to emerge. The cysteine-scanning mutagenesis approach is yielding key information regarding the structural determinants directly involved in facilitative sugar transport. With continued application of the cysteine-scanning mutagenesis approach to the remainder of the GLUT1 molecule, further insights will likely be made into the molecular mechanism of facilitative glucose transport within the GLUT proteins and other members of the facilitative monosaccharide transport protein superfamily.

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