Transmembrane Segment 6 of the Glut1 Glucose Transporter Is an Outer Helix and Contains Amino Acid Side Chains Essential for Transport Activity

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Experimental data and homology modeling suggest a structure for the exofacial configuration of the Glut1 glucose transporter in which 8 transmembrane helices form an aqueous cavity in the bilayer that is stabilized by four outer helices. The role of transmembrane segment 6, predicted to be an outer helix in this model, was examined by cysteine-scanning mutagenesis and the substituted cysteine accessibility method using the membrane-impermeant, sulphydryl-specific reagent, p-chloromercuribenzenesulfonate (pCMBS). A fully functional Glut1 molecule lacking all 6 native cysteine residues was used as a template to produce a series of 21 Glut1 point mutants in which each residue along helix 6 was individually changed to cysteine. These mutants were expressed in Xenopus oocytes, and their expression levels, functional activities, and sensitivities to inhibition by pCMBS were determined. Cysteine substitutions at Leu, Pro, Gln, and Gly resulted in inhibition of activity that ranged from ~35 to ~80%. Cysteine substitutions at Leu, Ser, and Leu moderately augmented specific transport activity relative to the control. These results were dramatically different from those previously reported for helix 12, the structural cognate of helix 6 in the pseudo-symmetrical structural model, for which none of the 21 single-cysteine mutants exhibited reduced activity. Only the substitution at Leu conferred inhibition by pCMBS, suggesting that most of helix 6 is not exposed to the external solvent, consistent with its proposed role as an outer helix. These data suggest that helix 6 contains amino acid side chains that are critical for transport activity and that structurally analogous outer helices may play distinct roles in the function of membrane transporters.

Members of the GLUT protein family are responsible for the transport of glucose and other monosaccharides across mammalian cell membranes (reviewed in Refs. 1–4). Glut proteins comprise a subset of the major facilitator superfamily (MFS), members of which are involved in the transport of many different solutes across cellular membranes and are expressed in virtually every species examined (5, 6).

Glut1, the first Glut protein identified, is one of the most thoroughly characterized membrane transporters (7). It is abundantly expressed in the human erythrocyte and is the only Glut protein to have been purified to near homogeneity (8, 9). Human Glut1 cDNA was cloned over two decades ago, and the deduced amino acid sequence of the protein suggested the presence of 12 transmembrane domains (10), a feature shared by most if not all members of the MFS. The results of a glycosylation-scanning mutagenesis study (11) and other experimental observations support this structural prediction. It was suggested that several of the 12 predicted amphipathic transmembrane helices form a water-filled cavity in the membrane-buried portion of the protein that participates in substrate binding and translocation across the lipid bilayer (10). The putative transmembrane helices possess many hydrogen bond-forming amino acid side chains that are capable of forming the sugar-binding site(s) of Glut1 via interaction with glucose hydroxyl groups (10), an hypothesis supported by earlier published data (12).

Current experimental evidence is consistent with a model for the exofacial configuration of Glut1 in which transmembrane segments 1 (13), 2 (14), 5 (15), 7 (14, 16), 8 (17), 10 (18), and 11 and 12 form an inner helical bundle that comprises a water-accessible cavity within the membrane. Data suggest that transmembrane segments 3 and 12 are outer helices that stabilize the inner bundle (20, 21). This experimentally derived model is largely consistent with homology modeling of Glut1 based on the high resolution structures recently described for the lac permease (22) and glycerol-3-P antiporter (23), two bacterial members of the MFS expressed in Escherichia coli. Homology modeling of the cytoplasmic conformation 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 site near the center of the bilayer, whereas helices 3, 6, 9, and 12 are predicted to encircle this inner helical bundle (24). The available experimental evidence suggests that the exofacial half of helix 4 may not be exposed to the external aqueous compartment in the exofacial configuration (25), although it comprises an inner helix in the endofacial confor-

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1 The abbreviations used are: MFS, major facilitator superfamily; pCMBS, p-chloromercuribenzenesulfonate; glycerol-3-P, glycerol-3-phosphate.

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TABLE 1
Cysteine scanning mutagenesis of helix 6

| Residue No. | Amino acid change | Codon change |
|-------------|-------------------|--------------|
| 186         | Trp → Cys        | TGG → TGC    |
| 187         | Pro → Cys        | CCG → TGC    |
| 188         | Leu → Cys        | CTG → TGC    |
| 189         | Leu → Cys        | CTG → TGC    |
| 190         | Leu → Cys        | CTG → TGC    |
| 191         | Ser → Cys        | AGC → TGC    |
| 192         | Ile → Cys        | ATC → TGC    |
| 193         | Ile → Cys        | ATC → TGC    |
| 194         | Phe → Cys        | TTC → TGC    |
| 195         | Ile → Cys        | ATC → TGC    |
| 196         | Pro → Cys        | CCG → TGC    |
| 197         | Ala → Cys        | GCC → TGC    |
| 198         | Leu → Cys        | CTG → TGC    |
| 199         | Leu → Cys        | CTG → TGC    |
| 200         | Glu → Cys        | CAG → TGC    |
| 201         | Gly → Cys        | GGC → TGC    |
| 202         | Ile → Cys        | ATC → TGC    |
| 203         | Val → Cys        | GTC → TGC    |
| 204         | Leu → Cys        | CTG → TGC    |
| 205         | Pro → Cys        | CCG → TGC    |
| 206         | Phe → Cys        | TTC → TGC    |

mations of the bacterial transporters. It is thus possible that the cytoplasmic and exoplasmic arrangements of the inner helical bundle of MFS transporters are not identical.

Herein we present data using cysteine-scanning mutagenesis and the substituted cysteine accessibility method to explore the role of transmembrane segment 6 in the exofacial structure of Glut1. The results demonstrate that most of helix 6 is not in contact with the external solvent, suggesting that this helix lies outside of the inner helical bundle. Substitution at several different sites resulted in a decrease or abolition of transport activity, suggesting that helix 6 possesses several residues that are essential for Glut1 function.

EXPERIMENTAL PROCEDURES

Materials—Imported female African Xenopus laevis frogs were purchased from Xenopus Express (Homosassa, FL), [3H]2-deoxyglucose and diguanosine triphosphate were purchased from Amersham Biosciences, Megascript™ RNA synthesis kits were purchased from Ambion Inc (Austin, TX), and Transform-er™ site-directed mutagenesis kits were obtained from Clontech.

General Procedures—Procedures for the site-directed mutagenesis and sequencing of human Glut1 cDNA and the in vitro transcription and purification of Glut1 mRNAs (26), isolation, microinjection, and incubation of Xenopus oocytes (27), preparation of purified oocyte plasma membranes and indirect immunofluorescence laser confocal microscopy (28), SDS-polyacrylamide gel electrophoresis and immunoblotting with Glut1 C-terminal antibody (29), and 2-deoxyglucose uptake measurements (30) have been described in detail previously.

Treatment with pCMBS—Stage 5 Xenopus oocytes were injected with 50 ng of wild-type, C-less, or mutant C-less mRNAs, and 2 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. Sham, sham-injected oocytes, 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 amount in ng of human erythrocyte Glut1 loaded in each lane as quantitative standards.

FIGURE 1. Expression of helix 6 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 2 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. Sham, sham-injected oocytes, 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 amount in ng of human erythrocyte Glut1 loaded in each lane as quantitative standards.
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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 GE Healthcare PhosphorImager SI. Analysis was performed using the ImageQuant NT program (Version 4.0). [3H]2-Deoxyglucose uptake (pmol/oocyte/30 min) 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 Glu1 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. In some cases (L190C, I192C, I195C, P196C, A197C), where unexpectedly elevated specific activities were initially measured for a particular mutant, usually in conjunction with greatly reduced levels of mutant protein expression relative to C-less Glu1, expression levels of C-less Glu1 and the mutant were titrated by injecting variable amounts of the corresponding mRNAs. The relative specific activity was then determined based on the calculations obtained at similar levels of protein expression.

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

RESULTS

A mutant Glu1 molecule in which all 6 native cysteine residues have been changed to either serine or glycine residues (C-less Glu1) has been previously characterized (31, 32). When expressed in Xenopus oocytes, the behavior and function of C-less Glu1 is indiscernible from the wild-type transporter. Thus, none of the 6 native Glu1 cysteine residues appear to be required for correct subcellular trafficking, stability, structural integrity, or transport activity (32). This parental C-less Glu1

FIGURE 2. 2-Deoxyglucose (2-DOG) uptake activity of helix 6 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 ± S.E. of 6–8 independent experiments, each experiment employing 15–20 oocytes per experimental group. a, raw uptake data (*, p < 0.01 for single-C mutants compared with parental C-less Glu1); b, the data were normalized per ng of each mutant protein expressed per 10 μg of total oocyte membrane (*, p < 0.05 for single-C mutants compared with parental C-less Glu1). Background values observed in sham-injected oocytes were subtracted prior to normalization.

3 C-less refers to a Glu1 molecule in which all 6 native cysteine residues were changed to either glycine or serine, and single-C, a Glu1 mutant construct using the C-less parent in which a single cysteine mutation was introduced in place of one of the transmembrane residues.
was used as a template to produce a series of 21 mutant transporters (single-C mutants) in which each amino acid residue within predicted transmembrane helix 6 was changed to a cysteine residue (Table 1). All of the mutant cDNAs were sequenced to verify the presence of the desired mutation and to confirm the absence of spurious mutations at undesired sites.

mRNAs encoding each of the single-C mutants were synthesized and then injected into stage 5 *Xenopus* oocytes. Indirect immunofluorescence laser confocal microscopy of frozen sections revealed that each of the mutants was expressed in the oocyte plasma membrane, although as observed previously during the analysis of other Glut1 helices, the expression levels varied considerably among the various mutants (Fig. 1a). This necessitated that the expression level of each mutant in the membrane be quantitated by immunoblot analysis of purified membrane preparations to calculate and directly compare their specific transport activities (Fig. 1b). When expressed in *Xenopus* oocytes, Glut1 protein appears as two broad bands by SDS-PAGE, the lower band being incompletely glycosylated forms of the protein present in the endoplasmic reticulum and in early Golgi compartments, and the upper band representing the fully glycosylated form of the protein that has largely reached the plasma membrane (26). Only the upper bands corresponding to the fully glycosylated proteins were used for quantitation.

The raw uptake data are shown in Fig. 2a, and the specific transport activities, normalized to the membrane content of each mutant, are presented in Fig. 2b. We have previously observed that specific activity measurements can vary depending on the level of protein expression achieved (28, 29). This has only been observed in a few instances with single-C mutants when increased relative specific activity of a mutant is initially calculated relative to the C-less control in conjunction with reduced levels of mutant protein expression relative to the control. This likely is a result of some inaccuracy in the quantification of the low level of mutant protein expression, which appears in the denominator of the specific activity calculation. In these instances, normalization is conducted by titrating the amount of mutant and control mRNAs that are injected to increase expression of the mutant and/or decrease expression of the control C-less protein. Relative specific activities are then determined at similar levels of expression of the mutant and control proteins. For helix 6, this titration procedure was conducted for L190C, I192C, I195C, P196C, and A197C. The immunoblot data in Fig. 1b represent relative expression levels obtained when equal amounts (50 ng) of the various mRNAs were injected.

Both the raw uptake values and the normalized specific transport activities differed between the control and the mutants in many cases. Cysteine substitutions at Leu204 and Pro205 abolished transport activity, whereas substitutions at Ile192, Pro196, Gln200, and Gly201 resulted in inhibition of activ-
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Figure 5. Low resolution model for the arrangement of the 12 transmembrane helices of Glut1. A proposed model of the exofacial glucose-binding site as viewed from the outside of the cell is shown. For simplicity, all transmembrane segments are drawn as perfect helices perpendicular to the plane of the membrane. 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. Helix 9 is shown in gray because it has not yet been analyzed by scanning mutagenesis.

pCMBS-mediated transport inhibition (Fig. 4). Note that W186C, L204C, and P205C could not be reliably analyzed by this method because of their extremely low transport activities. An attempt was made to measure the effect of pCMBS on the activity of W186C, but the standard error remained large for activity in the presence of pCMBS after 8 independent experiments involving ~160 oocytes. Although no statistically significant difference was observed in the activity of this mutant in the presence or absence of pCMBS, the very low specific transport activity and large standard error prevented us from reaching a definitive conclusion. Therefore, an “ND” designation is indicated in Fig. 4 for W186C.

DISCUSSION

Helix 6 is the structural cognate of helix 12 in the pseudo-2-fold symmetrical structure proposed for Glut1, in analogy to the lac permease and glycerol-3-P antiporter structures. Helices 6 and 12 are the most hydrophobic of all 12 Glut1 transmembrane helices, and both are proposed to be outer helices and thus to have minimal contact with the external solvent. This is consistent with our finding that only a single residue within helix 6 appears to react with pCMBS. In contrast, seven of the eight proposed inner helices, including helices 1 (13), 2 (14), 5 (15), 7 (14, 16), 8 (17), 10 (18), and 11 (19), possess multiple sites that are pCMBS-sensitive with regard to transport activity after cysteine substitution.

Helix 6 possesses 6 residues that are sensitive to cysteine substitution with respect to decreased transport activity. Helix 12 has no such residues. This observation suggests that helix 6, unlike helix 12, plays an active role in the transport mechanism. In fact, Leu<sup>204</sup> and Pro<sup>205</sup> are 2 of the most sensitive residues in this regard that we have thus far encountered. Interestingly, these 2 residues lie adjacent to one another near the cytoplasmic end of the helix, and 5 of the 6 sites where inhibition was observed lie in the cytoplasmic half of helix 6. Because none of the residues in this region of helix 6 appear to be accessible to solvent and because helix 6 is clearly an outer helix in the lac permease and glycerol-3-P antiporter structures, it is unlikely that any of the helix 6 residues are involved in substrate binding. Additionally, of the 6 residues whose cysteine substitution inhibits specific transport activity, only Gln<sup>200</sup> is capable of forming hydrogen bonds. Rather, it seems likely that the cytoplasmic end of helix 6 is involved in one or more conforma-
tional changes associated with the transport cycle. The data are consistent with helix 6 lying outside the inner helical bundle with multiple residues near its cytoplasmic end in contact with those in inner helices 1 and 5 (Fig. 5). The dramatically different sensitivities of helices 6 (this report) and 12 (21) to scanning mutagenesis also implies that two structurally homologous helices in Glut1 may play completely distinct roles in the structure and function of the molecule.

An updated simplified model for the helical bundling and exofacial binding site of Glut1 that incorporates all of the scanning mutagenesis and substituted cysteine accessibility method data is shown in Fig. 5. Note that the orientations of helices 4 and 12 are arbitrary because of the absence of reactivity in the former case or lack of periodicity in pCMBS sensitivity in the latter. Also, although helix 4 is shown as an inner helix in this diagram, its lack of pCMBS sensitivity suggests that its exoplasmic end may lie outside of the inner bundle in the exofacial orientation (25). It should also be emphasized that it is uncertain as to whether the structural and biophysical properties of Glut proteins are identical in Xenopus oocytes and in native mammalian cells.

Additionally, it is possible that the behavior of mutants constructed in an altered C-less parent molecule may not reflect the behavior of the same mutations introduced into a wild-type background. Specifically, unknown interactions between a specific single-C mutation and the C-less parental background may be the cause, in some instances, of altered levels of mutant transporter protein expression. Molecular modeling of the endofacial conformation of Glut1 based on homology modeling and energy minimalization (24) suggests similarity in structure to the lac permease (22) and glycerol-3-P antiporter (23). The accuracy of the model proposed in Fig. 5 must ultimately be determined by high resolution spectroscopic or crystallographic analysis of purified Glut1.

REFERENCES
1. Baldwin, S. A. (1993) Biochim. Biophys. Acta 1154, 17–49
2. Mueckler, M. (1994) Eur. J. Biochem. 219, 713–725
3. Pessin, J. E., and Bell, G. I. (1992) Annu. Rev. Physiol. 54, 911–930
4. Carruthers, A. (1990) Physiol. Rev. 70, 1135–1176
5. Saier, M. H., Jr. (2000) Mol. Microbiol. 35, 699–710
6. Pao, S. S., Paulsen, I. T., and Saier, M. H., Jr. (1998) Microbiol. Mol. Biol. Rev. 62, 1–34
7. Mueckler, M., Hresko, R. C., and Sato, M. (1997) Biochem. Soc. Trans. 25, 951–954
8. Kasahara, M., and Hinkle, P. C. (1977) J. Biol. Chem. 252, 7384–7390
9. Baldwin, S. A., and Lienhard, G. E. (1989) Methods Enzymol. 174, 39–50
10. Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E., and Lodish, H. F. (1985) Science 229, 941–945
11. Hresko, R. C., Kruse, M., Strube, M., and Mueckler, M. (1994) J. Biol. Chem. 269, 20482–20488
12. Barnett, J. E. G., Holman, G. D., and Munday, K. A. (1973) Biochem. J. 131, 211–221
13. Heinzé, M., Mondon, I., and Keller, K. (2004) Biochemistry 43, 931–936
14. Olsowski, A., Mondon, I., Krause, G., and Keller, K. (2000) Biochemistry 39, 2469–2474
15. Mueckler, M., and Makepeace, C. (1999) J. Biol. Chem. 274, 10923–10926
16. Hruz, P. W., and Mueckler, M. M. (1999) J. Biol. Chem. 274, 36176–36180
17. Mueckler, M., and Makepeace, C. (2004) J. Biol. Chem. 279, 10494–10499
18. Mueckler, M., and Makepeace, C. (2002) J. Biol. Chem. 277, 3498–3503
19. Hruz, P. W., and Mueckler, M. M. (2000) Biochemistry 39, 9367–9372
20. Mueckler, M., Roach, W., and Makepeace, C. (2004) J. Biol. Chem. 279, 46876–46881
21. Mueckler, M., and Makepeace, C. (2006) J. Biol. Chem. 281, 36993–36998
22. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003) Science 301, 610–615
23. Huang, Y., Lemieux, M. J., Song, J., Auer, M., and Wang, D. N. (2003) Science 301, 616–620
24. Salas-Burgos, A., Iserovich, P., Zuniga, F., Vera, J. C., and Fischbarg, J. (2004) Biophys. J. 87, 2990–2999
25. Mueckler, M., and Makepeace, C. (2005) J. Biol. Chem. 280, 39562–39568
26. Hresko, R. C., Murata, H., Marshall, B. A., and Mueckler, M. (1994) J. Biol. Chem. 269, 32110–32119
27. Marshall, B. A., Murata, H., Hresko, R. C., and Mueckler, M. (1993) J. Biol. Chem. 268, 26193–26199
28. Garcia, J. C., Strube, M., Leingang, K., Keller, K., and Mueckler, M. M. (1992) J. Biol. Chem. 267, 7770–7776
29. Mueckler, M., Wang, W., and Kruse, M. (1994) J. Biol. Chem. 269, 20533–20538
30. Keller, K., Strube, M., and Mueckler, M. (1989) J. Biol. Chem. 264, 18884–18889
31. Wellner, M., Mondon, I., and Keller, K. (1995) FEBS Lett. 370, 19–22
32. Mueckler, M., and Makepeace, C. (1997) J. Biol. Chem. 272, 30141–30146