A Conserved Amino Acid Motif (R-X-G-R-R) in the Glut1 Glucose Transporter Is an Important Determinant of Membrane Topology*

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The Glut1 glucose transporter is one of over 300 members of the major facilitator superfamily of membrane transporters. These proteins are extremely diverse in substrate specificity and differ in their transport mechanisms. The two most common features shared by many members of this superfamily are the presence of 12 predicted transmembrane segments and an amino acid motif, R-X-G-R-R, present at equivalent positions within the cytoplasmic loops joining transmembrane segments 2–3 and 8–9. The structural and functional roles of the arginine residues within these motifs in Glut1 were investigated by expression of site-directed mutant transporters in Xenopus oocytes followed by analyses of intrinsic transport activity and the membrane topology of mutant glycosylation-scanning reporter Glut1 molecules. Substitution of lysines for the cluster of 3 arginine residues in each of the 2 cytoplasmic pentameric motifs of Glut1 revealed no absolute requirement for arginine side chains at any of the 6 positions for transport of 2-deoxyglucose. However, removal of the 3 positive charges at either site by substitution of glycines for the arginines completely abolished transport activity as the result of a local perturbation in the membrane topology in which the cytoplasmic loop was aberrantly translocated into the exoplasm along with the two flanking transmembrane segments. Substitution of lysines for the arginines had no effect on membrane topology. We conclude that the positive charges in the R-X-G-R-R motif form critical local cytoplasmic anchor points involved in determining the membrane topology of Glut1. These data provide a simple explanation for the presence of this conserved amino acid motif in hundreds of functionally diverse membrane transporters that share a common predicted membrane topology.

Two of the most distinguishing features of the major facilitator superfamily are the presence of 12 predicted transmembrane segments and the occurrence of a highly conserved pentameric amino acid motif (R-X-G-R-R, where X can be any amino acid and R may be replaced by K) that lies within the cytoplasmic loops separating transmembrane segments 2–3 and 8–9. The presence of this conserved motif at equivalent positions in the two halves of these proteins lends support to the hypothesis that the members of this superfamily arose via the tandem duplication of a primordial ancestral gene (4).

The functional role of the pentameric motif within the 2/3 loop has been examined in the Escherichia coli lac permease (5) and the E. coli metal-tetracycline/H+ antiporter (6). None of the residues within the motif appeared to be critical for active transport by the lac permease when examined individually, and only 1 of the 5 residues (Arg70) within the motif was found to be critical for function of the metal-tetracycline/H+ antiporter. The role of the pentameric motifs in transport function has not yet been investigated for any eucaryotic member of the major facilitator superfamily, and a putative role of the motifs in structure or biosynthesis has not yet been addressed for any member of the superfamily.

The Glut1 transporter provides an excellent model system to study the role of the pentameric motif in the structure and biosynthesis of a major eucaryotic facilitator. Glut1 was the first membrane transporter proposed to possess 12 transmembrane segments (1). This topological model is strongly supported by protease mapping (1, 7), site-directed mutagenesis (8), antibody reactivity (9), and detailed glycosylation-scanning mutagenesis studies (10). Site-directed mutagenesis studies have demonstrated that the amino acid charge difference across the first transmembrane segment is important in determining the orientation of the first transmembrane segment only, suggesting that other sequence information is involved in specifying the membrane topology of downstream regions of the protein (11). Because most members of the major facilitator superfamily share the same predicted 12-transmembrane segment topology and the dual pentameric amino acid motifs, one possibility is that the motifs act as critical cytoplasmic anchor points for the 12-helix topology. In the present study we tested this hypothesis by the analysis of site-directed mutants expressed in Xenopus oocytes. Our data indicate that the positive charges in the R-X-G-R-R motifs located within loops 2 and 8 of Glut1 are important in determining the proper orientation of the two cytoplasmic loops as well as each of their two flanking transmembrane segments.

EXPERIMENTAL PROCEDURES
Constitution of Mutant Glut1 Transporters—Human Glut1 cDNA was previously subcloned into the oocyte expression vector pSP64T (12). The aglyco-Glut1 construct (Asn45 mutated to Thr), into which the glycosylated exofacial domain of rat Glut4 was inserted independently into each of the putative hydrophile soluble domains using engineered Kpn1 restriction sites, was previously subcloned into either the vector...
pSP64 or pSP64T and used as a probe for determining the Glut1 topology (10). The amino acid sequence of the inserted epitope is as follows: GTNAFPKVIEGQSYNTWGLRQGPGGPSIPQGTCTLTL WAGT.

The epitope contains an N-linked glycosylation site, underlined above and is flanked by Gly-Thr, the translated amino acid sequence corresponding to the Kpol restriction site. Two positively charged residues and 2 negatively charged residues shown in bold type were mutated to glycine residues to neutralize the charges within the reporter sequence. Site-directed mutagenesis was performed using the CLONTECH Transformer site-directed mutagenesis kit (CLONTECH Laboratories, Palo Alto, CA). The sequences of the mutant transporters were verified using the Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, OH).

In Vitro Transcription—Mutant Glut1 mRNAs were synthesized from pSP64 or pSP64T cDNAs linearized at a unique restriction site in the polylinker using the MEGAscript SP6 in vitro transcription kit (Ambion, Austin, TX). The manufacturer’s conditions were modified by adding 4 mM GpppG and 0.625 mCi/ml [35S]UTP (Ambion, Austin, TX). The manufacturer’s conditions were modified by adding 4 mM GpppG and 0.625 mCi/ml [35S]UTP (Ambion, Austin, TX). The sequences of the mutant transporters were verified using the Sequenase 2.0 kit (U.S. Biochemical Corp., Cleveland, OH).

Isolation and Microinjection of Xenopus Oocytes—Ovarian lobes were resected from adult female Xenopus laevis (Nasco, Fort Atkinson, WI) anesthetized with 3-aminobenzonic acid ethyl ester (1 g/liter) in ice-cold water. The lobes were digested with 2 mg/ml type I collagenase (Worthington, Freehold, NL) in calcium-free modified Barth’s saline (MBS)1 at room temperature for 2 h. The oocytes were extensively washed with MBS supplemented with 5 g/liter bovine serum albumin, 15 mM cold methionine, and 2 mM cysteine, and then intracellular membranes were prepared as described previously (13). Membranes were solubilized in 80 µl of 1% SDS and then diluted with 1 ml of 1% SDS and 20 µl of 1% Triton X-100, 1% deoxycholate, 1% bovine serum albumin in phosphate-buffered saline, pH 7.4. Transporters were immunoprecipitated overnight at 4 °C with 1.25 µg of a monoclonal Glut1 antibody (14) (a kind gift of Dr. G. E. Lienhard, Dartmouth Medical School) precoupled to 40 µl of goat anti-mouse IgG affinity gel (Cappel, Organon Teknika, West Chester, PA). After washing the resin six times in phosphate-buffered saline, transporters were eluted with 50 µl of 1% SDS and then incubated with 1 ml of 1% Triton X-100, 1% deoxycholate, 1% bovine serum albumin in phosphate-buffered saline, pH 7.4. Transporters were immunoprecipitated overnight at 4 °C with 1.25 µg of a monoclonal Glut1 antibody (14) (a kind gift of Dr. G. E. Lienhard, Dartmouth Medical School) precoupled to 40 µl of goat anti-mouse IgG affinity gel (Cappel, Organon Teknika, West Chester, PA). After washing the resin six times in phosphate-buffered saline, transporters were eluted with 50 µl of 1% SDS. 20 µl of eluted transporters were digested with endoglycosidase H at 37 °C for 1 h with the addition of 0.5 µl of 3 mM sodium acetate, pH 5.5, and 0.3 milliunits of enzyme (Roche Molecular Biochemicals). Samples were then subjected to SDS-polyacrylamide gel electrophoresis. Polyacrylamide gels were destained, radiochemically enhanced with 1 m salicylic acid, dried, and subjected to autoradiography.

Transport Measurements—Stage 5 Xenopus oocytes were injected with 50 ng of wild-type or mutant Glut1 mRNA. 3 days after injection, groups of ~30 oocytes were used for the determination of 2-1H/deoxy-

\[ \text{Designation} \quad \text{Amino Acid Changes} \]

| Designation | Amino Acid Changes |
|-------------|-------------------|
| L2RG        | R89, R92, R93 → glycines |
| L2RK        | R89, R92, R93 → lysines |
| L8RG        | R330, R333, R334 → glycines |
| L8RK        | R330, R333, R334 → lysines |

\[ ^{1} \text{The abbreviation used is: MBS, modified Barth’s saline.} \]
The positions of the pentameric amino acid motif, R-X-G-R-R, within loops 2 and 8 of Glut1 are illustrated in Fig. 1. Four mutant Glut1 cDNAs were produced by site-directed mutagenesis in which all 3 arginines within each motif were changed to either lysine or glycine (see Table I). These substitutions were designed to test the hypothesis that the positive charges within the motifs are critical determinants of the Glut1 membrane topology. The glycine substitutions eliminate the positive charge requirement, and should also reveal any strict requirement for an arginine side chain within the motif.

Messenger RNAs were transcribed from wild-type Glut1 and the four mutant Glut1 cDNAs, and the mRNAs were then injected into Xenopus oocytes. The relative level of expression of each of the proteins was determined by immunoblot analysis of purified oocyte plasma membranes (see Fig. 2) and transport activities were measured using 2-[3H]deoxyglucose (see Fig. 3A). These data allowed calculation of the transport activities of the mutant proteins relative to wild-type Glut1 (see Fig. 3B). Substitution of glycines for arginines within the loop 2 motif or the loop 8 motif completely abolished transport function. In contrast, substitution of lysines for the arginines in the loop 2 motif had little if any affect on transport activity, and the equivalent substitutions in the loop 8 motif decreased intrinsic transport activity by ~58%. These data suggest an absolute requirement for positively charged side chains within loop 2 and loop 8 but that the arginine side chain per se is not critical for transport activity.

The glycine and lysine mutations were next introduced into a series of Glut1 glycosylation-scanning reporter cDNAs. These reporter cDNAs encode chimeric Glut1 molecules in which a glycine substitutions eliminate the positive charges, and the lack of a side chain produced by this substitution should be structurally tolerated at these positions, which are predicted to form tight  bends between two transmembrane domains. The lysine substitutions act as a positive control for the positive charge requirement, and should also reveal any strict requirement for an arginine side chain within the motif.
Tran³⁵S-label and total membranes were then prepared. Glut1 porter molecule backgrounds were injected into Xenopus of loops 1, 6, 7, 8, 9, and 10. The two loop 8 motif mutants were each introduced into 5 reporter molecules providing the disposition of the N terminus and loops 1, 2, 3, and 4. The two loop 2 motif mutants were each introduced into 6 reporter molecules providing the disposition of the N terminus and loops 1, 3, and 4 were all properly localized to the correct compartment, but in the mutant molecules loop 2 was improperly localized to the exoplasmic compartment. The data also indicate that the aberrant topology in each case is localized to the mutated loop and the two flanking transmembrane segments, and that the remainder of the molecule upstream and downstream of the mutated loop exhibits a normal topological disposition. The abnormal structures that are most consistent with the pattern of glycosylation shown in Fig. 4 are illustrated in Fig. 5. Local perturbations in membrane topology as reported here for Glut1 resulting from the alteration of charges in flanking domains have been observed for bacterial (16) and eucaryotic (17) polytopic membrane proteins.

These data are consistent with our previous data indicating an important role for the amino acid charge difference across the first transmembrane segment in determining the local topological disposition of Glut1 (11). It is also consistent with our prediction that topological determinants are distributed throughout the Glut1 molecule, in contradiction to the original charge difference hypothesis (18). Additionally, these data are consistent with observations made for other bitopic (19) and polytopic (20) eucaryotic membrane proteins, indicating an important role for charged amino acid side chains in determining membrane topologies.

Our data also provide a simple explanation for the presence of the conserved pentameric motif in members of the major facilitator superfamily that differ radically in their substrate specificity and mode of transport, i.e. facilitated diffusion versus secondary active transport (3). The one striking characteristic shared by these otherwise distinct membrane transporters is their identical predicted membrane topologies. Thus, the

Fig. 5. Aberrant topologies of the arginine → glycine Glut1 mutants. The extramembranous loops are designated L1–L4 and L7–L10 as defined in Fig. 1. Mutating the 3 arginines in either loop 2 or loop 8 caused each loop to aberrantly translocate into the exoplasmic compartment along with its two flanking transmembrane segments.
R-X-G-R-R motifs likely play no direct role in the transport mechanism, but may only function as cytoplasmic anchor points for the generation of the correct membrane topology.

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