Analysis of the Transmembrane Topology of the Glycine Transporter GLYT1*

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A theoretical 12-transmembrane segment model based on the hydrophobic moment has been proposed for the transmembrane topology of the glycine transporter GLYT1 and all other members of the sodium- and chloride-dependent transporter family. We tested this model by introducing N-glycosylation sites along the GLYT1 sequence as reporter for an extracellular localization and by an in vitro transcription/translation assay that allows the analysis of the topogenic properties of different segments of the protein. The data reported herein are compatible with the existence of 12 transmembrane segments, but support a rearrangement of the first third of the protein. Contrary to prediction, hydrophobic domain 1 seems not to span the membrane, and the loop connecting hydrophobic domains 2 and 3, formerly believed to be intracellular, appears to be extracellularly located. In agreement with the theoretical model, we provide evidence for the extracellular localization of loops between hydrophobic segments 5 and 6, 7 and 8, 9 and 10, and 11 and 12.

Glycine is a major inhibitory neurotransmitter in the spinal cord and the brain stem of vertebrates. In addition, glycine can potentiate the action of glutamate, the main excitatory neurotransmitter in the brain, on postsynaptic N-methyl-D-aspartate receptors. The re-uptake of glycine into presynaptic nerve terminals or the neighboring fine glial processes provides one way of clearing the extracellular space of this neurotransactive substance and so constitutes an efficient mechanism by which the postsynaptic action can be terminated (1–3). This process is carried out by two different glycine transporters, named GLYT1 and GLYT2, which belong to the Na+ - and Cl−-dependent neurotransmitter transporter family (4–9). GLYT1 and GLYT2 present a differential expression pattern among central nervous system cells (7, 10–14).

The hydrophobic profiles of the Na+- and Cl−-dependent neurotransmitter transporters reveal the presence of 12 hydrophobic segments that have been suggested to form transmembrane α-helices (15). With the aid of sequence-specific antibodies, immunofluorescence, and electron microscopy, it has been shown that both the amino- and carboxyl-terminal ends of GLYT1 are intracellularly located (11, 16). Additional topological data have been obtained from the study of the glycosylation pattern of GLYT1. Site-directed mutagenesis has shown that GLYT1 is heavily glycosylated at four asparagine residues (Asn169, Asn172, Asn182, and Asn188) (17). This fact involves an extracellular localization of the hydrophilic loop placed between hydrophobic segments 3 and 4.

In this report, we present an extensive experimental evaluation of a neurotransmitter carrier protein transmembrane topology. The data included are compatible with a 12-membrane spanning segment model, with evidence for the extracellular localization of loops connecting hydrophobic domains 5 and 6, 7 and 8, 9 and 10, and 11 and 12. However, data herein favor an extracellular localization of the loop connecting HD2 and HD3, intracellularly located in the theoretical model. These observations, together with the previous demonstration of intracellular N and C termini and the extracellular localization of the loop placed between HD3 and HD4, provide an extensive experimental description of the GLYT1 transmembrane topology.

**EXPERIMENTAL PROCEDURES**

**Production of Antibodies against GLYT1**—The production and characterization of antibodies against a fusion protein containing the 76 C-terminal amino acids of GLYT1 have been described (12).

**Site-directed Mutagenesis**—The polymerase chain reaction (PCR)-based site-directed mutagenesis strategy followed to insert new glycosylation sites in GLYT1 was a modification of the method of Higuchi (18) as described (17). A deglycosylated form of the rB20a clone that had been previously subcloned in the XhoI-XbaI sites of pBluescript (construct N4) was used as template. Oligonucleotides (Isogen Bioscience) were designed to introduce the mutations listed in Fig. 2. The different mutated PCR fragments were introduced into construct N4 with the adequate flanking restriction sites. Mutant clones were identified either by sequencing or by restriction analysis: the glycosylation cluster sequence NNST introduced a SacI restriction site, and the cluster NNTS introduced a SpeI site. Mutant clones were characterized by sequencing. Finally, the full-length clones were transferred from pBluescript to the XhoI-XbaI sites of the pSVL expression vector.

**Expression in COS Cells**—Transient expression in COS cells was carried out using DEAE-dextran (Pharmacia Biotech Inc.) with dimethyl sulfoxide according to the method of Kaufman (19) with minor modifications (16).

**Electrophoresis and Blotting**—SDS-polyacrylamide gel electrophoresis (PAGE) and blotting were performed as described previously (16). Bands were visualized with the ECL detection method (Amersham Corp.) and quantified by densitometry (Molecular Dynamics ImageQuant Version 3.0).

**H+·K+-ATPase Cloning**—A cDNA coding for the 177 C-terminal amino acids of the rat H+·K+-ATPase was cloned by PCR from a rat gastric cDNA, obtained by reverse transcription from total RNA. cDNA was amplified with Taq polymerase, using as primers the following oligonucleotides: GCGGATCCAGCCTCTTGAGCCGCTACACCCCA-GCA (ATP1) and GCGGATCCACACTTCTGCTATTTGGAAGCTGAAC-T (ATP2). The 561-base pair fragment was digested with BamHI and...
EcoRI and ligated into a Bluescript vector digested with BamHI and EcoRI (vector ATPas-Bluescript).

Vector Construction—To analyze the topogenic properties of different GLYT1 fragments, a series of vectors was constructed using the ATPas-Bluescript vector as the starting point. To generate diverse GLYT1-ATPas fusion proteins, various GLYT1 fragments were produced by PCR and ligated in the correct reading frame upstream of the ATPas, under the control of the T7 promoter. All the GLYT1 fragments started with the 37 N-terminal amino acids, which would anchor the N terminus of the different fusion proteins to the cytoplasmic side of the membrane. Between the amino-terminal segment and the ATPas, we introduced different internal segments of GLYT1. Two possibilities existed. If the truncated GLYT1 segment was the natural continuation of the 37 N-terminal amino acids, a single GLYT1 PCR fragment including both the N-terminal segment and the desired amino acids was produced for each construct. These PCR fragments were flanked by restriction sites for XbaI and BamHI and were ligated in the XbaI-BamHI sites of the ATPas-Bluescript vector. Alternatively, if the variable GLYT1 segment was not contiguous to the N-terminal fragment, vectors were constructed in two steps. First, a fragment corresponding to the 37N-terminal amino acids was introduced in the GLYT1 PCR segment. These PCR fragments were flanked by the above-mentioned restriction sites, and after digestion and ligation, the reading frame of the fusion protein was always correct. Constructs were confirmed by DNA sequencing with the fmol™ kit (Promega).

In Vitro Transcription/Translation—Following linearization of the plasmids by EcoRI, RNA was synthesized using T7 RNA polymerase with the RNA synthesis and capping kit from Stratagene. Protein was synthesized from 0.5 μg of synthetic RNA using a reticulocyte lysate system (Promega) in the presence of [35S]methionine (Amersham Corp.) for 1 h at 30°C according to the manufacturer's instructions. Translation products were separated by 10% SDS-PAGE (mini-gels), fixed in methanol/acetic acid, dried, and subjected to autoradiography. Translation reactions containing canine microsomes (Promega) were centrifuged for 15 min at 15,000 × g through 100 mM KCl, 0.5 mM sucrose, and 50 mM HEPES, pH 7.5. The pellets were resuspended in electrophoresis loading buffer.

Enzymatic Deglycosylation—To confirm the variation in the electrophoretic mobility of the different COS cell-expressed glycosylation tagged mutants or in vitro translated fusion proteins, transfected COS cells or microsomal pellets were resuspended in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) and boiled for 10 min. Then, sodium phosphate and Nonidet P-40 were added (50 mM and 1% final concentrations, respectively), followed by 5 units of peptide N-glycosidase F (New England Biolabs Inc.). Samples were incubated for 2 h at 30°C prior to SDS-PAGE.

Alkaline Extraction of Microsomes—To confirm insertion of translated proteins, products obtained by translation in the presence of microsomes were diluted to 50 μl with HEPES, pH 11.5. After 10 min on ice, the samples are loaded onto a 100-μl cushion of 0.2 M sucrose, 50 mM phosphate and Nonidet P-40 were added (50 mM and 1% final concentrations, respectively), followed by 5 units of peptide N-glycosidase F (New England Biolabs Inc.). Samples were incubated for 2 h at 30°C prior to SDS-PAGE.

Patterns of GLYT1 immunoreactivity in transfected COS cells. COS cells were transfected with wild-type GLYT1 (clone rB20a) (WT) or with mutant N4 or were mock-transfected (MT). After 48 h of incubation at 37°C in a CO2 incubator, total cell protein was solubilized, and proteins were subjected to SDS-PAGE, electrophoresed onto nitrocellulose, and incubated with anti-GLYT1 antibody (1:250). Bands were visualized with the ECL detection method.

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Sites—We designed a series of mutants of GLYT1 by introducing N-glycosylation consensus sequences (NX(S/T)) (22) at different sites along the entire length of the protein and analyzed the mutant transporter for glycosylation at the newly engineered sites in a COS cell expression system. Glycosylation of membrane and secretory glycoproteins occurs only on the luminal side of the endoplasmic reticulum and Golgi apparatus membranes (23), which coincides with the extracellular face of the protein. Crude protein fractions obtained from transiently transfected COS cells were separated by SDS-PAGE, blotted onto nitrocellulose filters, and probed with an antibody directed against the carboxyl-terminal end of the protein (11, 12). In this experimental system, native GLYT1 is expressed as two bands (Fig. 1). Previous studies from our laboratory have shown that the upper band, ranging from 70 to 100 kDa, corresponds to the fully processed protein, whereas the lower band of 57 kDa corresponds to a partially glycosylated form of GLYT1 (17). Owing to the heterogeneous pattern of glycosylation of mature GLYT1, which could complicate interpretation of results, we did not use native GLYT1 as a starting point, but instead used a form of the protein in which the four native glycosylation sites (N169Q, N172Q, N182Q, and N188Q) had been eliminated by mutagenesis. This form of the protein (mutant N4) (Fig. 1), with a molecular mass of 47 kDa, has previously been shown not to be properly delivered to the cell surface, but is fully functional in transport assays after solubilization and reconstitution into liposomes, indicating that the oligosaccharide moiety is not required for proper folding of the protein (17). Since some of the newly introduced glycosylation sites were often not used, usually we had to introduce two to five clustered sites within a narrow region of the protein (Fig. 2) in order to maximize the probability that at least one of the sites would become glycosylated. When the mobility of the various GLYT1 mutants was compared with that of mutant N4, a variation was observed in constructs IL1b, EL3, EL4, and EL5 (named according to the theoretical model) (Fig. 3). However, the size of GLYT1 protein from mutants IL3, IL4, IL5, EL1, and EL6 was similar to that of N4 protein (Fig. 3). The increase in the size of the band observed in constructs IL1b, EL3, EL4, and EL5 was due to glycosylation, as treatment of the crude protein fraction with peptide N-glycosidase F prior to SDS-PAGE yielded the fully deglycosylated 47-kDa form of the protein (Fig. 4). Nega-
For each construct, the sequence of the fusion protein generated by in vitro transcription/translation is listed, connected by dashes. Amino acids are numbered according to the GLT1b isoform. Glycosylation sites of the GLYT1 region are listed, followed by the indicated transmembrane domains and the carboxyl terminus of the H\(^{+}\), K\(^{-}\)-ATPase \(\beta\)-subunit. Only the C and N termini of the variable GLYT1 region are listed, connected by dashes. Amino acids are numbered according to the GLT1b isoform. Glycosylation sites of the ATPase are indicated in boldface letters.

| Sequence | Position |
|----------|----------|
| GLYT1    | 1–37     |
| EL1      | 38–64    |
| EL2      | 62–104   |
| EL3      | 100–134  |
| EL4      | 216–244  |
| EL5      | 131–214  |
| EL6      | 62–124   |
| EL7      | 138–154  |
| EL8      | 218–244  |
| EL9      | 159–224  |
| EL10     | 220–244  |
| EL11     | 244–274  |
| EL12     | 275–295  |
| EL13     | 295–325  |
| EL14     | 326–355  |
| EL15     | 356–386  |
| EL16     | 387–418  |
| EL17     | 419–450  |
| EL18     | 451–470  |
| EL19     | 471–491  |
| EL20     | 492–523  |
| EL21     | 524–571  |

**Table II**

**Amino acid sequences of the GLYT1-ATPase fusion proteins**

For each construct, the sequence of the fusion protein generated by in vitro transcription/translation is listed, connected by dashes. Amino acids are numbered according to the GLT1b isoform. Glycosylation sites of the ATPase are indicated in boldface letters.

**Identification of Transmembrane Domains by in Vitro Transcription/Translation**—The absence of information about the topology of a part of the protein and the necessity of confirmation of the results obtained by the former technique prompted us to use an independent methodology that allowed us to analyze the topogenic properties of presumptive transmembrane segments. Vectors were constructed to allow insertion of different GLYT1 segments, presumed to be membrane spanning sequences, between the cytoplasmically anchored N-terminal sequence of GLYT1 and a reporter sequence containing several N-linked glycosylation consensus sequences (Fig. 5, A and B). mRNAs from the different constructs were obtained by in vitro transcription and then translated in a rabbit reticulocyte lysate system in an either the absence or presence of canine microsomes. The 177-amino acid carboxyl terminus of the H\(^{+}\), K\(^{-}\)-ATPase \(\beta\)-subunit, which contains five glycosylation sites, was chosen as a reporter because it has been previously used in a similar system and shown to contain neither anchoring nor stop transfer signals (24). Glycosylation of the fusion protein by the microsomes was visualized by a shift in relative molecular mass after SDS-PAGE and autoradiography of the gels. The increase in molecular mass indicates insertion of the protein in the microsomal membrane and translocation of the reporter present in the C-terminal part of the fusion protein. This involves the presence of at least an anchor signal within the analyzed sequence. Absence of glycosylation indicated the absence of membrane insertion or the presence of an even number of membrane spanning segments, the last one containing a stop transfer sequence (Fig. 5, A and B). Table II summarizes the amino acid sequences of the different constructs used in this study.

**Structure of the Amino-terminal Third of GLYT1**—To study the transmembrane topology of the segment of the protein placed between the intracellular N terminus and the extracellular loop containing the native N-glycosylation sites, we produced a series of plasmids as shown in Fig. 5C. First, we analyzed the ability of individual predicted membrane spanning segments to promote translocation of the ATPase (Fig. 6A). Our data indicated that, contrary to the prediction of the theoretic model, hydrophobic domain 1 (amino acids 38–64 in the variable region; construct 1) was not able to promote translocation of the ATPase to the microsomal lumen. Similar results were obtained with a longer construct (amino acids 38–69 in the variable region; construct 4) when assayed individually. This construct (21–24) was able to traverse the membrane, indicating that these segments on their own are able to act as signal anchor sequences. Interestingly, construct 5, which contained only the predicted loop EL2, was glycosylated, indicating the existence of an unpredicted anchor sequence in this loop. Next, we placed in the variable region of the ATPase vector segments containing several hydrophobic domains of GLYT1 (Fig. 6B). Translation products from constructs 6 and 7, carrying HD2 and HD3, were...
glycosylated independently of the inclusion of the natural glycosylation sites (construct 7) or not (construct 6). The inclusion of HD1 in the constructs did not affect the glycosylation pattern; for instance, fusion proteins containing HD1 and HD2 (construct 9) or HD1, HD2, and HD3 (construct 10) were glycosylated. The inability of HD1 to traverse the membrane and the presence of an anchor signal in EL2 strongly support a signal anchor sequence. According to the theoretical model, the even-numbered hydrophobic domains should act as stop transfer signals. However, this signal was not identified in our experiments, as glycosylation of the reporter was not prevented by HD3 in constructs 6 and 9.

The insertion into the membrane of all the constructs was routinely assessed by alkaline extraction (at pH 11.5) of the microsomes. This method is known to strip the membrane of peripheral proteins and hence is a useful empirical procedure to distinguish between integral membrane proteins and soluble or peripheral proteins. Products from the constructs, depicted in Fig. 6 (A and B), either glycosylated or not, were recovered in the microsomal pellet, indicating that these fusion proteins are integral membrane proteins. The elevation in the relative molecular mass, when observed, was due to glycosylation, as integral membrane proteins. The elevation in the relative molecular mass, when observed, was due to glycosylation, as treatment of translated protein in microsomal membranes with peptide N-glycosidase F reduced the molecular mass to that observed in the absence of microsomes. Fig. 6C shows the deglycosylation pattern produced by peptide N-glycosidase F on fusion proteins obtained from constructs 1–3 and 5. Similarly, deglycosylation was observed with constructs 4 and 7–10 (data not shown).

Structure of the Central and Carboxyl-terminal Parts of GLYT1—The topogenic properties of sequences representing HD5 through HD12 were analyzed first by inserting each individual hydrophobic sequence into the ATPase vector. The results are summarized in Fig. 7. Fusion proteins containing any of the individual segments from HD5 to HD12, except HD6, were inserted into the membrane and glycosylated. The results indicate that all these hydrophobic sequences, except HD6, individually considered were able to act as signal anchor sequences. According to the theoretical model, the even-numbered hydrophobic domains should act as stop transfer signals for the previous domain. We analyzed the glycosylation pattern of fusion proteins produced by translation of constructs carrying pairs of hydrophobic domains: constructs 19 (HD5 and HD6), 20 (HD7 and HD8), 21 (HD9 and HD10), and 22 (HD11 and HD12). None of these fusion proteins resulted glycosylated (Fig. 7) in the presence of microsomes, but they were all inserted into the membrane as they were resistant to alkaline extraction. These results indicate that, as expected, HD6, HD8, HD10, and HD12 acted as stop transfer sequences when placed after HD5, HD7, HD9, and HD11, respectively. These data support results obtained by the glycosylation scanning technique, confirming the extracellular localization of EL3, EL4, and EL5 and supporting as well the extracellular localization of EL6.

**FIG. 7.** Schematic representation of constructs used for elucidating the topology of the central and C-terminal regions of GLYT1. Symbols are as described in the legend of Fig. 5. Results obtained in the presence of microsomes are summarized in the right column.
DISCUSSION

We have made a systematic investigation of the membrane topology of GLYT1 by inserting new glycosylation consensus sequences along the entire length of this protein, followed by analysis of the mutant protein expressed in COS cells. The analysis has been complemented by an independent biochemical method consisting of the insertion of a glycosylation reporter after proposed transmembrane domains and analysis of the glycosylation pattern in an in vitro translation system. On the basis of hydrophathy plots, it has been proposed that GLYT1, as all other members of its gene family, contains 12 transmembrane segments, a number also shared by carrier proteins without sequence homology to the sodium- and chloride-dependent transporter family (25–27). Our results are consistent with a 12-transmembrane model, but support a rearrangement in the first third of the protein.

As with most of the available techniques to study the transmembrane topology of proteins, the two techniques used in this report present some limitations. For instance, the introduction of new glycosylation sites throughout the protein has the potential to alter the topology. In addition, the transmembrane topology could also be altered by truncation and addition of the large reporter protein. Thus, the most compelling conclusions would be those supported by convergent results of independent methods. In this report, data from both methodologies are coincident with respect to the structure of the second third of the protein, and they support the theoretical model. The glycosylation tagging technique shows that loops EL3, EL4, and EL5 are glycosylated after the introduction of a new glycosylation consensus sequence, suggesting an extracellular location. In vitro transcription experiments support this structure, with HD6, HD8, and HD10 acting as stop transfer signals for the respective preceding hydrophobic domains HD5, HD7, and HD9, which would act as membrane anchor signals. Consistent with these observations, loop EL4 of the norepinephrine transporter has also been shown to be extracellular by using antipeptide antibodies (28). With the glycosylation tagging technique, we did not observe glycosylation of loop EL6. However, absence of glycosylation does not disprove an extracellular localization, as it may simply be a consequence of steric hindrance (22, 29, 30).

In fact, data from in vitro transcription support an extracellular localization for this loop, as both HD11 and HD12 are able to traverse the membrane, with acting HD12 as a stop transfer signal when placed after HD11. Moreover, in the accompanying paper, Bennett and Kanner (31) obtained evidence for glycosylation of EL6 of the γ-aminobutyric acid transporter GAT-1 by using the glycosylation tagging technique. These results are consistent with the intracellular location of the C terminus of GLYT1 (17).

As far as the first third of the protein is concerned, previous studies on GLYT1, GLYT2, dopamine, and serotonin transporter topologies have shown that the N terminus of this family of proteins is intracellular (11, 16, 27, 32). In addition, the four native glycosylation sites of GLYT1 and those of the serotonin transporter, in the loop connecting HD3 and HD4, are extracellularly located (17, 33). These observations imply an odd number of transmembrane domains preceding the first natural glycosylation site, Asn169. The glycosylation tagging technique supports an extracellular localization of the loop connecting HD2 and HD3, a structure that is contrary to that predicted by the theoretical model (Fig. 8, A and B). Similar observations have been made in the accompanying paper by Bennett and Kanner (31) for the γ-aminobutyric acid transporter GAT-1. Consistent with this model, in vitro translation indicates that HD1 on its own is not able to traverse the membrane. Moreover, we have been able to identify three segments with ability to span the membrane in this region of the protein: HD2, HD3, and a stretch of amino acids in the glycosylated loop EL2. Nevertheless, still some caution should be observed in the interpretation of these results, as the model in Fig. 8A, which fulfills the above-mentioned observations, predicts HD3 containing a stop transfer signal. However, constructs carrying HD2 and HD3 (constructs 6 and 9) were glycosylated in the presence of microsomes, indicating the absence of stop transfer signals in the assayed sequence. Nevertheless, one has to keep in mind the limitation of the in vitro translation technique, and perhaps the behavior of these sequences in the context of the native protein would be as predicted by this model. Model in Fig. 8A proposes HD1 as a reentrant loop, similar to the one described for the glutamate receptors (34, 35) or the potassium channel (36), as opposed to a completely intracellular HD1.

Although the data in this report are insufficient to support this arrangement of HD1, the fusion protein obtained from translation of construct 1 was not separated from the membrane by the alkaline wash of the microsomes, indicating a close association of this domain with the membrane. Moreover, some data obtained for other members of the gene family support the idea that HD1 is associated with the membrane. This domain is highly conserved among the members of the sodium- and chloride-dependent transporter family. Arginine 69 of the γ-aminobutyric acid transporter GAT-1, which is located in this domain and also present in GLYT1, seems to be important for the control of the permeation process (37). Moreover, aspartate 59, present in the equivalent domain of the dopamine transporter, has been suggested to interact with the amine group of dopamine during the translocation process (38). These data suggest that this domain must be close to the permeation pore and thus
probably is associated with the membrane.

In spite of the above-mentioned methodological limitations, the transmembrane structure of various eukaryotic proteins has been analyzed with these techniques. The glycosylation tagging method has been used to study the structure of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type of glutamate receptor (34), the cystic fibrosis transmembrane conductance regulator (39), and the Na+/glucose cotransporter SGLT1 (40). The gene fusion system has been used extensively for mapping the transmembrane topology not only of bacterial proteins, but also of eukaryotic proteins. For instance, a similar gene fusion technique using the ATPase β-subunit as reporter has been used to determine the topology of the H⁺,K⁺-ATPase α-subunit (24). The same technique has also been employed with other reporters, notably the large prolactin epitope, used to determine the topology of the αδ-subunits of the nicotinic receptor (41), or the structure of the Na⁺/glucose cotransporter

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