Glycine transporter GLYT2 is an axonal glycoprotein involved in the removal of glycine from the synaptic cleft. To elucidate the role of the carbohydrate moiety on GLYT2 function, we analyzed the effect of the disruption of the putative N-glycosylation sites on the transport activity, intracellular traffic in COS cells, and asymmetrical distribution of this protein in polarized Madin-Darby canine kidney (MDCK) cells. Transport activity was reduced by 35–40% after enzymatic deglycosylation of the transporter reconstituted into liposomes. Site-directed mutagenesis of the four glycosylation sites (Asn-345, Asn-355, Asn-360, and Asn-366), located in the large extracellular loop of GLYT2, produced an inactive protein that was retained in intracellular compartments when transiently transfected in COS cells or in nonpolarized MDCK cells. When expressed in polarized MDCK cells, wild type GLYT2 localizes in the apical surface as assessed by transport and biotinylation assays. However, a partially unglycosylated mutant (triple mutant) was distributed in a nonpolarized manner in MDCK cells. The apical localization of GLYT2 occurred by a glycolipid rafts independent pathway.

To terminate synaptic transmission in the central nervous system, neurotransmitters are inactivated by either enzymatic degradation or active transport into neuronal and/or glial cells (1, 2). The removal of neurotransmitters is accomplished by transporter proteins located in the plasma membrane of the presynaptic nerve terminals and surrounding glial processes. Glycine transporters are encoded by two genes (glyt1 and glyt2) that produce several alternative isoforms (3–7). GLYT2 is predominantly expressed in the spinal cord and the brainstem, associated with the presynaptic aspect of glycnergic synapses (8–11). Similarly, GLYT1 is expressed at higher levels in glycnergic areas, but it could also participate in N-methyl-D-aspartate-mediated glutamatergic neurotransmission (5, 9–14).

GLYT1 and GLYT2 share a common predicted structure comprising 12 putative transmembrane domains and a large hydrophilic loop between transmembrane domains III and IV which contains several potential N-linked glycosylation sites. Previous data have shown that mature GLYT1 and GLYT2 are heavily glycosylated proteins (15, 16). N-Glycosylation of proteins has been demonstrated to play a variety of roles including modulation of biological activity, regulation of intracellular targeting, protein folding, and maintenance of protein stability. In the case of GLYT1, we have previously shown that glycosylation is necessary for proper trafficking of the protein, but it is not indispensable for the transport activity itself (16).

The subcellular localization of transporters seems to be an important determinant of the impact of transport activity on synaptic function. In this sense, various transporters show a differential distribution among membrane domains of the cells where they are expressed. For instance, the GABA transporter, GABA transporter 1, the transporters for biogenic amines, and the glycine transporter GLYT2 are concentrated in presynaptic terminals (17–20). In non-neural cells, the betaine transporter, a member of the GABA transporter subfamily, is expressed in the basolateral membrane of epithelial MDCK cells (21). The specific mechanisms of such a nonuniform distribution have been studied in experimental systems of polarized cultured cells, especially in the epithelial MDCK cell line, since somatodendritic neuronal proteins use to be localized in the basolateral domain, whereas axonal proteins are in the apical one (22). N-Glycosylation has been shown to be involved in the apical localization of a number of secretory and membrane proteins (23), but the molecular mechanisms on apical delivery are yet unclear.

In the present report we have analyzed the consequences of the disruption of the potential N-glycosylation sites of GLYT2 on the transport of solute, in transport of the protein to the cell surface, and in the asymmetrical distribution of GLYT2 in a model of polarized cells.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and G418 were from Life Technologies, Inc. BHK medium was from Life Technologies, Inc. Bovine serum albumin was from Sigma. [2-3H]Glycine (1757.5 GBq/mmol) was from Amersham Pharmacia Biotech. Recombinant N-glycosidase F (PNGaseF) was from New England Biolabs. Transwell inserts were from Costar. Culture dishes were from Nunc (Denmark). All other reagents were obtained in the purest form available.

cDNA Constructs and Site-directed Mutagenesis—All mutants were performed as described previously (16, 24). Mutant and wild type cDNAs were cloned in the HindIII/XbaI or XhoI/XbaI restriction sites of pcDNA3 for expression in MDCK cells. Site-directed mutagenesis was performed using a modification of the method of Higuchi (25) as described (24). Mutants and wild type cDNAs were cloned downstream of the neomycin resistance gene of pcDNA3.
from the cytomegalovirus promoter of pcDNA3 mammalian expression vector (Invitrogen). To disrupt the glycosylation sequences (NX/S/T), codons coding for Asn were mutated to Asp. Glycosylation sites were eliminated sequentially, starting with single mutations as follows: N345D, N355D, N360D, and N366D. Mutants devoid of two, three, and four glycosylation sites were generated, using as a template DNA sequences mutated at one, two, or three glycosylation sites, respectively. All mutations were confirmed by DNA sequencing.

**Transient Transfection in COS Cells—**COS-7 cells (American Type Culture Collection) were grown at 37 °C and 5% CO2 in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulphate. Transient expression in COS cells was carried out by electroporation with AFFECTAMINE Plus (Life Technologies, Inc.) following the procedure indicated by the supplier. Cells were incubated for 48 h at 37 °C and then used to assay transport activity, surface expression, and immunofluorescence.

**Transfection and Stable Expression of MDCK Cells—**The parental MDCK type II cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37 °C. 7% CO2. MDCK cells were transfected by electroporation with pcDNAs that carries the neomycin resistance gene for selection. After selection with 0.6 μg/liter G418 for 12–15 days, single colonies were isolated with cloning cylinders and tested for transporter expression by immunocytochemistry. Clones stably transfected were then assayed for transport activity shown by mock-transfected cells or proteoliposomes obtained from mock-transfected cells.

**Glycine Transport Assay—**Transport assays in transfected COS-7 cells were performed at 37 °C in HEPES-buffered saline (150 mM NaCl, 10 mM HEPES-Tris, pH 7.4, 1 mM CaCl2, 2.5 mM KCl, 2.5 mM MgSO4, 10 mM glucose) as described previously (26). Cells were incubated for the indicated times with 0.5 ml of an uptake solution that contained an isotopic dilution of 3H-labeled glycine in the former solution yielding a 10 3H final glycine concentration (or the desired concentration). Kinetic analysis was performed by varying glycine concentration in the uptake medium between 10 μM and 1 mM. Transport into reconstituted liposomes was measured at room temperature using an inwardly directed NaCl gradient in the presence of a negative membrane potential, as described (27, 29). Nonspecific transport was defined as the glycine transport shown by mock-transfected cells or proteoliposomes obtained from mock-transfected cells.

**Vectorial Glycine Uptake Assay—**Parental and transfected MDCK cells were plated at 50% confluence on Transwell tissue culture inserts (6.5 mm diameter, 0.4 μm filter pore size, Costar Co., Cambridge, MA) and grown for 5–7 days. Glycine uptake was performed at room temperature according to a modification of the method of Yamamura et al. (29). A wash of each chamber with complete phosphate-buffered saline (PBS/Ca/Mg: 137 mM NaCl, 2.7 mM KCl, 0.89 mM CaCl2, 0.49 mM MgCl2, 4.3 mM Na2HPO4·7H2O, 1.4 mM KH2PO4, pH 7.3) and another wash with uptake buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM (+-)glucose, 10 mM HEPES-Tris, pH 7.4), the cells were incubated with 0.2 μM [1H]glycine in uptake buffer either in the upper or in the lower chamber for 10 min. At the end of the incubation, the cells were washed with ice-cold PBS/Ca/Mg three times from the side they were incubated with the 3H-labeled substrate and once from the opposite side. The cells were dried and lysed in 0.2 μM NaOH. Aliquots were taken and counted in scintillation fluid, and protein concentration was determined with the Bio-Rad protein assay. Basal glycine uptake was defined as glycine transport shown by nontransfected MDCK cells.

**Solubilization and Reconstitution Procedure—**For each reconstitution experiment, transfected cells from one dish (10-cm diameter) were used. Cells were scraped, collected by centrifugation, and adjusted at a protein concentration of 5–10 mg/ml with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4·7H2O, 1.4 mM KH2PO4, pH 7.3). Solubilization of cells was performed using sodium cholate at 1:1 detergent/protein ratio. After 10 min on ice, solubilized membranes were pelleted with a Beckman microfuge (14,000 rpm, 30 min) and proteoliposomes were kept on ice until use.

**Glycosidase Treatment—**Wild type GLYT2 protein transiently transfected in COS cells was solubilized and reconstituted into liposomes as described above. Proteoliposomes were treated with or without PNGase F (150 units/mg protein) in the presence of protease inhibitors (4 μM pepstatin A and 0.4 mM phenylmethylsulfonyl fluoride) for 3 h at 37 °C with gentle agitation. After treatment, proteoliposomes were washed and immediately used for glycine transport assays or for SDS-PAGE and immunoblotting.

**Cell Surface Biotinylation—**MDCK cells were plated at 50% confluence on 0.4-μm pore size, 25-mm Transwell cell culture filter inserts for 24 h and grown for 7 days. After the cells were washed with 2 ml of ice-cold PBS/Ca/Mg, cell surface proteins were excised, and cells were lysed with 1 ml of lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM HEPES-Tris, 0.25% deoxycholate, 1% Triton X-100, 0.1% SDS, pH 7.4) for 30 min. The cell lysate was clarified by sedimentation at 14,000 × g for 10 min. COS cell proteins were labeled following the same procedure except they were plated on regular culture dishes. Biotinylated proteins were recovered by adding streptavidin-agarose beads to a portion of the cell lysate followed by incubation for 2 h with gentle agitation at room temperature. Beads were pelleted by centrifugation. After three washes with 1 ml of lysis buffer, proteins bound to the beads were eluted in 2 × Laemmli sample buffer, separated by SDS electrophoresis, and transferred to nitrocellulose filters. The filters were probed with antibodies recognizing GLYT2 (10, 11) as described previously (10). Bands were visualized with the ECL detection method (Amersham Pharmacia Bio-tech) and quantified by densitometry (Molecular Dynamics ImageQuant version 3.0) by using film exposures that were in the linear range.

**Isolation of Low Density Membrane Domains—**The procedure used to prepare Triton X-100-insoluble membranes by centrifugation to equilibrium in sucrose density gradients was essentially as described by Brown and Rose (30). Cells grown to confluency in four 100-mm dishes were rinsed with PBS and lysed for 20 min in 1 ml of 150 mM NaCl, 25 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1% Triton X-100 at 4 °C. The lysate was scraped from the dishes with a rubber policeman, and the dishes were rinsed with 1 ml of the same buffer at 4 °C, and the lysate was homogenized by passing the sample through a 22-gauge needle. The lysate was brought to 40% sucrose in a final volume of 4 ml and placed at the bottom of an 8-ml 5–30% linear sucrose gradient. Samples were centrifuged for 18 h at 39,000 rpm at 4 °C in a Beckman SW 41 rotor. Fractions of 1 ml were harvested from the bottom of the tube, and aliquots were subjected to immunoblot analysis. MAL proteolipid was detected by the dot-blot technique by spotting 100 μg of protein onto a nitrocellulose filter. The filter was probed with a highly specific antibody for MAL kindly provided by Dr. M. A. Alonso (Centro de Biología Molecular) and previously characterized (31).

**Protein Concentration—**Protein concentration was determined by the method of Bradford (24).

**RESULTS AND DISCUSSION**

GLYT2 is an axonal glycoprotein involved in the re-uptake of the neurotransmitter glycine in the glycinergic synapses. Based on its deduced amino acid sequence, the protein has four consensus sites (NX/T/S) for N-glycosylation in the second extracellular loop (Asn-345, Asn-355, Asn-360, and Asn-366) as depicted in Fig. 1. Glycosylation has been shown to play a functional role in other members of the sodium- and chloride-dependent neurotransmitter transporter family (24, 33). To address the role of the carbohydrate moiety on different functional aspects of GLYT2, we disrupted the glycosylation consensus sequences by site-directed mutagenesis replacing the asparagine residues with aspartate. Then we analyzed the effects of these mutations on transport activity, on transport of the protein from the intracellular compartments to the cell surface, and on asymmetrical distribution of the protein in the surface of polarized cells. First, we produced a series of mutants deficient in one glycosylation site. We prepared four single mutants (N345D, N355D, N360D, and N366D), a double mutant (N355D/N360D), a triple mutant (N345D/N355D/N360D), and the quadruple mutant (N345D/N355D/N360D/N366D). Wild type or mutant forms of GLYT2 were then transiently transfected into COS cells, and their transport activities were assayed. As shown in Fig. 2, mutants deficient in one glycosylation site sustained glycine transport.
transport activities similar to those displayed by wild type transporter, indicating that no single N-linked carbohydrate chain is specifically required for the GLYT2 function. The double and triple mutants retained 74 and 58% of wild type glycine transport activity, respectively. When all four potential glycosylation sites were mutated, a complete loss of activity was observed. These results indicate that the effects of disrupting N-glycosylation sites on GLYT2 activity are cumulative. The progressive decrease in transport activity was not due to a change in the affinity of the transporter for the substrate, as deduced from kinetic analysis of the triple mutant. The calculated $K_m$ for glycine was nearly identical in wild type and triple mutant of GLYT2 (102 ± 4 and 93 ± 3 μM, respectively). However, there were decreases in the $V_{max}$ values (3.3 ± 0.5 and 2.0 ± 0.2 nmol of glycine/mg of protein/6 min, respectively).

To determine whether the impaired transport of the mutants was due to a poor expression or to a deficient transport of the protein to the plasma membrane, we performed cell surface biotinylation experiments followed by immunodetection (Fig. 3).
results. Moreover, when the wild type was deglycosylated by treatment with PNGaseF, the activity of the glycine transporter was completely inactive after reconstitution. The quadruple mutant was completely inactive after reconstitution.

GLYT2 were expressed in COS cells, solubilized with detergent, and reconstituted into liposomes. Fig. 4 shows enzymatic removal of N-linked sugar chains of GLYT2 reconstituted into liposomes. COS cells expressing the transporter were solubilized, and membrane proteins were reconstituted into liposomes. Proteoliposomes containing wild type transporter were treated with or without PNGaseF as described under “Experimental Procedures.” Glycine transport activity was expressed as a percentage of GLYT2 transport into proteoliposomes that was 7.32 pmol of glycine/mg of protein/15 min. Error bars represent S.E. of three measurements.

We quantified the relative amounts of the mutants on the plasma membrane compared with their total amount in cell extracts by a densitometric analysis considering as 100% the fraction of total wild type protein that reached the membrane (Fig. 3C). For the mutants, a parallelism between the difficulty to arrive to the cell surface and the decrease in transport activity was observed. No significant differences were detected in cell and membrane expression of all single and double mutants as compared with the wild type. However, mutants devoid of three or four N-glycosylation sites showed low expression levels and difficulties reaching the plasma membrane. The quadruple mutant was not detected in the surface, which is consistent with the absence of transport activity shown when this protein is expressed in COS cells (Fig. 2).

The subcellular localization of mutants was additionally examined by immunocytochemistry on transfected COS cells. Whereas the plasma membrane of wild type transfected cells was clearly labeled, a poor presence or total absence of labeling was observed at the surface of cells expressing the triple and quadruple mutants, respectively. Immunofluorescence together with biotinylation experiments led us to conclude that, although each carbohydrate chain considered individually is not specifically essential for GLYT2 function, the progressive removal of carbohydrate chains produced an accumulative decrease in surface expression and, consequently, in transport activity of the glycine transporter. Similar results have been described for the norepinephrine transporter (33) and GLYT1 (24).

To determine whether the inactivation and intracellular retention of the transporter produced by the total disruption of N-glycosylation sites could be explained by misfolding of the protein, the quadruple mutant and the wild type form of GLYT2 were expressed in COS cells, solubilized with detergent, and reconstituted into liposomes. Fig. 4A shows that whereas the reconstructed wild type was fully active, the quadruple mutant was completely inactive after reconstitution. Moreover, when the wild type was deglycosylated by treatment with PNGaseF after being reconstituted into liposomes, the transporter reduced the transport activity by 35–40%. The removal of N-linked sugar chains by PNGaseF produced a shift in the electrophoretic mobility of GLYT2 protein as shown on Western blot analysis (Fig. 4B, lanes 1 and 2) and the quadruple mutant N345DN355DN366DNS366D (lane 3) were incubated without (lanes 1 and 3) or with PNGaseF (lane 2). Glycine transport activity is expressed as a percentage of GLYT2 transport into proteoliposomes that was 7.32 pmol of glycine/mg of protein/15 min.

These results indicate that the loss of carbohydrates renders a GLYT2 transporter that is in an inactive conformation, probably misfolded, and that is unable to reach the cell surface. The importance of glycosylation for a proper surface delivery has been previously stated for other transporters (24, 33–37) and for various plasma membrane proteins (38). Moreover, the reduction in the wild type GLYT2 activity after enzymatic deglycosylation indicates that the oligosaccharide chains stabilize the optimal active conformation of the transporter once it has been synthesized. Interestingly, these results contrast with those reported for GLYT1, norepinephrine transporter, and serotonin transporter where surface trafficking but no transport activity impaired in the nonglycosylated mutant (24, 33, 34) but are consistent with results obtained with a glycine transporter purified from pig spinal cord (15).

GLYT2 is expressed mainly in axons and terminals of neurons in the spinal cord and other glycineric areas but is absent in most of dendrites and cell bodies. A number of transporters of this gene family also show an asymmetrical distribution on the surface of cells where they are expressed, and thus, it is supposed that the specific subcellular distribution of transporters must be essential to accomplish their biological functions. To study the molecular mechanism involved in asymmetrical

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distribution of neuronal proteins, a frequently used experimental model is found in the epithelial MDCK cell line. We recently developed MDCK cell lines constitutively expressing GLYT2 in which most of the wild type transporters were localized in the apical domain of polarized cells (39). Because glycosylation is known to be an apical determinant for a number of secreted and membrane proteins (40), we decided to analyze the distribution of deglycosylated GLYT2 mutants in this experimental system. However, in transiently transfected MDCK cells, like in COS cells, the quadruple mutant remained accumulated in intracellular compartments (not shown), and thus, this mutant could not be further analyzed. Again like in COS cells, the triple mutant was partially active, and thus we generated stable cell lines with this construct. The distribution of the protein between the apical and basolateral domains was analyzed by vectorial transport assays and by biotinylation experiments (Fig. 5). In agreement with our previous observations, 80 ± 8% of the transport activity determined in MDCK cells stably transfected with wild type GLYT2 was located in the apical side (Fig. 5A), and most of the protein (89 ± 6%) was labeled when the Sulfo-NHS-SS-Biotin reagent was added from the apical side (Fig. 5B). However, in MDCK cells transfected with the triple GLYT2 mutant, the transport activity and the labeled protein were detected at about the same levels at both cell surfaces (60 ± 3% apical in transport assays and 55 ± 7% apical in biotinylation experiments) (Fig. 5, A and B). The high intracellular retention of this mutant precluded the production of reliable confocal microscopy images.

These results clearly indicate that full glycosylation is not only necessary for proper conformation and cell surface delivery but also for asymmetrical distribution in polarized cells, a property that must be physiologically relevant for neuronal proteins that need to be asymmetrically distributed. The mechanisms for apical sorting are diverse, and signals have been found in the extracellular (41), in the transmembrane (42), and in the intracellular protein domains (43, 44). The carbohydrate moiety is involved in apical localization of other proteins (45), but this has not been reported for neurotransmitter transporters. In fact, the mechanism for apical localization of the GABA transporter GABA transporter 3 in MDCK cells has been investigated, and it was shown to depend on a THF motif located in the intracellular carboxyl end of the protein (44). However, intracellular ends of GLYT2 have been previously shown not to be important in its apical distribution (39).

Some apically directed membrane proteins are associated with specialized membrane domains that are rich in glycosphingolipids and are detergent-insoluble (46). These structures, termed “rafts,” can be recovered from low density fractions after centrifugation to equilibrium in sucrose density gradients. To investigate whether GLYT2 used this sorting mechanism, we centrifuged Triton X-100 extracts from MDCK cells stably transfected with the wild type GLYT2 to equilibrium (30). Under these experimental conditions, GLYT2 was completely solubilized by a brief treatment of the cells with Triton X-100 at 4 °C and appeared in higher density fractions (fractions 1–4) (Fig. 6A). As a control of the fractionation procedure, we analyzed in parallel the distribution of the proteolipid MAL (Fig. 6B), a protein associated with detergent-insoluble membranes that was found in the buoyant, membrane-insoluble fractions (fractions 7–8). These experiments indicated that GLYT2 is not associated with membrane rafts and must be polarized by an alternative mechanism. Proteins that utilize the glycolipid pathway are heterogeneous in their apical sorting signal that can be found in N-linked carbohydrates, in the glycosylphosphatidylinositol anchor, or even in transmembrane domains. Among them, there are a number of N-glycosylated intestinal hydrolases (47), the alkaline phosphatase (48), or the influenza virus hemagglutinin (42). Also this pathway is used by other integral membrane proteins like the MAL proteolipid (31) or caveolin (49). However, other membrane proteins including both glycosylated (enteropeptidase) (50) and nonglycosylated proteins (CD3-e) (43) are apically sorted by a proteolipid-independent pathway.

The nature of the molecular machinery involved in the recruitment of glycoproteins to the apical membrane is largely unknown. A first model propose that N-glycans constitute the apical signal by themselves. Indeed the insertion of ectopic glycosylation sites in secretory or membrane proteins converted nonpolarized secretory proteins or intracellularly retained membrane proteins into apical ones (41, 45). In this...
model N-glycans would interact with lectins sorters in the trans-Golgi network that would mediate incorporation of glycosylated proteins into apical transport vesicles. Although some candidate lectins have been identified (51, 52), their role in this process is still unclear. Our observation that the triple GLYT2 mutant is distributed in a nonpolarized manner despite the presence of a glycosylation site would not support this model for GLYT2 except that the massive reduction in the glycosylation observed in this mutant would also reduce the affinity for lectins and, consequently, the efficiency of apical recruitment. Indeed, a similar observation has been performed for erythropoietin, a secretory protein where not all its glycosylation sites are equally effective in promoting apical sorting (53). An alternative model where N-glycans would not constitute a sufficient sorting signal per se but would play an indirect role by allowing the correct folding and structural stabilization of proteinaceous sorting signals has been proposed (40). Our observation on the triple GLYT2 mutant would also be compatible with this alternative model.

In summary, in this report we describe the importance of the sugar moiety of GLYT2 for the transport of the solute and for the arrival to the membrane in a polarized manner. This could be relevant for the physiology of the glycine neuron, as GLYT2 has to be asymmetrically distributed to be finally settled in the axonal domain where it develops its biological function.

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The Role of N-Glycosylation in Transport to the Plasma Membrane and Sorting of the Neuronal Glycine Transporter GLYT2
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