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Localization of N-glycosylation sites and functional role of the carbohydrate units of GLAST-1, a cloned rat brain L-glutamate/L-aspartate transporter

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The L-glutamate transporter GLAST-1 belongs to the newly discovered family of Na^+-dependent, high-affinity glutamate transporters, which are involved in the regulation of synaptic excitatory neurotransmitter concentration in mammalian brain. The members of this family have a similar topological organisation with at least six transmembrane helices (TMHs) and two putative N-glycosylation sites located in the extracellular loop connecting TMH 3 and TMH 4. Besides these two conserved N-glycosylation motifs at Asn206 and Asn216, GLAST-1 possesses an additional one at Asn35. The putative N-glycosylation consensus motifs (Asn-Xaa-Ser/Thr) were deleted by replacement of Asn206 and/or Asn216 by Thr using site-directed mutagenesis (mutants N206T, N216T and N206,216T). The cDNAs encoding wild-type GLAST-1 and the three glycosylation-defective transport proteins were expressed in the *Xenopus laevis* oocyte system. Immunoprecipitation of the [^{35}S]methionine-labeled and glycopeptidase-F-treated transporter molecules indicates that GLAST-1 is glycosylated at Asn206 and Asn216, whereas Asn35 remains unglycosylated. To assess a possible functional role of the two glycosylation sites wild-type and glycosylation-deficient GLAST-1 were expressed in *Xenopus* oocytes and characterized functionally by using the whole-cell voltage-clamp technique. The results prove that N-glycosylation has no impact on the transport activity of GLAST-1.

Keywords. GLAST-1; L-glutamate/L-aspartate transporter; site-directed mutagenesis; N-glycosylation; functional analysis.

L-Glutamate is the predominant neurotransmitter at most excitatory synapses in the central nervous system [1]. Its concentration in the synaptic cleft increases to about 1 mM during neurotransmission. This concentration is rapidly reduced to a micromolar level in the resting state by a high-affinity Na^+-dependent uptake system, thus terminating the overall process of neurotransmission and protecting neurons from the excitotoxic effect of L-glutamate upon prolonged action [2, 3]. Glutamate can be reaccumulated from the synaptic cleft by glial uptake [4, 5] or by the presynaptic nerve terminal [6]. GLAST-1 was the first purified and cloned brain-specific L-glutamate transporter expressed in glial cells, e.g. Bergmann glia cells of cerebellum [7]. We have isolated GLAST-1 from rat brain as a protein species of 64 and 70 kDa (Schulte, S. and Stoffel, W., unpublished results). The difference in molecular mass is due to heterogeneity in the oligosaccharide chain. Deglycosylation of the two species with glycopeptidase F (PNGase F) gave a single polypeptide of 56 kDa. The 70-kDa product is the predominant, probably mature, form of GLAST-1. The two forms were separated by lectin affinity chromatography using lentil lectin which is specific for the α-1,6-linked fucose to the innermost GlcNAc of the core and wheat germ agglutinin for the 70-kDa species of GLAST-1 (Schulte, S. and Stoffel, W., unpublished results). Two additional glutamate transporters have been discovered, GLT-1, supposedly a glial transporter [8, 9], and EAAC-1, expressed more strongly in kidney and gut, than in brain [10]. These excitatory amino acid transporters form a new family of integral membrane proteins displaying ≈50% amino acid identities. The neutral amino acid carrier ASCT-1 [11] also called SATT [12] with ≈37% sequence identity to the glutamate transporter family appears to be a further member of this transporter family. The enlarged L-glutamate transporter family GLAST-1, GLT-1, EAAC-1, and ASCT-1/SATT exhibit a unique membrane topology (Fig. 1) deviating from the supposed 12 transmembrane domain motif of previously discovered neurotransmitter transporters such as the 4-aminobutyric acid (GABA) transporter [13]. One common structural feature is the presence of putative glycosylation sites in the extended extracellular loop joining the transmembrane helices TMH 3 and TMH 4 (Fig. 1). It is remarkable that all cloned neurotransmitter transporters possess this loop with several possible N-glycosylation motifs being a clue to the importance of glycosylation for the transporters [14].

Within the known members of the enlarged L-glutamate transporter family these N-glycosylation sites are localized at similar positions within the amino acid alignment of GLAST-1: GLAST-1, Asn206, Asn216; GLT-1, Asn205, Asn215; EAAC-1, Asn178, Asn195; and ASCT-1/SATT, Asn201, Asn206. GLAST-1 and EAAC-1 possess further putative N-glycosylation

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Abbreviations. GLAST-1, L-glutamate/L-aspartate transporter; TMH, transmembrane helices; N206T, N216T and N206,216T, mutants of GLAST-1 in which Asn206, Asn 216 or both have been replaced by Thr; PNGase F, glycopeptidase F.

Enzyme. Glycopeptidase F, peptide–N^4–(*N*-acetyl-β-glucosaminyl)-asparagine amidase (EC 3.5.1.52).

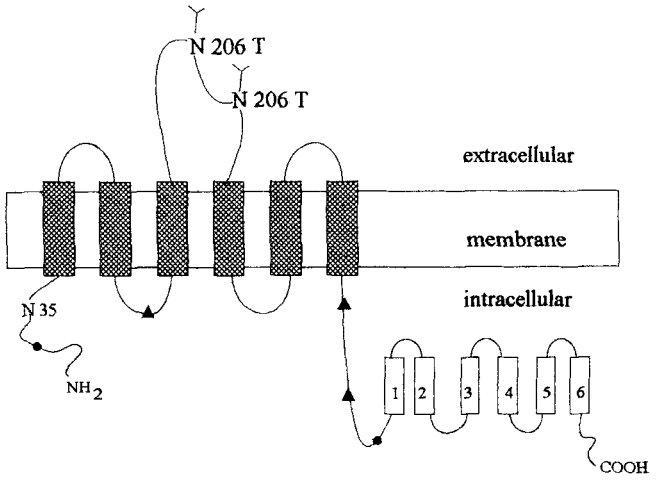

Fig. 1. Localisation of posttranslational modification sites in the putative secondary structure model of GLAST-1. The amino acid substitutions investigated here are indicated and the N-glycosylation sites represented as branched lines. Within the enlarged glutamate transporter family there is a consensus regarding the N-terminal six transmembrane helices (shown as striped rectangles) with N- and C-termini on the cytoplasmic side of the membrane. Six short hydrophobic domains indicated as small rectangles are highly conserved throughout the family of glutamate transporters. The exact topology of the C-terminus awaits experimental clarification. (●) Two putative protein kinase A sites of GLAST-1; (▲) three putative kinase C sites.

motifs at Asn35 and Asn43, Asn85, respectively. The function of the carbohydrate groups of these transport proteins is not clear. However, several studies suggest that carbohydrate moieties may play an important role in transporter function. A study in which sialidase treatment of synaptosomes resulted in a 40% decrease in the V_{max} of dopamine transport indicates that carbohydrate groups might be required for optimal dopamine uptake [15]. Recent studies have shown that glycosylation is essential for the activity of the glucose transporter GLUT-1 and a purified glycine transporter [16, 17].

In the work described in this paper, we have localized the glycosylation sites of GLAST-1 to Asn206 and Asn216, using GLAST-1 mutants in which Asn206 or Asn216 or both were replaced by Thr (N206T, N216T or N206,216T). Our results strongly suggest that N-glycosylation of GLAST-1 has no detectable influence on the affinity of L-glutamate uptake. The Na^+ and voltage dependence of glutamate uptake and the sensitivity of GLAST-1 to L-glutamate uptake inhibitors is unaffected by the absence of one carbohydrate chain (N206T, N216T) as well as by the total lack of carbohydrate groups (N206,216T). The strong tendency of GLAST-1 to form homodimers is most probably mediated by the carbohydrate moieties. Whether the homodimerisation is functionally important under *in vivo* conditions has not yet been determined.

MATERIALS AND METHODS

Materials. U.S.E. mutagenesis kit was purchased from Pharmacia. T4 DNA ligase, SP6 polymerase and restriction enzymes were obtained from Gibco-BRL or Boehringer Mannheim. PNGase F was from Boehringer Mannheim, and protein-A−Sepharose CL-4B was purchased from Sigma. The voltage-clamp amplifier of Warner Instruments Corp. and the hard- and software package ISO2 from MFK (Frankfurt) were used in the whole-cell voltage-clamp experiments.

Site-specific mutagenesis. The plasmid pSP-GLAST [3] served as template in the site-specific mutagenesis performed by the unique-site elimination procedure [18]. The procedure applies two mutagenic oligonucleotide primers. One primer (SP-*Eco*RV: 5′-CAGCAAATGAGATCTCTGGAAC-3′) eliminates a unique *Eco*RV site in the 3′-untranslated region of rat GLAST-1 cDNA and creates a unique *Bgl*II site instead. This primer served as selection primer in all three mutagenesis reactions. Two mutant oligonucleotides were designed to replace the codons for Asn at the putative glycosylation sites Asn206 (N206T: 5′-CAGGCCACCGAAACACTGTTG) and Asn216 (N216T: 5′-CGTGATCAACACCGTGTCAG) by a Thr codon. The mutagenic primers N206T and N216T were used separately to introduce point mutations. Both oligonucleotides were employed during an additional mutagenesis reaction to create a double mutant. The three resulting mutated cDNAs were subcloned into pSP-GLAST by using the restriction enzymes *Mlu*NI and *Kpn*I. The nucleotide sequences of the subcloned DNA fragments were verified by sequencing between the sites of the indicated restriction enzymes.

cRNA preparation and expression in oocytes. Wild-type and mutant pSP-GLAST-1 cDNA were linearized with *Eco*RI. The respective cRNAs were synthesized from their corresponding templates using SP6 RNA polymerase. Stage V−VI oocytes were defolliculated with collagenase and injected with 40 nl cRNA (0.5 μg/μl). Oocytes were maintained in Barth's modified saline at room temperature [19]. cRNA preparation and injection of the oocytes has been described previously [7].

Electrophysiology. Electrogenic transport was assayed by voltage-clamp using a two-electrode voltage-clamp amplifier. Briefly, oocytes were voltage-clamped at −90 mV and continuously superfused with Barth's modified saline. Microelectrodes filled with 3 M KCl had resistances ranging over 1−2 MΩ. In experiments in which the $[Na^+]$ of the bath solution was varied, Na^+ was equimolarly substituted by choline. The data of the concentration/response curves were fitted by non-linear regression to minimize the squared errors to the Hill equation $I = I_{max} \times [S]^n/([S]^n + K_m^n)$, where I_{max} is the normalized maximal current, [S] the substrate concentration, K_m the apparent affinity constant, and n the cooperativity parameter. Data were expressed as mean ± SD.

Immunoprecipitation and glycosidase treatments. Oocytes were injected with GLAST-1-specific cRNAs and incubated in Barth's modified saline containing [^{35}S]methionine (2.5 mCi/ml) for 24 h. Oocytes were washed three times with Barth's modified saline, homogenized in a solution composed of 20 mM Tris/HCl pH 7.6, 0.1 M NaCl, 1% Triton X-100, 0.5% SDS, and 1 mM phenylmethanesulfonyl fluoride, then centrifuged at 12000*g* for 5 min at 4°C. The supernatants were incubated at 4°C overnight with anti-GLAST antibody [7]. Antigen-antibody complex was bound by protein-A−Sepharose at 4°C for 2 h. Precipitated proteins were dissociated and reduced by incubating the samples dissolved in Laemmli sample buffer (0.0625 M Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.001 bromphenol blue) for 2 min at 95°C. The proteins were analysed by polyacrylamide gel electrophoresis [20] followed by fluorography [21].

The glycosidase treatment was performed in the homogenisation buffer without SDS after the centrifugation step. PNGase F (0.2 U/oocyte) from *Flavobacterium meningosepticum* was added and incubated for 2 h at 37°C. Reactions were stopped by adding 0.05 vol. 10% SDS. The deglycosylated transporter was subjected to immunoprecipitation as described above.

RESULTS

GLAST-1 is glycosylated in *Xenopus laevis* oocytes. X. *laevis* oocytes perform most of the co- and post-translational modifica-

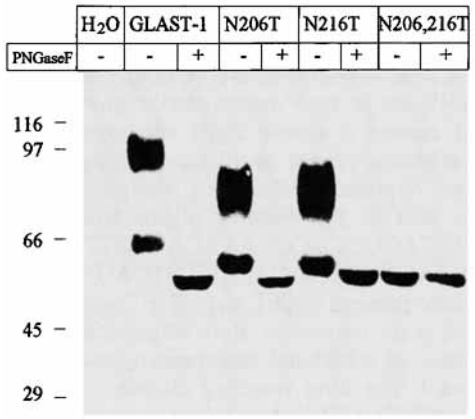

Fig. 2. Deglycosylation of cloned wild-type and mutant GLAST-1 expressed in oocytes with PNGase F. Wild-type and mutant GLAST-1 cRNAs were injected into *Xenopus* oocytes, incubated in Barth's medium supplemented with [^{35}S]methionine. The oocytes were homogenized and immunoprecipitated before (lanes 1, 2, 4, 6, 8) and after treatment with PNGase F (lanes 3, 5, 7, 9) as described under Experimental Procedures. The samples were analyzed by SDS/PAGE and fluorography. Sizes of marker proteins are indicated in kDa.

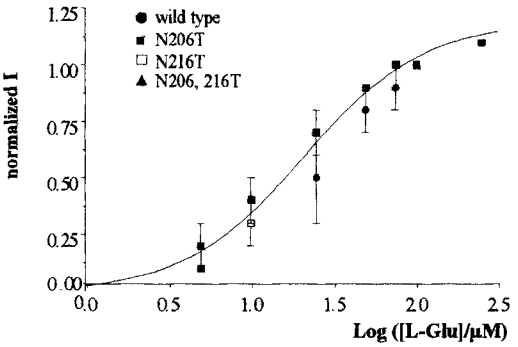

Fig. 3. Apparent K_m value for glutamate of wild-type and glycosylation-site mutant GLAST-1. The values represent the means, the bars the standard deviation of the GLAST-1 evoked currents (wild-type or mutants) of three different oocytes. The current of each oocyte was normalized to the current amplitude at 100 μM L-Glu. The solid line is fitted to the data of wild-type GLAST-1 by minimizing squared errors according to the equation $I = I_{max} \times [S]^n/([S]^n + K_m^n)$ with an apparent K_m value of 20 ± 1 μM L-glutamate. The best fit to the data of the glycosylation mutants N206T, N216T and N206,216T yielded nearly identical concentration/response curves with K_m values of 20 ± 1 μM, 21 ± 1 μM and 19 ± 1 μM for L-glutamate respectively (not plotted). The cooperativity coefficients n were between 1 and 1.2 ± 0.1. The holding potential was −90 mV (90 mM $[Na^+]_o$).

tions of proteins [19]. Wild-type and mutant GLAST-1 was functionally expressed in *X. laevis* oocytes in the presence of [^{35}S]methionine upon microinjection of the respective cRNAs. The SDS/PAGE pattern of labeled wild-type and mutant GLAST-1 after immunoprecipitation is shown in Fig. 2. Water-injected control oocytes expressed no immunoprecipitable protein. Injection of GLAST-1 cRNA into oocytes led to the expression of the transporter manifested as a broad band with an apparent molecular mass of 60–65 kDa (lane 2) in SDS/PAGE (10%). The higher-molecular-mass band represents the dimeric form of the transporter. The genuine mature transporter isolated from rat brain also tends to dimerize (Schulte, S. and Stoffel, W., unpublished results). A similar high tendency to aggregate has been reported for GLT-1, which is one of the three recently cloned glutamate transporters [22]. Whether this is a common feature of the members of the glutamate transporter family remains to be studied. The glycosylation of GLAST-1 in the oocyte expression system was proven by treatment of the labeled transporter with PNGase F, an enzyme which hydrolyzes the GlcNAc-Asn linkage of most types of N-linked carbohydrate groups from glycoproteins. The PNGase F treatment reduced the apparent molecular mass of GLAST-1 to 56 kDa (lane 3).

Glycosylation sites were localized by site-directed mutagenesis. The primary structure of GLAST-1 derived from the nucleotide sequence of its cloned cDNA suggests N-glycosylation at three putative canonical sites, Asn35, Asn206, and Asn216. The absence of a cleavable signal sequence suggests a cytosolic localisation of the N-terminus. Therefore the predicted six transmembrane helices would place Asn35 to the cytosol and leave only Asn206 and Asn216 for glycosylation (Fig. 1). We deleted the glycosylation sites by replacing the Asn residues at amino acid position 206 and/or 216 by Thr. The wild-type and mutant cDNAs (N206T, N216T, and N206,216T) were *in vitro* transcribed to their respective cRNAs. The resulting cRNAs were injected into oocytes for expression. Fig. 2 indicates that the mutants N206T and N216T were only monoglycosylated with an apparent molecular mass of 57–60 kDa (lanes 4 and 6). Expression of the double mutant N206,216T yielded a 56-kDa transporter (lane 8) which did not change its electrophoretic mobility after treatment with PNGase F (lane 9). The double mutant therefore represents the nonglycosylated form of GLAST-1 with an apparent molecular mass which is in good agreement with the PNGase-F-treated wild-type transport protein (lane 3). These results also exclude the utilization of the putative N-terminal glycosylation site at Asn35. GLAST-1 is glycosylated only at positions Asn206 and Asn216.

Nonglycosylated GLAST-1 does not dimerize. Mature native GLAST-1 isolated from rat brain (Schulte, S. and Stoffel, W., unpublished results) or the wild-type cRNA expressed in oocytes are glycosylated and tend to form homodimers. Since gel electrophoresis was performed under reducing condition most probably disulfide bridges are not required for dimerisation of GLAST-1. When the two mutants N206T and N216T were expressed in oocytes these monoglycosylated forms also dimerized (Fig. 2, lanes 4 and 6). In contrast to the glycosylated transporters, the immunoprecipitation of the double mutant N206,216T with the deleted glycosylation sites Asn206 and Asn216 or the PNGase-F-treated polypeptides yielded a single, sharp band representing the monomeric form of GLAST-1 (lanes 8, 3, 5 and 7). Therefore we presume that glycosylation is required for homodimerization of GLAST-1.

The mutant nonglycosylated GLAST-1 is functionally active. The impact of glycosylation on the L-glutamate transport function of GLAST-1 was analyzed in *Xenopus* oocytes expressing wild-type and glycosylation-deficient transporter by using the voltage-clamp technique. Our previous results provide evidence that GLAST-1-mediated L-glutamate uptake is electrogenic with a proposed stoichiometry of three Na^+ ions cotransported/molecule L-glutamate into the cell accompanied by the counter transport of one K^+ ion [23].

To investigate the influence of *N*-glycosylation on the affinity of GLAST-1 for L-glutamate, the apparent K_m values of wild-type and mutant GLAST-1 were determined (Fig. 3). At an extracellular Na^+ concentration ($[Na^+]_o$) of 90 mM, the half-maximal current was obtained at a L-glutamate concentration of 20 ± 1 μM, which is in good agreement with our previously published results [24]. The affinity for L-glutamate of the glycosyla-

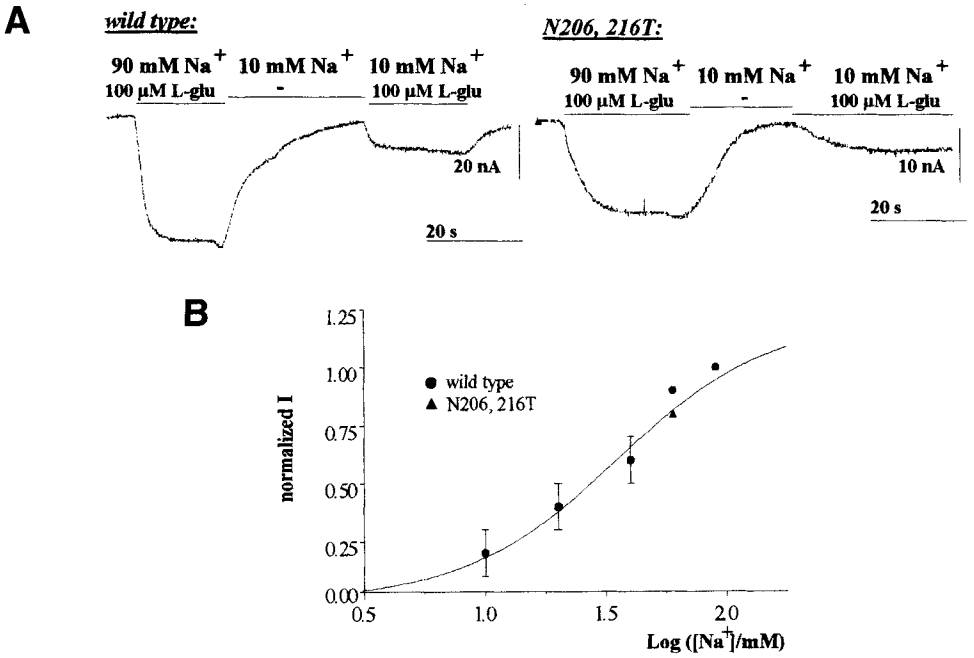

Fig. 4. Sodium dependence of $I_{GLAST-1}$ and $I_{N206,216T}$. (A) Original traces recorded at the indicated $[Na^+]_o$. Changing the extracellular sodium concentration diminished I_{max} of $I_{GLAST-1}$ and $I_{N206,216T}$ in the same manner. (B) Concentration/response curve for $[Na^+]_o$. The values represent the means, the bars the standard deviation from three oocytes. The current of each oocyte was normalized to the current amplitude at 90 mM $[Na^+]_o$. The solid line was fitted to the data of wild-type GLAST-1 by minimizing squared errors according to the equation in the Experimental Procedures with an apparent K_m value of 29 ± 1 mM (cooperativity coefficient $n = 1.8$). The best fit to the data obtained from the glycosylation mutant N206,216T yielded a nearly identical concentration/response curve with a K_m value of 31 ± 1 mM (cooperativity coefficient $n = 1.8$) (not plotted). The currents were recorded at 100 µM L-Glu (holding potential −90 mV).

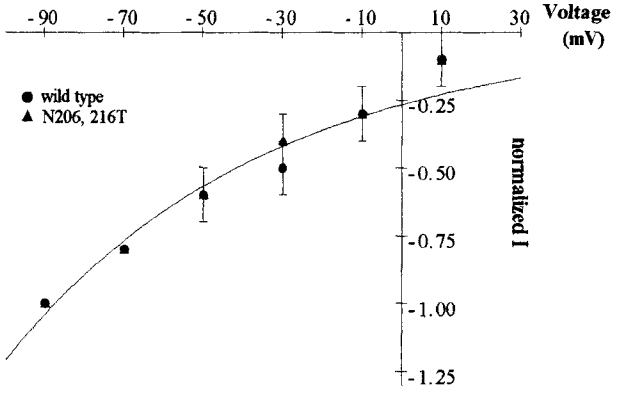

Fig. 5. Voltage dependence of $I_{GLAST-1}$ and $I_{N206,216T}$. Data shown are mean ± SD obtained from three different oocytes. Currents were normalized to the current amplitude at −90 mV. The solid line was fitted by eye. $[L\text{-Glu}]_o$ and $[Na^+]_o$ were 100 µM and 90 mM respectively.

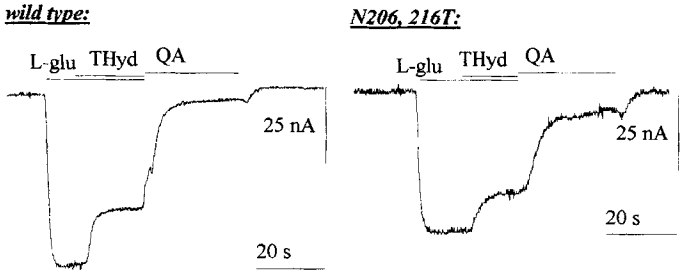

Fig. 6. Inhibitors of L-glutamate uptake. Simultaneous application of 1 mM DL-*threo*-3-hydroxyaspartate (THyd) and 100 µM L-Glu reduced the inward current by 24%. Application of 100 µM quisqualic acid (QA) induced a current with less than 10% of the amplitude as compared with $I_{GLAST-1}$ at 100 µM L-glutamate concentration. Oocytes expressing the wild-type or mutant cRNA show similar sensitivity in response to the different inhibitors.

tion-deficient transporters N206T, N216T, and N206,216T showed no significant differences with apparent K_m values of 20, 21, and 19 ± 1 µM for L-glutamate, respectively.

Fig. 4 A shows that the replacement of extracellular Na^+ by choline$^+$ blocked the currents $I_{GLAST-1}$ and $I_{N206,216T}$ to the same extent. Fitting the inward currents recorded at stepwise increased $[Na^+]_o$ (constant L-Glu concentration of 100 µM) to a Hill equation yielded K_m values for the wild-type and double mutant N206,216T of 35 and 33 ± 1 mM Na^+, respectively (Fig. 4B). The strict Na^+-dependence of GLAST-1, therefore, is not affected by the total lack of carbohydrate moieties. Next we studied the voltage dependence of the glycosylation-deficient transporter N206,216T. The peak currents from wild-type and mutant N206,216T plotted as a function of voltage were roughly superimposed, underlining our findings that glycosylation has no impact on the functional properties of GLAST-1 (Fig. 5).

The sensitivity of wild-type and glycosylation-defective transporter N206,216T to L-glutamate-uptake blockers was analyzed as described before [24]. Earlier studies have shown that all known glutamate-uptake inhibitors were either substrates or had no significant effect on transport [4, 6, 24, 25]. DL-*threo*-3-Hydroxyaspartate is a well known competitive inhibitor of L-glutamate transport [26]. Its potency to inhibit $I_{GLAST-1}$ or $I_{N206,216T}$ was not distinguishable (Fig. 6). Quisqualic acid also induced similar inward currents in GLAST-1- and N206,216T-expressing oocytes. Kainic acid was not a substrate for the wild-type trans-

porter nor for the mutant N206,216T. Summarizing the electrophysiological measurements, we conclude that the presence or absence of N-glycosylation has no significant influence on GLAST-1-mediated L-glutamate uptake.

DISCUSSION

The recently discovered three L-glutamate transporters of the central nervous system [7, 8, 10] and the closely related transporter protein ASCT-1/SATT form a family of integral membrane proteins sharing a common membrane topology different from known neurotransmitter transporters such as the 4-aminobutyric acid, 5-hydroxytryptamine, noradrenaline, and glycine transporters. Secondary structure prediction of GLAST-1 suggests six transmembrane α-helices at the N-terminus which were proposed for GLT-1, EAAC-1, and ASCT-1/SATT at similar positions. No obvious topology can be derived for the C-terminus. Experimental approaches are needed to delineate the C-terminal topology. Within the extracytoplasmic loop between TMH 3 and TMH 4 all known members of the glutamate transporter family including ASCT-1/SATT possess two putative N-glycosylation sites localized in similar positions. Beyond these two conserved carbohydrate attachment sites GLAST-1 possesses a further possible N-glycosylation site at Asn35. The ability of the carbohydrate groups to modulate the physical properties of the protein to which they are attached, especially the overall folding of the nascent polypeptide chain as well as the protection against proteolysis, is well documented [27, 28]. More importantly there is increasing evidence that carbohydrate groups modulate the intrinsic functional properties of a wide variety of membrane proteins [29]. These include the modulation of signal transduction as suggested for rhodopsin [30], recognition processes at the cell surface [29], and transporter activity in the case of the mammalian dopamine, glucose, and glycine transporter [13–15].

We report here experimental evidence for the utilization of only two of the three glycosylation consensus motifs of GLAST-1, namely those between TMH 3 and TMH 4. The high sequence identity and the topological similarity within the enlarged L-glutamate transporter family (GLAST-1, GLT-1, EAAC-1, and ASCT-1/SATT) suggests that the other members of this family are also glycosylated at the conserved putative N-glycosylation sites between TMH 3 and TMH 4. Carbohydrate groups of plasma membrane proteins are always located at the extra-cytoplasmic surface. Our experiments prove that the N-terminal glycosylation motif Asn35-Ile-Thr of GLAST-1 is not utilized. This supports the idea that the N-terminus is located on the cytosolic side of the plasma membrane.

The mammalian Na^+-dependent L-glutamate transporters show significant similarity ranging between 27–32% to the proton-coupled GLTP L-glutamate transporters of *Escherichia coli* [33], *Bacillus stearothermophilus*, and *Bacillus caldotenax*. The most prominent difference between GLAST-1 and the proton-coupled prokaryotic L-glutamate transporters is the insertion of a sequence of approximately 50 amino acid residues in the extracytoplasmic loop which allows the attachment of the carbohydrate groups of GLAST-1 and might play a role in the selective ion dependence of the prokaryotic and eukaryotic transporters. However, our data underline the Na^+-dependence of both the glycosylated wild type and the nonglycosylated mutant N206,216T of GLAST-1 and therefore rule out the involvement of the carbohydrate structures in the selectivity for Na^+ or H^+ ions.

In this study we present evidence that the strong homodimerization tendency *in vitro* of GLAST-1 is due to the presence of the glycan structure. Whether this aggregation also occurs in the plasma membrane by lateral diffusion in the lipid bilayer is not clear. It is conceivable that the strong tendency to aggregate facilitates the enrichment of GLAST-1 in astroglia processes bordering glutamatergic synapses. Clustering of GLAST-1 was shown in the plasma membrane of astroglia and was proposed to participate in synaptic plasticity [9]. Indeed, astroglia has been directly connected with synaptic plasticity of the developing visual cortex [31, 32]. However, since the nonglycosylated GLAST-1 mutant N206,216T does not dimerize but yet catalyzes an unimpaired L-glutamate neurotransmitter uptake, glycosylation and dimer formation do not seem to be involved in the intrinsic transport function of GLAST-1.

In this paper we present data characterizing the impact of N-glycosylation on the cloned neurotransmitter transporter GLAST-1. The results reported here contribute to our understanding of the relationship between structure and function of the L-glutamate transporters.

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