The reuptake of excitatory amino acids, such as glutamate, terminates excitatory signals and prevents the persistence of excitotoxic levels of glutamate in the synaptic cleft. The L-glutamate/L-aspartate transporter (GLAST-1) is the first member of the recently discovered glutamate transporter family, which includes GLT-1 and EAAC1. The neutral amino acid carrier ASCT1 is structurally closely related to this new family of membrane proteins. Transmembrane transport of neutral amino acids is expected to differ in its binding site from that of the acidic excitatory amino acids glutamate and aspartate. Three positively charged amino acid residues, Arg-122, Arg-280, Arg-479, and one polar Tyr-405 are conserved in all glutamate transporters. They are replaced by apolar amino acid residues in the ASCT1 sequence. We exchanged these residues in the GLAST-1-specific cDNA by site-directed mutagenesis. cRNAs of these mutants were expressed in the Xenopus oocyte system. The functional characterization of the mutants R122I and R280V and the double mutant R122I/R280V revealed that the mutations have no influence on the intrinsic properties and kinetics of glutamate transport but alter the $K_m$-values for L-aspartate and the competitive inhibitor D,L-threo-3-hydroxy aspartate. Substitutions of Tyr-405 by Phe (Y405F) and Arg-479 (R479T) by Thr completely inactivate the glutamate transporter. Immunoprecipitations of $[^{35}S]$methionine-labeled transporter molecules indicate similar expression levels of wild-type and mutant transporters. Immunostaining of oocyte sections clearly proves the correct targeting to and integration of the mutant GLAST-1 proteins in the plasma membrane. Our results suggest the pivotal function of the hydroxy group of the highly conserved Tyr-405 and the positively charged Arg-479 in the binding of the negatively charged acidic neurotransmitter glutamate.

The regulation of the neurotransmitter concentration in the synaptic cleft is an important component of the synaptic transmission process (1, 2). It is mediated by high affinity, Na$^+$-dependent uptake systems. The neurotransmitter transporters have been characterized by the uptake of radiolabeled substrates in brain slices (1), synaptosomes (3), and isolated cells (4) for their substrate specificity and ion dependence. A number of neurotransmitter carriers have been cloned on the basis of structural homologies and found to form a family of related proteins (5, 6). The recently discovered Na$^+$-dependent glutamate transporters (GLAST-1, GLT-1, and EAAC1) represent a new family of integral membrane proteins (7–9) with approximately 50% amino acid identities. They show significant similarities ranging between 27 and 32% to the proton L-glutamate transporter protein (GLTP) of Escherichia coli (10), Bacillus stearothermophilus, and Bacillus caldothermace and to the dicarboxylate transporter (DCTA) of Rhizobium meliloti (11). 

Models proposed for the membrane integration of the glutamate transporters show a consensus regarding six N-terminal transmembrane helices with a large extracellular loop between the proposed transmembrane helices 3 and 4. We have shown that two of these putative N-glycosylation sites of GLAST-1 localized in this extracellular loop are N-glycosylated. These sites are also common to GLT-1, EAAC1, and ASCT1, and it is reasonable to suggest that they are glycosylated in a similar manner (12). On the other hand, the topology of the highly conserved C-terminal part of the protein awaits experimental clarification (13).

The cloned transporter GLAST-1 cotransports glutamate with three sodium ions across the plasma membrane, whereas one potassium ion is countertransported out of the cell (14). There is evidence from studies in the salamander retinal glia that a pH-changing anion, probably a hydroxyl ion, is countertransported (15). Despite the extensive electrophysiological characterization of the transport process (16–18), it remains unclear which amino acid residues of GLAST-1 are involved in the uptake process. It is reasonable to assume that charged or polar residues of the glutamate transporter are involved in the binding and translocation of glutamate and its cosubstrates. Mutagenesis has been carried out on Lys-298 and His-326 of the glutamate transporter GLT1. Substitution of these two residues with polar and positively charged residues leads (in the case of Lys-298) to a reduced transport activity, which has been interpreted as a targeting defect, whereas the severely impaired transport by GLT1 mutated at His-326 has been referred to a participation of His-326 in the putative proton translocation process of the transporter (19).

Neutral amino acids in mammalian cells are predominantly transported by two Na$^+$-dependent uptake systems ASC (predominantly Ala, Ser, and Cys) and A (predominantly Ala). The recently cloned neutral amino acid transporter ASCT1 (20) also called SATT (21) resembles the properties of the ASC transporter and displays $\sim$37% sequence identity with the structurally related excitatory amino acid transporter family.

In the present study, we analyzed the contribution of positively charged or polar residues of GLAST-1 to the binding and/or transport of the negatively charged neurotransmitter glutamate. We considered charged or polar residues of GLAST-1, which are conserved throughout the eukaryotic and prokaryotic glutamate transporters but are replaced in the neutral amino acid transporter by apolar residues. These res-
ides were substituted by the amino acid residues present at the respective sites of the sequence of ASCT1. We determined the $K_m$ values for glutamate and Na$^+$ and measured the voltage dependence of these mutant GLAST-1 in the Xenopus oocyte system. Mutagenized GLAST-1 with the substitutions R122I and R280V and the double mutant R122I, R280V exhibits nearly unaffected transport kinetics as compared to the wild type. However the $K_m$ values for THA$^+$ and aspartate of all three mutants are changed in a similar manner. GLAST-1 mutants Y405F and R479T and the quadruple mutant R122I, R280V, Y405F, R479T (Q) revealed that Tyr-405 and Arg-479 are essential for glutamate transport activity. We propose that the hydroxy group of the conserved Tyr-405 and the positively charged Arg-479 contribute to the binding of the acidic neurotransmitter glutamate.

**EXPERIMENTAL PROCEDURES**

Site-specific Mutagenesis—The plasmid pSP-GLAST-7 (22) served as template in the site-specific mutagenesis (22). Primer SP-EcoRV (5'-CAGCAGAATGAGACTCTCGGAAC-3'), which eliminates a unique EcoRV site in the 3'-untranslated region of rat GLAST-1 cDNA but creates a unique BglII site was selected as sequence primer in all mutagenesis reactions. Four mutant oligonucleotides replaced the codons as follows: Arg-122 by Ile (R122I), GGAAGATGGGGATGATAGCTGTGGTC; Arg-280 by Val (R280V), GCCATCATGGTATTGGTAGCGGTG; Tyr-405 by Phe (Y405F), GCCCTCCTGAAGAAATGGCTG; and Arg-479 by Thr (R479T), GACGCCCTCAACACCCACCAACG. The four resulting mutated cDNAs were subcloned into pSP-GLAST, and the nucleotide sequences of the subcloned DNA fragments between the sites of the used restriction enzymes were determined. The double mutant R122I, R280V and the quadruple mutant Q was constructed by standard cloning procedures using the synthesized mutant constructs bearing the respective point mutations.

dRNA Preparation and Expression in Oocytes—Wild-type and mutant pSP-GLAST-1 cDNA were linearized with restriction enzymes and injected with 40 nl of cRNA (0.5 μg/μl) RNA polymerase. Stage V-VI oocytes were defolliculated with collagenase and injected with 40 nl of cDNA (0.5 μg/μl). RNA preparation and injection of the oocytes has been described previously (7, 23).

**Flux Measurements**—24–72 h after injection of GLAST-cRNA, single oocytes were placed in 1.5 ml reaction tubes and incubated for 15 min at 21–23°C in Barth’s modified saline supplemented with 0.6 μM [35S]methionine (2.5 mCi/ml) for 24 h. Oocytes were further processed and blocked with 3% bovine serum albumin in PBS at 4°C for 1 h. Sections were incubated for 1.5 h at 4°C with anti-GLAST antibody in 1% bovine serum albumin/PBS, washed five times with PBS for 5 min, and incubated for 30 min at room temperature with fluorescein 5'-isothiocyanate-conjugated goat anti-rabbit IgG. After washing the sections five times with PBS for 10 min, they were sealed with coverslips, examined with a Zeiss Axioskop fluorescence microscope, and photographed with a Zeiss MC100 camera.

Materials—U. S. E. mutagenesis kit was purchased from Pharmacia Biotech Inc. T4 DNA ligase, SP6 polymerase, and restriction enzymes were obtained from Life Technologies, Inc. or Boehringer Mannheim. [35S]Glutamate and L-[3H]alanine was from Amersham Corp. Fluorescein isothiocyanate-gaot anti-rabbit IgG and protein A-Sepharose CL-4B were purchased from Sigma. The voltage clamp amplifier of Warner Instruments Corp. and the hardware and software package IS02 from MFK (Frankfurt) were used in the whole cell voltage clamp experiments.

**RESULTS**

Site-directed mutagenesis of GLAST-1 cDNA and Expression of the Mutant Transporters—The amino acid sequences of the known mammalian glutamate transporters GLAST-1, GLT-1, and EAAC1 and the prokaryotic dicarboxylate transporter DCTA of R. meliloti on the one hand and the neutral amino acid transporter ASCT1 on the other exhibit remarkable similarity (Fig. 1). There are three conserved positively charged arginines (Arg-122, -280, and -479) and the polar Tyr-405, which are common to the glutamate transporter, that are replaced by apolar residues in the ASCT1 sequence. They are marked by filled circles in Fig. 1. We used site-directed mutagenesis for the substitution of Arg-122 (R122I), Arg-280 (R280V), Tyr-405 (Y405F), and Arg-479 (R479T) of GLAST-1 by the corresponding amino acid residues of the ASCT1 sequence: Ile, Val, Phe, and Thr, respectively. The double mutant R122I, R280V and the quadruple mutant Q were constructed as described under “Experimental Procedures.” The mutations were verified by DNA sequencing and transcribed in vitro to their respective cRNAs. Wild-type and mutant GLAST-1 cRNAs were micro-injected for functional expression in Xenopus oocytes in the presence of [35S]methionine. The autoradiogram of labeled wild-type and mutant GLAST-1 protein immunoprecipitated with GLAST-1-specific antibodies (7) and separated by SDS-polyacrylamide gel electrophoresis is shown in Fig. 2. Wild-type GLAST-1 cRNA was translated into a protein visible with an apparent molecular mass of 60–65 kDa (lane 2), whereas water-injected control oocytes expressed no immunoprecipitable protein (lane 1). The expression rate of the mutant transporter cRNAs are similar (lanes 3–8). Therefore, the amino acid exchanges have no influence on the expression efficiency and the stability of the proteins. The expressed GLAST-1 (12) as well as the mature transporter isolated from rat brain tend to dimerize. The strong and broad signal at high molecular mass (90–110 kDa) represents the dimeric form of the transporter. A similar high tendency to aggregate has been reported for GLT-1 (19).

**Functional Characterization of the Mutants R122I and R280V and the Double Mutant R122I, R280V**—The impact of the point mutations on the function of the glutamate transporter GLAST-1 was analyzed in Xenopus oocytes expressing wild-type and mutant transporter by uptake studies of radioactive amino acids and with whole cell voltage clamp technique. To investigate the influence of the positively charged residues on the affinity of GLAST-1 for L-glutamate, the apparent $K_m$ values of wild-type and mutant GLAST-1 were determined (Fig. 3A). At an extracellular Na$^+$ concentration ([Na$^+$]o) of 90 mM, the half-maximal current was obtained at a L-glutamate concentration of 21 ± 3 μM (n = 11), which is in good agreement with our previously published results (12). The affi-
corded at stepwise increased $[\text{Na}^+]_o$ and constant $\text{L-Glu}$ concentration (100 $\mu$M). Currents were fitted to a Hill equation, which yielded $K_m$ values for $\text{Na}^+$ of wild type and the double mutant R122I, R280V of 32 ± 3 mM (n = 6) and 29 ± 5 mM (n = 4), respectively (Fig. 3B). The values of the mutant transporter R122I and R280V for $\text{Na}^+$ are neither significantly affected (R122I, 36 ± 5 mM, n = 4; R280V, 31 ± 1 mM, n = 2).

Glutamate transport is thought to be associated with conformational changes within the electrical transmembrane field. The influence of positively charged side chains as compared to neutral amino acid residues on the voltage dependence of the transport process has been explored in the experiment depicted in Fig. 3C. The peak currents of wild type and the double mutant R122I, R280V were plotted as a function of voltage. The resulting curves are roughly superimposed. This suggests that the elimination of two positive charges at amino acid positions 122 and 280 of GLAST-1 has no detectable effect on the voltage dependence of the transport process. The results for the mutants R122I and R280V were quite similar (data not shown).

However, the expressed mutant GLAST-1 transporters R122I and R280V as well as the resulting double mutant show a significant decrease (paired t test, p < 0.05) in the apparent $K_m$ value for the competitive inhibitor THA from 22 ± 6 $\mu$M (n = 6) for wild-type GLAST-1 to 10 ± 2 $\mu$M (n = 6), 11 ± 2 $\mu$M (n = 6), 10 ± 2 $\mu$M (n = 6) for R122I, R280V and R122I, R280V, respectively (Fig. 4A). The increase of the apparent affinity for $\text{L}$-aspartate is similar to that for the value for THA (Fig. 4B). The $K_m$ value for $\text{L}$-aspartate of wild-type GLAST-1 is 14.5 ± 2 $\mu$M (n = 4) and 9.5 ± 0.9 $\mu$M (n = 4) (significant smaller paired t test, p < 0.05) for the double mutant R122I, R280V. $\text{L}$-[14C]Alanine uptake experiments shown in Fig. 5C and electrophysiological measurements (data not shown) revealed that substitution of Arg-122 and 280 by Ile and Val, respectively does not enable GLAST-1 to transport neutral amino acids (Ala, Ser, Cys, and Thr) with a higher efficiency than the controls.

Functional Characterization of the Mutants Y405F and R479T and the Quadruple Mutant Q—The transporter mutants (Y405F, R479T, and Q) exhibited no detectable glutamate transport activity. Fig. 5A shows that superfusion of oocytes with $\text{L}$-[Glutamate] up to 500 $\mu$M evoked no detectable current in the oocytes expressing the mutagenized transporters Y405F, R479T, or Q. Additionally the $\text{L}$-[14C]glutamate uptake measurements depicted in Fig. 5B revealed that substitution of Tyr-405 (mutant GLAST-1 Y405F) and Arg-479 (mutant GLAST-1 R479T) by Phe and Thr, respectively abolishes glutamate transport activity. Furthermore, we assayed neutral amino acid transport activity of the mutant transporters Y405F, R479T, and Q by $\text{L}$-[14C]Alanine uptake experiments (Fig. 5C) and whole cell patch clamp recordings (data not shown). The two techniques revealed that there is no enhanced neutral amino acid transport activity as compared to the wild type.
Site-directed Mutagenesis of Glutamate Transporter

Fig. 3. Comparison of the basic L-glutamate transport properties of wild-type and mutant GLAST-1. A, L-Glu $K_m$ values. The values represent the means, and the bars represent the standard deviation of $I_{GLAST-1}$ ($n = 4–11$). The current of each oocyte was normalized to the current amplitude at 100 mM L-Glu. The solid line is fitted to the data of wild-type GLAST-1 by minimizing squared errors according to the equation $I = I_{\text{max}} \times [S]/(K_o + [S])$ with an apparent $K_o$ value of 21 ± 3 mM L-glutamate. The best fit to the data of the mutant transporters yielded nearly identical concentration response curves ($K_o$ values for L-glutamate: R122I, 22.5 ± 2.5 μM; R280V, 18 ± 4 μM; R122I, R280V, 18 ± 3 μM). The cooperativity coefficients were between 1 and 1.3 ± 0.1. The holding potential was −90 mV (90 mM Na$^+$). B, concentration response curve for Na$^+$. The values represent the means, and the bars represent the standard deviation from 4–6 oocytes. The current of each oocyte was normalized to the current amplitude at 90 mM Na$^+$. The solid line was fitted to the data of wild-type GLAST-1 by minimizing squared errors according to the equation mentioned under “Experimental Procedures” with an apparent $K_o$ value of 32 ± 3 mM (cooperativity coefficient = 1.8). The best fit to the data obtained from the mutants R122I, R280V, and R122I, R280V yielded a nearly identical concentration response curve with $K_o$ values of 36 ± 5, 31 ± 1, and 29 ± 5 mM, respectively (cooperativity coefficient = 1.8 ± 0.3). The currents were recorded at 100 μM L-Glu (holding potential, −90 mV). Only the data of the double mutant were plotted. C, voltage dependence of $I_{GLAST-1}$. Data shown are mean ± S.D. obtained from 4–6 different oocytes. Currents were normalized to the current amplitude at −90 mV. The solid line was fitted by eye. L-Glu$\text{L}$ and Na$^+$, were 100 μM and 90 mM, respectively.

Fig. 4. $K_m$ values of THA (A) and L-aspartate (B) for wild-type and mutant GLAST-1 transporters. Dose response curves as described in Fig. 3A, except that THA and L-Asp were applied instead of L-glutamate. The $K_m$ values for THA and L-Asp are significantly smaller for the mutants R122I and R280V and the double mutant R280V, R122I (paired t test; $p = 0.03$ and $p = 0.01$, respectively). The $K_m$ value for THA of wild-type GLAST-1 is 22 ± 6 μM ($n = 6$); for the mutants R122I, R280V, and R122I, R280V, $K_m$ values are 10 ± 2 μM ($n = 6$), 11 ± 3 μM ($n = 6$), and 10 ± 2 μM ($n = 6$), respectively. The apparent $K_m$ value for aspartate of wild-type GLAST-1 is 14.5 ± 2 μM ($n = 4$), and the value determined for the double mutant R122I, R280V is 9.5 ± 0.9 μM ($n = 4$). The cooperativity coefficients were between 1 and 1.3 ± 0.1. The holding potential was −90 mV (90 mM Na$^+$).
ies are presented in Fig. 6. In oocytes expressing wild-type GLAST-1 or mutants Y405F, R479T, or Q (Fig. 6, B, C, D, and E, respectively) a bright ring of fluorescence with similar intensity was observed at the perimeter of the oocytes, consistent with the localization of the transporter at or close to the cell surface. No accumulation of fluorescent-labeled proteins was detectable within the cell. In the case of water-injected control oocytes, no fluorescence was observed (Fig. 6A). The immunocytochemistry clearly indicates that the mutant transporters are correctly targeted to the plasma membrane. Therefore, Tyr-405 and Arg-479 might play an essential role in glutamate transport.

DISCUSSION

The recently discovered three L-glutamate transporters of central nervous system (7–9) and the neutral amino acid transport protein ASCT1 (20, 21) form a family of integral membrane proteins. They exhibit significant similarity to the prokaryotic proton L-glutamate transporter protein (GLTP) (10) and to dicarboxylate transporter (DCTA) (11). The hydropathy plots of the related eukaryotic transporters suggest a conserved membrane topology implicating a similar transport mechanism (26). The striking difference between the substrates of the neutral (e.g. alanine) and the acidic amino acid transporters (e.g. glutamate and aspartate) is the negatively charged carboxy group of the acidic neurotransmitter. Intriguing amino acid residues important for the recognition and discrimination of the different substrates are positively charged or polar. The amino acid residues Arg-122, Arg-280, Tyr-405, and Arg-479 are conserved throughout the eukaryotic and prokaryotic glutamate transporters. They are substituted by apolar residues in the neutral amino acid transporter ASCT1 (Fig. 1). In the study described here, we exchanged these amino acid residues of GLAST-1 for the residues of the neutral amino acid transporter ASCT1. The mutants R122I and R280V as well as the double mutant R122I,R280V expressed in the Xenopus oocyte
system show no significant differences in their apparent affinity for L-Glu and their Na\(^+\) and voltage dependence. GLAST-1 mutants R122I and R280V and the double mutant do not transport any substrate of the neutral amino acid carrier. This argues against a contribution of Arg-122 and Arg-280 to the substrate specificity of GLAST-1. Interestingly, the apparent \(K_m\) values for aspartate and the competitive inhibitor THA are decreased significantly. Arg-122 and Arg-280 are positioned at the boundaries between the putative intracellular hydrophilic loops and transmembrane domains 3 and 5, respectively. Although Arg-122 and -280 seem to be localized intracellularly, we included these residues in our investigations because transport is possibly mediated by conformational changes sequentially exposing the substrate binding site to the external and internal surfaces of the protein (27, 28). Charged residues frequently border hydrophobic regions of integral membrane proteins and thus contribute to their correct positioning within the membrane (29). A conformational change evoked by the lack of the charged residues in the mutant transporters might facilitate the transport of the less bulky substrates THA and aspartate in contrast to glutamate.

In contrast to Arg-122 and -280, which are localized in the N-terminal part of GLAST-1, Tyr-405 and Arg-479 are positioned in the C-terminal part of the protein. The topology of the C terminus derived from hydropathy plots is undefined (13, 26). Therefore, predictions concerning the extra- or intracellular localization of Tyr-405 or Arg-479 are not possible. Arg-122 and Arg-280 are positioned at identical positions in a highly conserved region of the related neutral amino acid transporter ASCT1, completely abolished L-glutamate transport. Since the expression level of the mutant transporter and the targeting to the plasma membrane is unimpaired (Figs. 2 and 6), our results strongly suggest that Tyr-405 and Arg-479 are essential for the glutamate transport process. A drawback to mutagenesis studies might be conformational changes of the protein introduced by amino acid substitution, which could complicate the interpretation of the direct role of the amino acid residue in the binding and transport of glutamate. The exchange of Tyr for Phe is nearly conservative. In addition, Phe (Y405F) as well as Thr (R479T) represent amino acids that are localized at identical positions in a highly conserved region of the related neutral amino acid transporter exhibiting a similar hydropathy plot. These facts argue against conformational changes of the affected protein domains.

Our interpretation is supported by modeling studies on the Clostridium symbiosum glutamate dehydrogenase structure. They implicate interactions of the \(\gamma\)-carboxylate group of glutamate with the \(\beta\)-OH of Ser-380 and Thr-193 and the \(\epsilon\)-NH\(_3\)\(^+\) of Lys-89 (30). This is in line with our results that the hydroxy group of Tyr-405 and the positively charged Arg-479 are essential for glutamate transport. It is conceivable that these amino acid residues, strongly conserved in the glutamate transporters but not in the neutral amino acid transporter ASCT1, interact with the \(\gamma\)-carboxylate group of glutamate.

The studies presented here provide new insights into the structure-function relationship of the glutamate transporter family. Further experiments will unravel the structural motifs involved in binding, substrate specificity, and translocation of the L-glutamate transporter.

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Functional Analysis of the High Affinity, Na\(^+\)-dependent Glutamate Transporter GLAST-1 by Site-directed Mutagenesis
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