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

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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 caldotenax* 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 three 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 GLT-1 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 ~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-

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idues 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[1] 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) served as template in the site-specific mutagenesis (22). Primer SP-*Eco*RV (5′-CAGCAAATGAGATCTCTGGAAC-3′), which eliminates a unique *Eco*RV site in the 3′-untranslated region of rat GLAST-1 cDNA but creates a unique *Bgl*II site served as selection 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), GGACCGCCCTCTTCGAAGCTTGGC; and Arg-479 by Thr (R479T), GGACCGCCTCACAACCACCACCAACG. 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.

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 of cRNA (0.5 $\mu g/\mu l$). cRNA 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 100 μM L-[^{14}C]glutamate or L-[^{14}C]alanine at a specific activity of 45 mCi/mmol. The oocytes were washed three times with 1 ml of Barth's modified saline, homogenized in 0.1 ml of 1% SDS, and transferred into 10 ml of Bray's solution. Radioactivity was determined in a Beckman liquid scintillation counter.

Electrophysiology—Electrogenic transport was assayed by voltage clamp using the 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 a resistance ranging from 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 ± S.D.

Immunoprecipitations—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 further processed for immunoprecipitation of wild-type and mutant GLAST-1 as described before (12). Proteins were analyzed by polyacrylamide gel electrophoresis (24) followed by fluorography (25).

Immunohistochemistry—Oocytes were embedded in Tissue-Tek® OC at −20 °C 24–72 h after injection of wild-type, mutant cRNA, or H_2O as control. 12-μm sections were prepared. They were fixed at room temperature with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min (PBS: 6.5 mM Na_2HPO_4, 1.45 mM KH_2PO_4 (pH 7.2), 136 mM NaCl, 2.68 mM KCl), washed three times with PBS for 5 min, 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 Axioscope fluorescence microscope, and photographed with a Zeiss MC 100 microscope 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. L-[^{14}C]Glutamate and L-[^{14}C]alanine was from Amersham Corp. Fluorescein isothiocyanate-goat 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 ISO2 from MFK (Frankfurt) were used in the whole cell voltage clamp experiments.

RESULTS

Site-directed Mutagenesis of the 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, 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 microinjected for functional expression in *Xenopus* oocytes in the presence of [^{35}S]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.[2] 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. 3*A*). At an extracellular Na^+ concentration ($[Na^+]_0$) 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 af-

[1] The abbreviations used are: THA, D,L-threo-3-hydroxyaspartate; PBS, phosphate-buffered saline; Q, the quadruple mutant R122I, R280V,Y405F,R479T.

[2] Schulte, S., and Stoffel, W. (1995) *Eur. J. Biochem.*, in press.

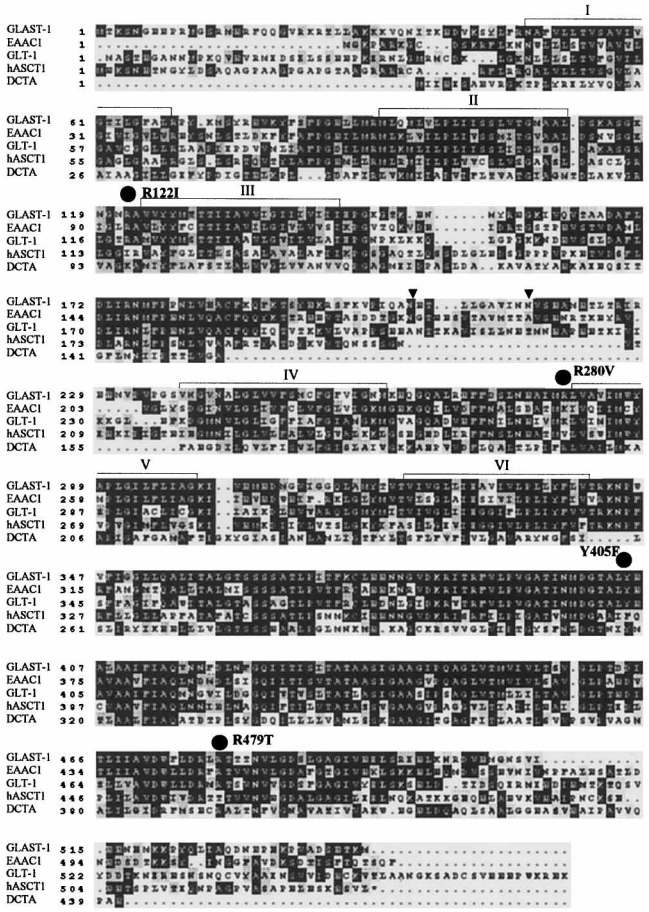

FIG. 1. **Alignment of the amino acid sequences of mammalian glutamate transporters GLAST-1, EAAC1, GLT-1, neutral amino acid transporter ASCT1, and prokaryotic DCTA dicarboxylate transporter of *R. meliloti*.** The amino acid sequences are deduced from the cDNA nucleotide sequences. Sequence identities/similarities are indicated by using *white on black lettering/black on gray lettering*, respectively. The experimentally verified *N*-glycosylation sites of GLAST-1 are marked by *black triangles*. *Lines* are drawn over the N-terminal six hydrophobic regions, which are proposed to span the plasma membrane in a α-helical manner. The amino acid substitutions are indicated by *black circles*. Sequences of GLAST-1, EAAC1, GLT-1, ASCT1, and DCTA are taken from the EMBL/GenBank data base under accession numbers X6374, L12411, X67857, L14595, and P20672 respectively.

finity for L-glutamate of the mutant transporters R122I, R280V, and R122I,R280V showed no significant differences and had apparent K_m values of 22.5 ± 2.5 μM (n = 4), 18 ± 4 μM (n = 8), and 18 ± 3 μM (n = 7), respectively. Likewise, the L-[^{14}C]glutamate uptake experiments shown in Fig. 5*B* revealed that the transport velocity of L-glutamate seems not to be influenced significantly by the amino acid substitutions.

We studied the Na^+ dependence of the mutant transporters to probe for electrostatic forces between the sodium ion and the two positively charged arginines. Inward currents were recorded at stepwise increased $[Na^+]_0$ and constant L-Glu concentration (100 μM). Currents were fitted to a Hill equation, which yielded K_m values for $Na^+$of wild type and the double mutant R122I,R280V of 32 ± 3 mM (n = 6) and 29 ± 5 mM (n = 4), respectively (Fig. 3*B*). The values of the mutant transporter R122I and R280V for 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

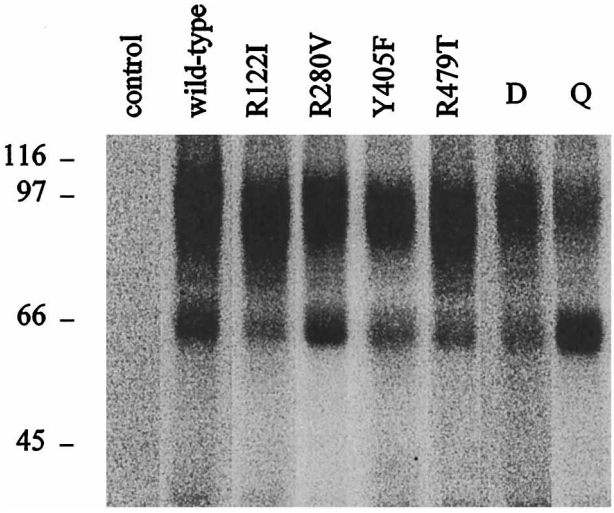

FIG. 2. **Immunoprecipitation of wild-type and mutant GLAST-1 proteins expressed in *Xenopus* oocytes.** Wild-type and mutant GLAST-1 cRNAs were injected into *Xenopus* oocytes and incubated in Barth's medium supplemented with [^{35}S]methionine. Oocytes were homogenized and immunoprecipitated as described under "Experimental Procedures." Samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Sizes of marker proteins are indicated in kDa. *Lane 1*, water-injected control oocytes; *lanes 2–6*, indicated mutants; *lane 7*, double mutant R122I,R280V (*D*); *lane 8*, quadruple mutant Q.

in Fig. 3*C*. 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 μM (n = 6) for wild-type GLAST-1 to 10 ± 2 μM (n = 6), 11 ± 3 μM (n = 6), 10 ± 2 μM (n = 6) for R122I, R280V and R122I,R280V, respectively (Fig. 4*A*). The increase of the apparent affinity for L-aspartate is similar as to the value for THA (Fig. 4*B*). The K_m value for L-aspartate of wild-type GLAST-1 is 14.5 ± 2 μM (n = 4) and 9.5 ± 0.9 μM (n = 4) (significant smaller paired t test, $p < 0.05$) for the double mutant R122I,R280V. L-[^{14}C]Alanine uptake experiments shown in Fig. 5*C* 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. 5*A* shows that superfusion of oocytes with L-$[Glu]_o$ up to 500 μM evoked no detectable current in the oocytes expressing the mutagenized transporters Y405F, R479T, or Q. Additionally the L-[^{14}C]glutamate uptake measurements depicted in Fig. 5*B* 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 L-[^{14}C]alanine uptake experiments (Fig. 5*C*) 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

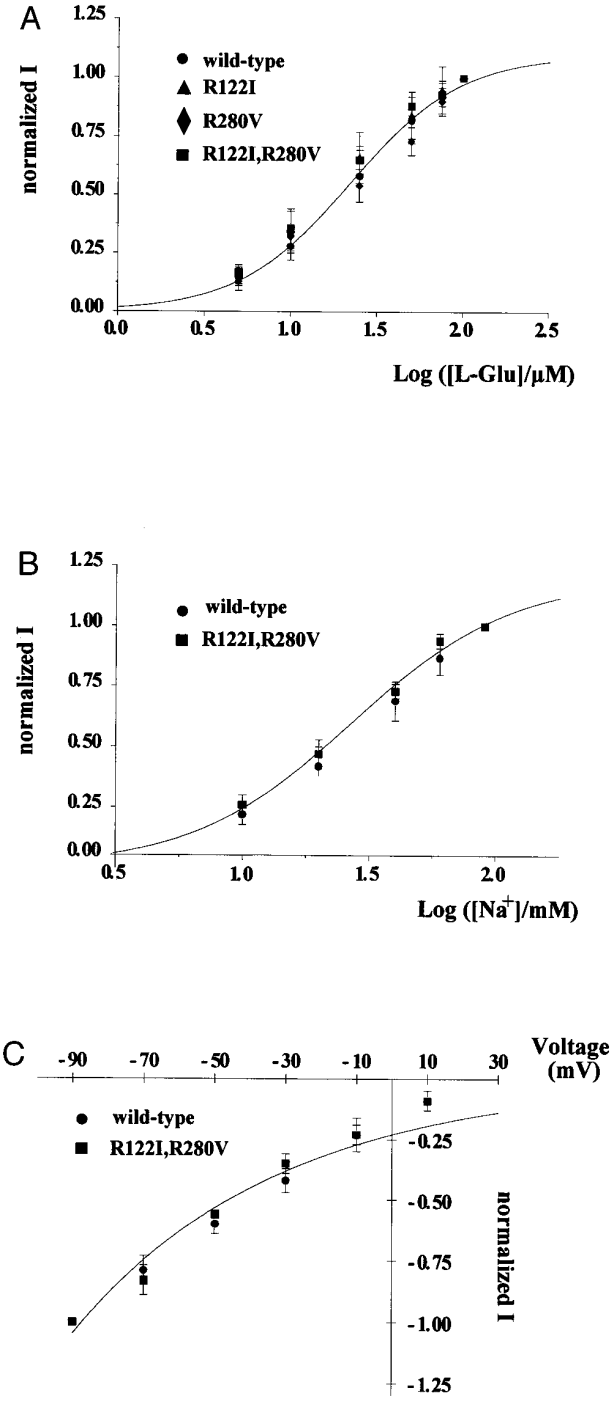

FIG. 3. **Comparison of the basic L-glutamate transport properties of wild-type and mutant GLAST-1.** *A*, L-Glu K_m values. The values represent the means, and the *bars* represent the standard deviation of $I_{GLAST-1}$ (n = 4–11). The current of each oocyte was normalized to the current amplitude at 100 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 21 ± 3 μM L-glutamate. The best fit to the data of the mutant transporters yielded nearly identical concentration response curves (K_m 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 $[Na^+]_o$). *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 $[Na^+]_o$. 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_m 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_m 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. $[L\text{-Glu}]_o$ and $[Na^+]_o$ were 100 μM and 90 mM, respectively.

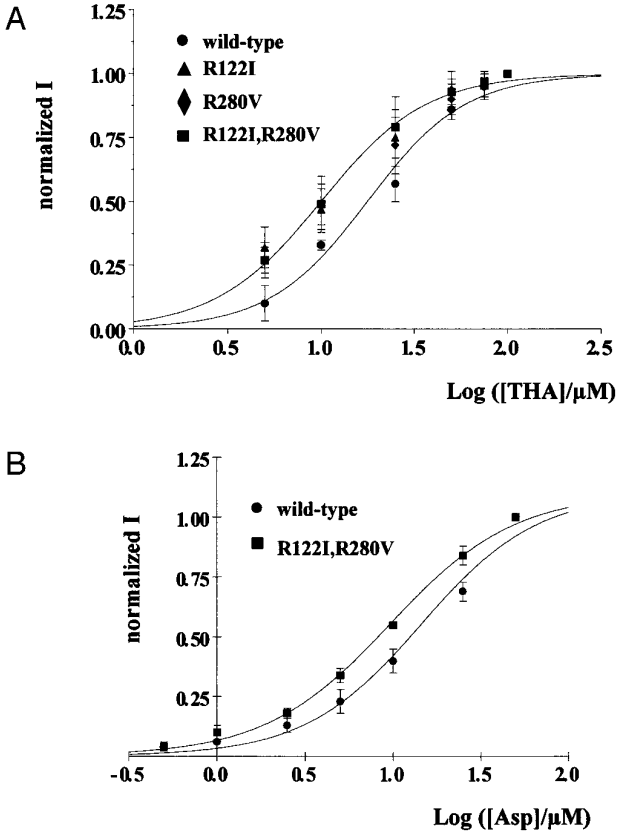

FIG. 4. **K_m values of THA (*A*) and L-aspartate (*B*) for wild-type and mutant GLAST-1 transporters.** Dose response curves as described in Fig. 3*A*, except that THA and L-Asp were applicated instead of L-glutamate. The K_m values for THA and L-Asp are significantly smaller for the mutants R122I and 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 $[Na^+]_o$).

type. The loss of glutamate uptake activity by oocytes expressing the GLAST-1 mutants Y405F, R479T, and Q might have several reasons: 1) a reduced expression level or stability of the mutant transporters, 2) impaired targeting of the mutated transporters to the plasma membrane, and 3) the substituted tyrosine 405 and arginine 479 have important intrinsic function in the transport process. The first possibility was ruled out by comparison of the [^{35}S]methionine-labeled translation products of wild-type and mutant GLAST-1 expressed in the *Xenopus* oocyte. The immunoprecipitation of the recombinant transporters revealed that their expression level is comparable to the wild type (Fig. 2). The targeting of the transporters to the plasma membrane was analyzed by immunostaining of oocyte sections using rabbit anti-GLAST-1-peptide antibodies as a first antibody and a fluorescein isothiocyanate-conjugated secondary antibody. The results of the immunofluorescence stud-

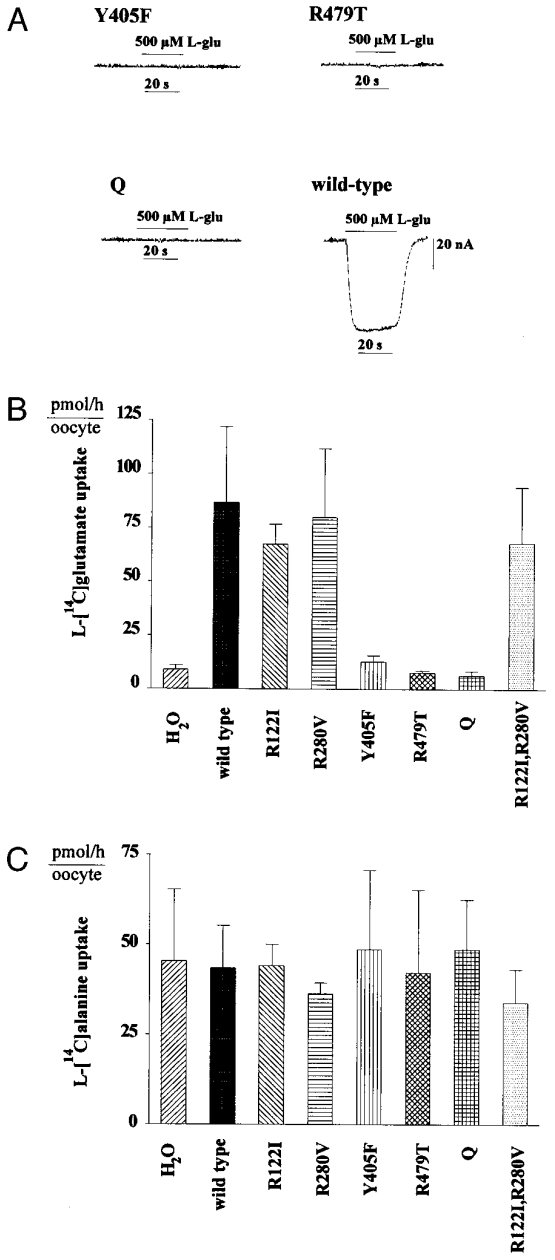

FIG. 5. **Functional characterization of the mutant transporters Y405F and R479T.** *A*, original traces recorded at indicated L-Glu concentrations. Superfusion of oocytes expressing Y405F, R479T, and Q with 500 μM L-Glu evoked no detectable currents in contrast to wild-type GLAST-1-expressing oocytes. [L-Glu] up to 500 mM revealed the same results (data not shown). *B*, L-[^{14}C]glutamate uptake mediated by wild-type GLAST-1 and mutant transporters. The L-[^{14}C]glutamate uptake rates of R122I and R280V and the double mutant R122I,R280V are quite similar as compared to the L-[^{14}C]glutamate uptake of wild-type GLAST-1. Y405F, R479T, and the quadruple mutant Q-expressing oocytes show L-[^{14}C]glutamate uptake rates similar to water-injected controls. *C*, L-[^{14}C]alanine uptake by wild-type GLAST-1 and mutant transporters is similar to the water-injected control oocytes. Each column represents the mean ± S.D. (n = 4).

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. 6*A*). The immunocytochemistry clearly indicates that the mutant transporters are

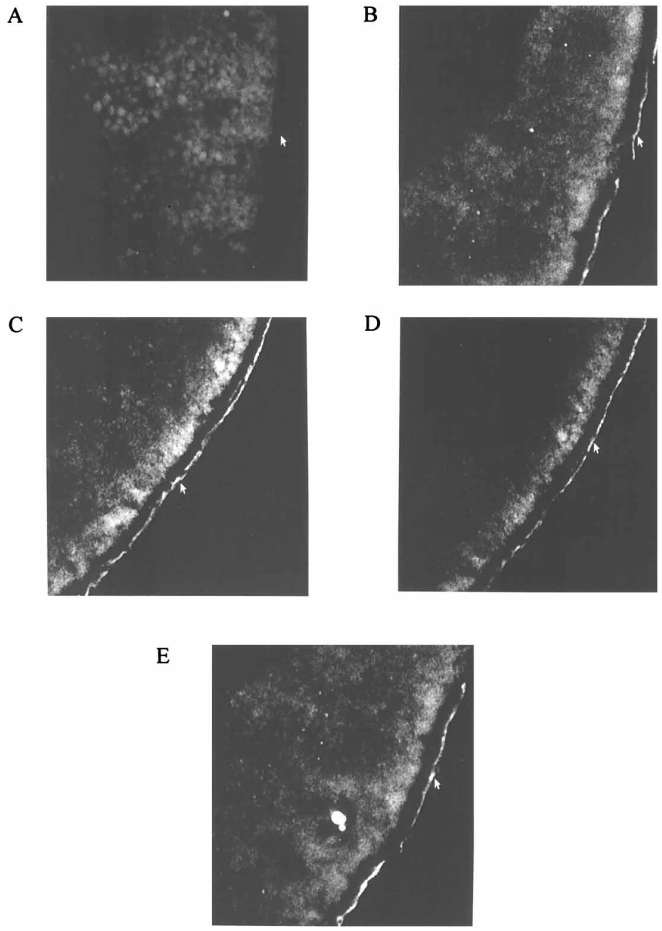

FIG. 6. **Wild-type and mutant GLAST-1 are located in the plasma membrane.** Cryosections (10–12 μm) of *Xenopus* oocytes were incubated with anti-GLAST-1 antibody and stained with fluorescein isothiocyanate-conjugated secondary antibody. Surface expression was noted in wild-type GLAST-1 (*B*), Y405F (*C*), R479T (*D*), and Q (*E*) expressing oocytes with similar intensity (marked by *arrow*). In the case of water-injected control oocytes (*A*), no fluorescence staining was observed (marked by *arrow*).

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. The C-terminal domain between residues 360–497 is highly conserved, which might indicate a functional importance of this region. This is supported by our data. Substitution of the Tyr-405 and the Arg-479 by Phe and Thr, which are the respective amino acid residues of the 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 γ-carboxylate group of glutamate with the β-OH of Ser-380 and Thr-193 and the ϵ-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 γ-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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