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Cloning of the Human Glycine Transporter Type 1: Molecular and Pharmacological Characterization of Novel Isoform Variants and Chromosomal Localization of the Gene in the Human and Mouse Genomes

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SUMMARY

We report the molecular cloning of a cDNA encoding a high affinity human glycine transporter. An open reading frame of 1914 nucleotides encodes a 638-amino acid protein that transports glycine in a Na^+/Cl^--dependent manner. In common with other Na^+/Cl^--dependent transporters, it possesses 12 putative transmembrane domains, according to its hydropathicity profile. This protein is the human homologue of a glycine transporter previously isolated from rat [glycine transporter type 1b (GlyT-1b)]. In addition to the human GlyT-1b, we also characterized a novel functional isoform produced by alternative splicing. This isoform, GlyT-1c, which is distinct from GlyT-2 recently characterized in rat, contains an additional exon encoding 54 amino acids in the amino-terminal part of GlyT-1b and is mainly expressed in brain. These two isoforms are products of the same gene and are localized on human chromosome 1p31.3, as well as on mouse chromosome 4, close to the locus for the spontaneous mouse neuromuscular mutation *clasper*. When expressed in COS-7 cells, both the human GlyT-1b and GlyT-1c display a time- and dose-dependent uptake of glycine, which is abolished when either Na^+ or Cl^- is substituted with other ions. For both GlyT-1b and GlyT-1c the affinities for glycine are similar, with K_m values of 70–90 μM, and this uptake is inhibited by sarcosine with similar potencies. In addition to the three transporter isoforms present in the human genome, i.e., GlyT-1a, GlyT-1b, and GlyT-1c, point-mutated variants, which appear to be totally devoid of glycine uptake activity when expressed in COS-7 cells, were obtained by polymerase chain reaction amplification of mRNA from human substantia nigra. These variants point to regions of the glycine transporter that might be important in the processing or transport function of this protein.

The only amino acid without an asymmetric center, glycine, has been found to have many important functions in the central nervous system since it was first proposed as a neurotransmitter in 1965 (for review, see Ref. 1). Together with GABA, it is a major postsynaptic inhibitory neurotransmitter, and it acts by increasing the chloride channel conductance of target neurons (2, 3). In addition, it is an essential modulator of glutamate/NMDA receptor-gated channels (4, 5).

The reuptake of neurotransmitter substances from the synaptic cleft into presynaptic nerve endings or glial cells is a major pathway for terminating the actions of neurotransmitters (6, 7). This reuptake is performed by neurotransmitter-specific transporters. Molecular biology has now revealed that most of these transporters belong to a gene superfamily. These transporters are proteins of roughly 600 amino acids in length that possess 12 putative transmembrane domains. The overall sequence homology among the various members of this superfamily is very high, and several stretches of sequence in various regions of these proteins are even more highly conserved. Thus, low stringency hybridization using cDNA probes or PCR amplification has led to the isolation of several related members of the family. Expression of their cDNAs in cells establishes sodium- and chloride-dependent uptake of various substrates. In addition to transporters for classical neurotransmitters, i.e., dopamine, norepinephrine, serotonin, and GABA, transporters

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ABBREVIATIONS: GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; GlyT-1 and -2, glycine transporter types 1 and 2; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RFLV, restriction fragment length variations; kb, kilobase(s); bp, base pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ASC, alanine, serine, cysteine.

for proline, betaine, and taurine have also been cloned and characterized (for review, see Refs. 8–10).

Two different glycine transporters belonging to this family, GlyT-1a and GlyT-1b, have been cloned from rat and mouse (11–14). The two proteins GlyT-1a and GlyT-1b are essentially identical, except in their amino-terminal sequences, with the first 10 amino acids of GlyT-1a differing from the first 15 amino acids of GlyT-1b. These two isoforms show no difference in their uptake properties but display distinct patterns of expression in the central nervous system and peripheral tissues (14).

In the work reported here, screening of a cDNA library from human substantia nigra with a probe derived from the rat dopamine transporter (15) yielded a partial cDNA clone encoding a putative member of this Na^+/Cl^--dependent transporter family. Using PCR techniques, a full length clone was isolated and transfected into COS-7 cells, revealing high affinity glycine transporter activity. This glycine transporter clone is highly homologous (95%) to the rat and mouse transporters and represents the human homologue of GlyT-1, according to the classification of Borowsky *et al.* (14). In addition, we report the characterization of a novel isoform of the glycine transporter, GlyT-1c, which arises by alternative splicing of a 54-amino acid exon in the amino-terminal tail of human GlyT-1b. Northern blot analysis revealed that expression of this isoform is mainly restricted to brain. We determined that the gene encoding GlyT-1 subtypes (official designation, *Glyt1*) is localized on human chromosome 1p31.3 and mouse chromosome 4. Interestingly, *Glyt1* maps to the same region of mouse chromosome 4 as the locus for the neurological mutant *clasper* (16). The *clasper* phenotype is similar to that of the *spastic* mouse mutation, which is associated with a decrease in the number of glycine receptors (17).

Very recently a novel glycine transporter was identified by cDNA cloning and referred to as GlyT-2 (18). GlyT-2 is the product of a gene distinct from that coding for any of the previously characterized glycine transporter isoforms and, therefore, represents a distinct glycine transporter subtype. In light of these results, the novel human isoform reported here is referred to as GlyT-1c, whereas the rat isoforms formerly called GlyT-1 and GlyT-2 (14) are referred to as GlyT-1b and GlyT-1a, respectively, according to the order of exon usage in the gene (18).

Materials and Methods

Library screening. A human substantia nigra cDNA library (Clontech) was screened at low stringency using a cDNA probe obtained from the amino terminus to the third transmembrane domain of the rat dopamine transporter (as described in Ref. 19). Phages were transferred to nitrocellulose filters (BAS 85; Schleicher & Schuell). Filters were prehybridized at 42° for 2 hr in 30% formamide, 4× saline sodium citrate (0.6 M NaCl, 60 M sodium citrate, pH 7.0), 0.01% sodium dodecyl sulfate, 20 mM Tris·HCl, pH 7.4, 1× Denhardt's solution, 20 μg/ml salmon sperm DNA, 20 μg/ml yeast tRNA. Hybridization was performed overnight at 42° in the same solution containing 10% dextran sulfate and the nick-translated probe (5×10^5 cpm/ml). Subsequent washes were performed as described previously (20). One of several positive clones, SNH7, was plaque purified and sequenced after subcloning into pBluescript SK^+ (Stratagene). SNH7 displayed a strong homology with other members of the Na^+/Cl^--dependent transporter family, but its sequence comprised only the first to the seventh transmembrane domains. Eight additional clones were then isolated by rescreening the same library at higher stringency (40% formamide for prehybridization and hybridization), using SNH7 as a probe. Overlapping clones comprising sequences from the putative start codon to the 11th putative transmembrane domain were obtained. A full length clone was obtained as described below.

RACE-PCR and full length cDNA cloning. To obtain the 3′ part of the coding sequence, we used the 3′-RACE-PCR technique (21). Total RNA was prepared from human substantia nigra, and single-stranded cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). Oligonucleotide primers A (located just after the ninth putative transmembrane domain) and B were used for the first round of PCR (see Table 1). For the second and third rounds of RACE-PCR, two additional oligonucleotide primers (primers C and D) closer to the 3′ end of the clone were sequentially used, together with primer E, an oligonucleotide directed against the polylinker and connected to an oligo(dT) primer (these sequences are not shown in Fig. 1 because these primers are located on incompletely processed intronic sequences). After the third round of RACE-PCR, a 1.6-kb cDNA was amplified, and this sequence possesses an in-frame stop codon.

The full length cDNA used for expression in eukaryotic cells was amplified by reverse transcription-PCR from RNA of human substantia nigra. This amplification was done in two fragments by creating an artificial *Xho*I site at position 1026 without modification of the amino acid sequence. Primers F and G were used to amplify the 5′ half of the transporter, and primers H and I were used to amplify the 3′ half (see Table 1). The amplified DNA fragments were subcloned into the expression vector pRc/CMV (Invitrogen) and used for pharmacological characterization. Amplification of human GlyT-1a was done with primers G and J. It should be mentioned that primer J was designed from the rat coding sequence (11), because we were unable to amplify any cDNA by using primers in the 5′ untranslated sequence.

Northern blot analysis. A specific cDNA probe for GlyT-1c was obtained by PCR amplification of plasmid DNA with primers K and L (Table 1), as described (20). A cDNA probe common for GlyT-1a, -1b, and -1c was obtained with primers H and I (Table 1). Probes were radiolabeled with [^{32}P]dCTP and [^{32}P]dATP by nick-translation (Boeringher Mannheim) and were hybridized to a human multiple-tissue Northern blot (Clontech, Palo Alto, CA). Hybridization and washing were performed as described (20).

Pharmacological characterization. DEAE-dextran transfection was used for transient expression. Typically, 2×10^6 COS-7 cells were plated in 100-mm diameter dishes 1 day before transfection. Transfection was started by washing the cells with phosphate-buffered saline, and cells were incubated for 30 min at 37° with 5 ml of phosphate-buffered saline containing 2.5 mg of DEAE-dextran and 15 μg of DNA. After this incubation, 20 ml of Dulbecco's modified Eagle medium containing 100 mM chloroquine were added, and the cells were further incubated for 2.5 hr. After removal of the medium, cells were treated for 2.5 min with 5 ml of medium containing dimethylsulfoxide and were incubated overnight in fresh medium. On the next morning, cells from two 100-mm diameter dishes were trypsinized, distributed into three 24-well plates for uptake studies (usually at a density of $\approx 10^5$ cells/well), and allowed to grow for 48 hr (up to $\approx 2.5 \times 10^5$ cells/well). For uptake experiments, the Dulbecco's modified Eagle medium was replaced with uptake buffer containing 5 mM Tris base, 7.5 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM ascorbic acid, and 5 mM D-glucose, final pH 7.1. At the end of the incubation, the cells were washed three times with 0.5 ml of uptake buffer, resuspended in 0.4 ml of 1% sodium dodecyl sulfate solution, and left at 37° with gentle shaking for 1 hr. An aliquot was taken for scintillation counting. For uptake inhibition studies, cells were preincubated for 2 min in the presence of compounds to be tested. Radiolabeled [^{3}H]glycine (50 nM) was added in a final volume of 0.5 ml and the incubation was continued for 4–5 min. To determine the Na^+ dependence NaCl was replaced with 120 mM LiCl, and to determine the Cl^- dependence NaCl was replaced with 120 mM sodium acetate.

TABLE 1

Oligonucleotide primers for PCR

Oligonucleotides are 5′ to 3′. Underlined sequences are for the restriction sites indicated. Numbers for primers F–I, K, and L are designated according to the numbering of GlyT-1b in Fig. 1. Numbers for primer J are designated according to the numbering of GlyT-1a (GlyT-2 in Ref. 13).

Primer	Sequence	Endonuclease	Position
A (Sense)	CTTGTCGACTAAGAGCTGCGTAAGGGAAGGGC	*Sal*I	
B (Antisense)	GACTCGAGTCGACATCGTTTTTTTTTTTTTTTTT	*Xho*I	
C (Sense)	ATTGTCGACACGTGCAGGGAAGGGTTGGGGAG	*Sal*I	
D (Sense)	TATAGTCGACTGCAGGCGCCTCCCACAG	*Sal*I	
E (Antisense)	GACTCGAGTCGACATCGT	*Xho*I	
F (Sense)	ATATAAGCTTGCTGCGATCGCTCGCCCCAGG	*Hind*III	−21 to −1
G (Antisense)	TGGCCTCGAGGATCTTGTCCCACTGCGGGG	*Xho*I	1004 to 1033
H (Sense)	GATCCTCGAGGCCAAGGTGTGGGGTGATG	*Xho*I	1020 to 1048
I (Antisense)	TATATCTAGAGGTGGCACTCCCCTGGCAGCTG	*Xba*I	2082 to 2104
J (Sense)	ATATAAGCTTATGGTAGGAAAAGGTGC	*Hind*III	1 to 17
K (Sense)	TCTTCCCCAGAACAGGTGACG		31 to 52
L (Antisense)	CTCGCTGGGCACAGCACCATTCTGGG		103 to 129

Human chromosomal assignment. Chromosomal assignment of the glycine transporter gene was accomplished by somatic cell hybrid analysis and *in situ* hybridization. The cDNA probe used corresponds to nucleotides 236–1454 in Fig. 1. For somatic cell hybridization a mapping panel consisting of 17 mouse-human (NA09925-NA09938, NA09940, NA10324, and NA10567) and two Chinese hamster-human (NA10611 and GM07298) hybrids was obtained from the National Institute of General Medical Sciences Mutant Cell Repository (Camden, NJ). Characterization of these hybrids and their human chromosome contents are described in detail in the National Institute of General Medical Sciences Mutant Cell Repository catalog. The human glycine transporter-hybridizing fragments showed perfect segregation with chromosome 1. *In situ* hybridization to human metaphase chromosome spreads, posthybridization wash, emulsion autoradiography, and silver grain analysis were carried out as described previously (22).

Genetic mapping of the glycine transporter in the mouse genome. C3H/HeJ-*gld*, *Mus spretus*, and [(C3H/HeJ-*gld* × *M. spretus*)F_1 × C3H/HeJ-*gld*] backcross mice were bred and maintained as described previously (23). RFLV were determined by Southern blot hybridization of DNA from C3H/HeJ-*gld* and (C3H/HeJ-*gld* × *M. spretus*)F_1 parental mice, digested with various restriction endonucleases, as described previously (23). *M. spretus* was chosen as the second parent because of the relative ease of detection of informative RFLV, in comparison with crosses of inbred strains.

DNA isolated from mouse organs by standard techniques was digested with restriction endonucleases, and 10-μg samples were subjected to electrophoresis in 0.9% agarose gels, transferred to Nytran membranes (Schleicher & Schuell), hybridized at 65° with gene probes labeled with [α-^{32}P]dCTP by the hexanucleotide priming technique, and washed under stringent conditions, all as described previously (23). Informative RFLV for the reference locus probes *Jun* and L*myc* were as described previously (24–27). The *Glut-1* probe, derived from pGEM (28), hybridized to 2.0-kb and 2.8-kb *Pst*I fragments in C3H/HeJ DNA and to a 4.6-kb *Pst*I fragment in *M. spretus* DNA.

Results

Structural features of the human glycine transporter. Fig. 1 shows the nucleotide and deduced amino acid sequences for the human glycine transporters GlyT-1b and GlyT-1c. The boxed nucleotide and amino acid sequences represent the alternatively spliced exon unique to the GlyT-1c isoform. As shown in Fig. 2A, this insert of 162 bp occurs at the same codon where the amino-terminal sequence of GlyT-1a diverges from the amino acid sequence of the GlyT-1b isoform (14). Fig. 2B defines the sequence of the exon-intron junction flanking this 162-nucleotide insert. Typical donor-acceptor sites are found at both junctions. GlyT-1c differs from the GlyT-1b amplified from human substantia nigra in that it contains an additional 162-bp insert in the amino-terminal region of the transporter and two amino acid changes in the putative fourth extracellular loop (Fig. 3).

The human glycine transporter has four consensus *N*-linked glycosylation sites in the second extracellular loop (Fig. 1). Interestingly, a consensus recognition site for protein kinase C phosphorylation is found in the presumed intracellular loop between putative transmembrane segments 4 and 5. Similar consensus phosphorylation sites exist between transmembrane domains 4 and 5 in several other members of this transporter family (10).

Sequence information for the cDNA encoding the glycine transporter was obtained by two different approaches, i.e., cDNA library screening and RACE-PCR amplification from mRNA isolated from human substantia nigra. For the 15 clones chosen for analysis, four distinct restriction enzyme patterns were apparent (data not shown). Whereas the majority of the clones characterized had the same restriction pattern and were highly homologous to the rat and mouse (95%) glycine transporter GlyT-1 (11–14), four other variant transporters were obtained. As shown in Fig. 3, three of these variants clones, i.e., clones 3, 5, and 8, were found to be the likely products of spurious splicing accidents in which an exon had been deleted. The locations of the deletions in clones 3, 5, and 8 correspond to the locations of the intron-exon junctions delineated in the GABA transporter gene (29). The fourth variant, clone 7, contained four point mutations, two in the amino-terminal region and two between putative transmembrane regions 5 and 8. All of these variants were characterized for their ability to transport glycine (see below).

Pharmacological characterization of GlyT-1b. To determine the substrate for this transporter, sodium-dependent uptake activity was assessed by comparing GlyT-1b-transfected COS-7 cells with mock-transfected COS-7 cells in the presence of NaCl or LiCl. These experiments were carried out before the identification of the rat and mouse glycine transporters, and thus >16 compounds were tested as potential substrates. Radiolabeled glycine, L-serine, taurine, L-alanine, choline, L-aspartic acid, GABA, and prostaglandin E_1 were found to be endogeneously taken up by COS-7 cells in a sodium-dependent manner. However, only in GlyT-1b-transfected COS-7 cells was there a 10-fold increase in uptake of [^{3}H]glycine, compared with the mock-transfected cells. The affinity and uptake veloc-

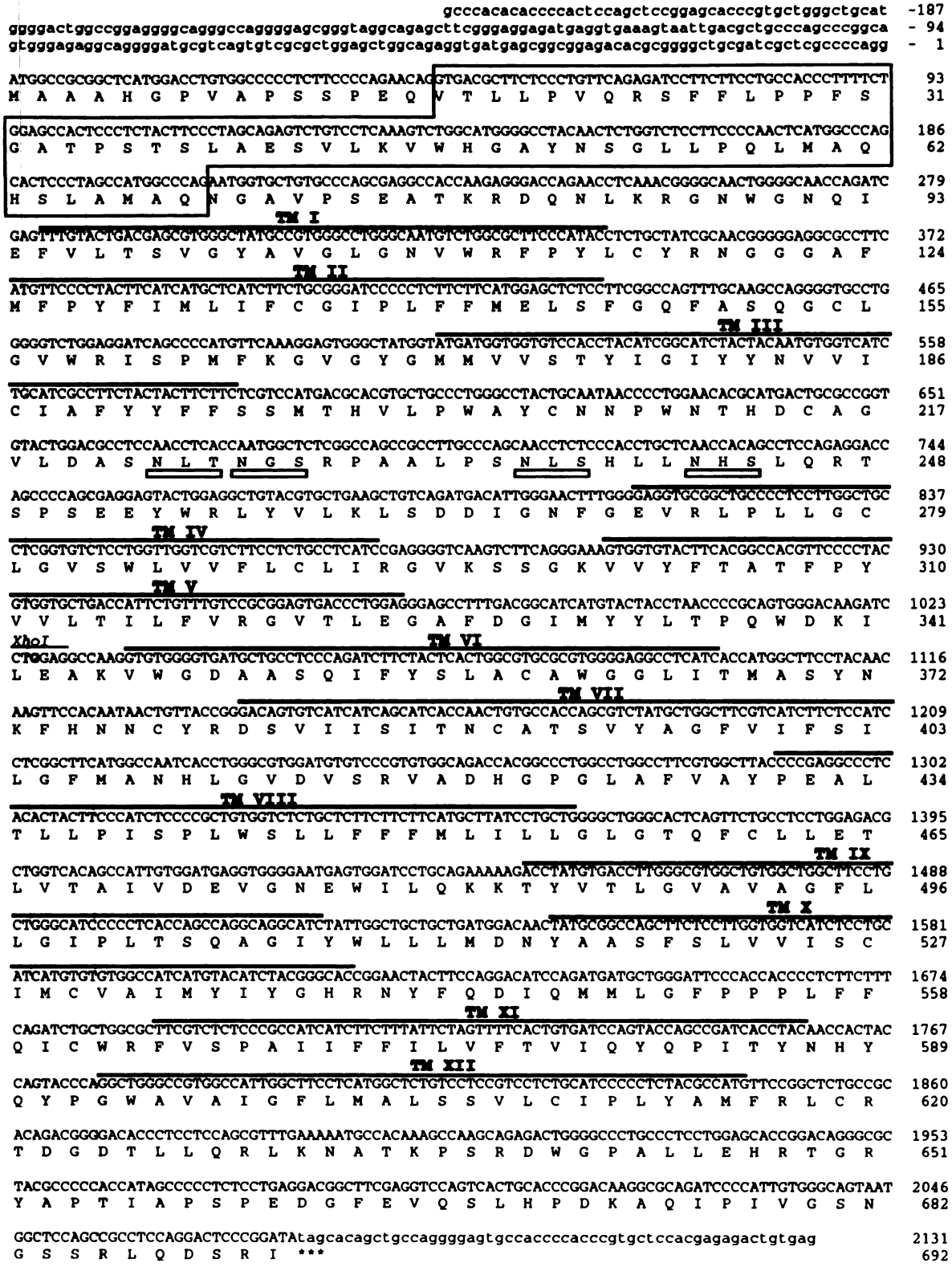

Fig. 1. Nucleotide and deduced amino acid sequences of GlyT-1b and GlyT-1c. The coding region is numbered starting from the putative translation initiation codon. *Solid bars*, putative transmembrane (*TM*) domains; *open bars*, consensus sites for *N*-glycosylation. *Solid box*, the alternatively spliced 162 nucleotides (54 amino acids) in the amino-terminal region.

ity were determined using the isotopic dilution method. Data analysis using linear regression analysis (LIGAND program) indicated that the kinetics of uptake were best fit using a two-site model, compared with a one-site model. Glycine uptake in mock-transfected cells displayed only the low affinity component of the uptake observed in GlyT-1b-expressing cells. Because this low affinity uptake of glycine (K_m = 3–5 mM) was found to be endogenous to the COS-7 cells, it was subsequently

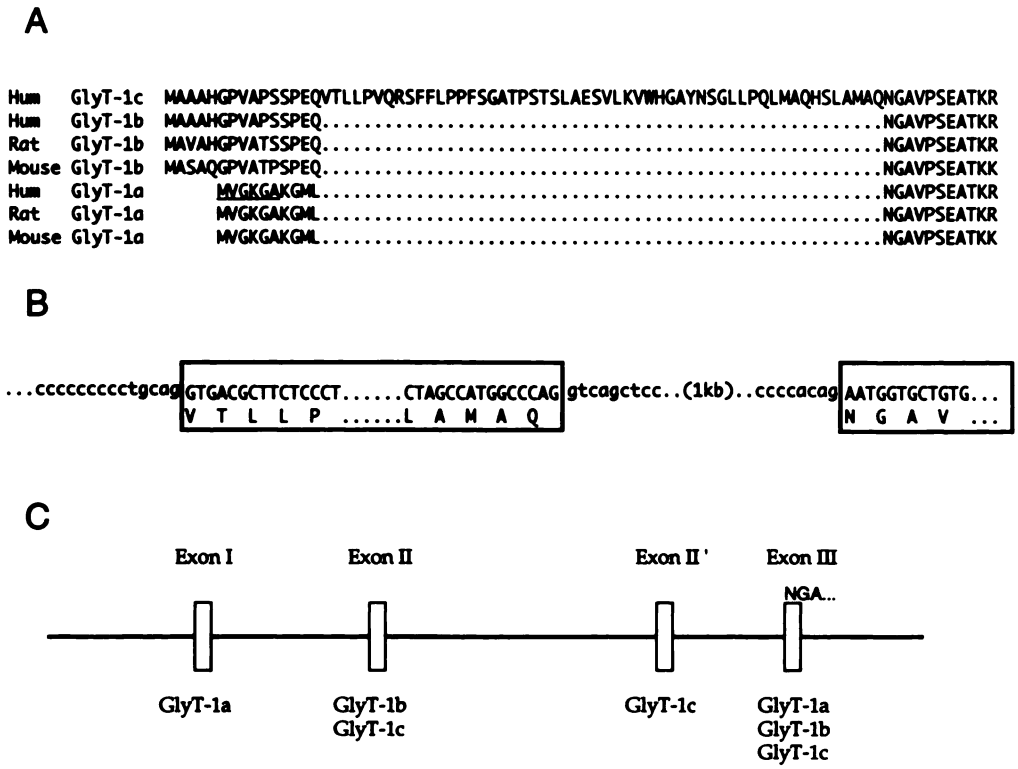

Fig. 2. A, Alignment of amino acid sequences in the amino-terminal region of human, rat, and mouse glycine transporter isoforms. For human GlyT-1a the sequence MVGKGA is *underlined*, because the nucleotide sequence for these residues is part of PCR primer J synthesized from the published rat sequence. B, Genomic organization of the GlyT-1 gene at the alternatively spliced 162 nucleotide exon. *First solid box*, 54-amino acid insert in GlyT-1c. *Second solid box*, first exon that is common to all isoforms. *Sequences in lower case letters*, introns that flank the 162-nucleotide insert. C, Schematic representation of the exon-intron organization in the 5′ part of the GlyT-1 gene. *Solid line*, the gene; *open boxes*, exons. Organization for exons I and II is taken from the mouse gene (18). Length for introns and exons are not to scale, because they were determined on different species.

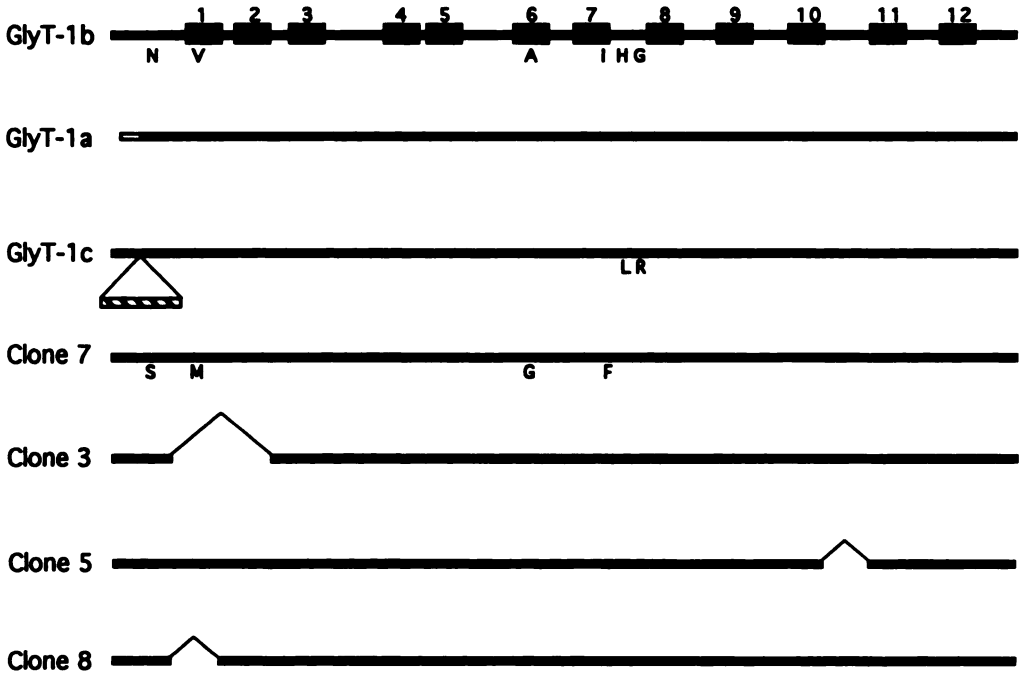

Fig. 3. Human glycine transporter variants are shown in relation to GlyT-1. *Thick bars in GlyT-1b*, locations of the putative transmembrane domains. *Hatched box in GlyT-1c*, 54-amino acid additional exon. *Open box in GlyT-1a*, first 10 residues that are specific. Point mutations from the corresponding amino acid in the sequence of GlyT-1b are noted with specific one-letter code designations. *Thin lines in clone 3, clone 5, and clone 8*, deleted regions in these clones that correspond to nucleotides 282–496, 282–364, and 1613–1677, respectively, in Fig. 1. Amino acids that are changed in the various isoforms are *highlighted below the line of GlyT-1b* and are located at positions N_{69}, V_{104}, A_{359}, I_{403}, H_{421}, and G_{422}.

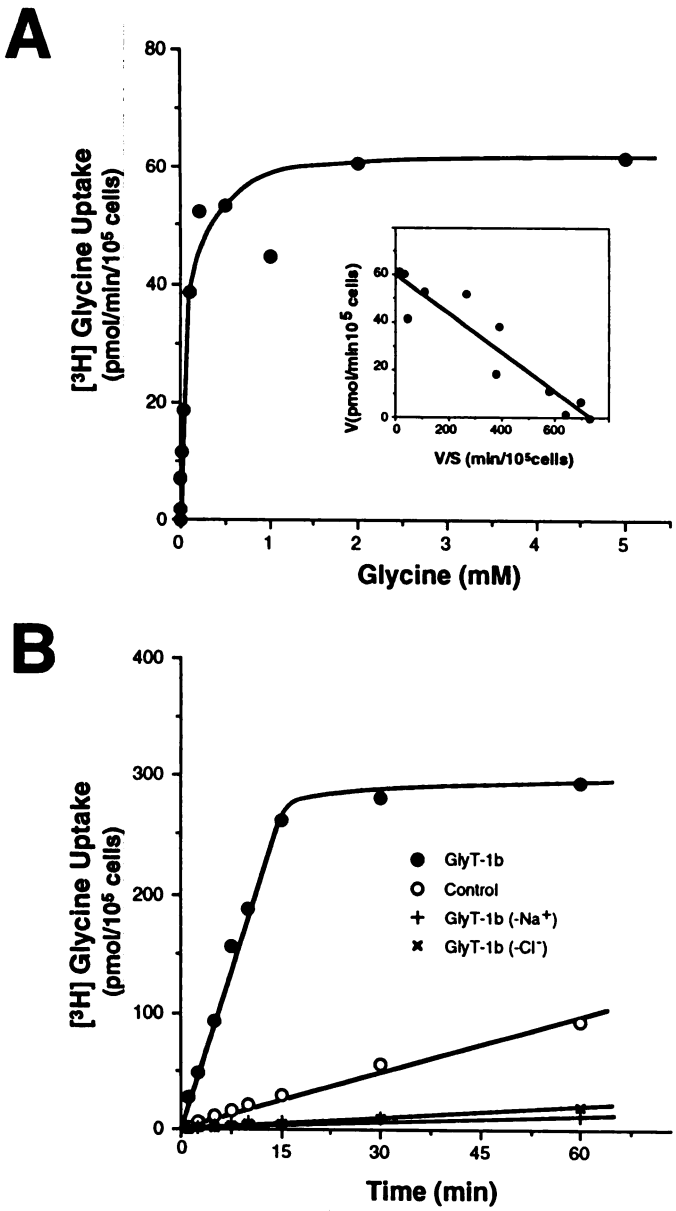

Fig. 4. Kinetic properties of GlyT-1b transiently expressed in COS-7 cells. A, Saturation curve for [^{3}H]glycine uptake by isotopic dilution (0–5 mM). and Eadie-Hofster representation (inset). B, Time course of [^{3}H] glycine (50 nM) uptake in control and transfected COS-7 cells. The control group is COS-7 cells transiently transfected with the dopamine transporter in the same expression vector (19). *GlyT-1b (–Na$^+$)* and *GlyT-1b (–Cl$^-$)*, COS-7 cells transfected with GlyT-1b but in which NaCl was substituted with 120 mM LiCl and 120 mM sodium acetate, respectively. Routinely, 10 μg of DNA were used to transfect 2×10^6 cells. Results are from a typical experiment in triplicate, which was replicated four times with similar results.

subtracted from the total uptake in the transfected cells. As shown in Fig. 4A, the dissociation constant for the high affinity site was 72 ± 9 μM (four different experiments, in triplicate). The time course of uptake (Fig. 4B) was linear for the first 10–15 min and then reached a plateau during the next hour, followed by a decrease (data not shown), probably as a consequence of cell death. When either Na^+ or Cl^- was substituted in the incubation medium with other ions, the uptake activity was totally abolished, including the constitutive uptake in mock-transfected cells. Twenty-three compounds (17 amino acids, three glycine derivatives, and two structural analogues)

TABLE 2

Pharmacological characterization of human glycine transporter transiently expressed in COS-7 cells

Competion experiments were conducted in COS-7 cells transiently transfected with GlyT-1b. Various compounds (1 mM final concentration) were tested for the inhibition of glycine uptake in the presence of 50 nM [^{3}H]glycine.

Tested compound[a]	Inhibition
	%
Control	0
Glycine	85
Sarcosine	65
Proline	30
Thiamine	26
Cysteine	25
Adenine	20
Glutamine	19
Alanine	9
Glutamate	9
Valine	8
Arginine	5
Glycylglycine	0
Glycinamide	0

[a] The following amino acids did not inhibit [^{3}H]glycine uptake when tested at 1 mM: isoleucine, leucine, lysine, serine, methionine, phenylalanine, threonine, thymidine, tryptophan, and tyrosine.

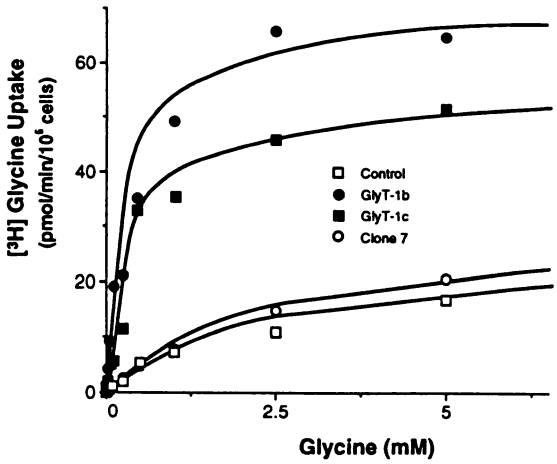

Fig. 5. Kinetic properties of human glycine transporter isoforms and variants. Each construct was expressed transiently in COS-7 cells, and uptake of [^{3}H]glycine was measured as described in Materials and Methods. Control COS-7 cells were transfected with the dopamine transporter (19).

were used to inhibit the high affinity glycine uptake mediated by GlyT-1b (Table 2). At 1 mM concentrations, L-glutamine, L-cysteine, L-proline, thiamine, and adenine showed some inhibition, whereas glycine and sarcosine were most potent.

Pharmacological features of glycine transporter variants. In addition to GlyT-1b, other cDNA variants of the glycine transporter were also functionally tested. GlyT-1c was functionally active, displaying a dose-response curve almost identical to that observed for GlyT-1b (Fig. 5), with a K_m value for glycine of 90 ± 9 μM (three experiments). This larger glycine transporter also showed a dependence on Na^+ and Cl^- ions, with the glycine uptake being abolished when Na^+ and Cl^- were substituted with Li^+ or acetate, respectively. The two amino acid changes (Fig. 3) in the fourth extracellular loop of GlyT-1c do not appear to have functional consequences. Three alternatively spliced variants, clone 3, clone 5, and clone 8 (Fig. 3), were also expressed in COS-7 cells but did not show any glycine uptake above the level of the endogenous uptake in the COS-7

cells. Clone 7, which has four point mutations (Fig. 3), was devoid of uptake activity (Fig. 5). Because this lack of uptake was somewhat unexpected, more extensive studies on this variant were conducted. First, we tested whether the lack of functional activity was due to lower expression of its mRNA or a deficit in the uptake mechanisms. For this, COS-7 cells were transiently transfected with GlyT-1b, GlyT-1c, or clone 7. Half of the transfected cells were used for uptake experiments and the other half were used to prepare RNA for dot blot analysis. When hybridized to a radiolabeled cDNA probe specific for a common part of these three transporters, transfected COS cells all clearly displayed similar levels of RNA transcripts for these three different constructs (data not shown). In an attempt to determine which amino acid changes affected the glycine transport, two chimeric transporters were constructed by combining clone 7 with GlyT-1b at the *Xho*I site that was artificially created between the fifth and sixth transmembrane domains (see Materials and Methods and Fig. 1). Interestingly, the first chimeric transporter (amino-terminal half of clone 7 and carboxyl-terminal half of GlyT-1) was totally nonfunctional, whereas the reverse chimera showed about 30% of the glycine uptake displayed by GlyT-1, suggesting that point mutations in the amino-terminal part of the glycine transporter (asparagine to serine and valine to methionine) impair protein expression or transporter function, whereas the double point mutation (alanine to glycine in transmembrane region 6 and isoleucine to phenylalanine in transmembrane region 7) appears to interfere less markedly. Finally, we examined whether these mutations might be the product of different alleles of the glycine transporter gene. The DNA fragment encompassing the asparagine to serine change in the amino-terminal part was amplified from human genomic DNA by PCR and sequenced. However, no such changes were found in eight unrelated human DNA samples (data not shown).

Northern hybridization analysis. Using a cDNA probe specific for the 162-bp insert of GlyT-1c, a single band at 3.6 kb was detected in brain mRNA (Fig. 6A) and a weaker signal was also apparent in kidney. A cDNA probe directed against the common part of GlyT-1a, -1b, and -1c displayed strong labeling in most tissues, with hybridization intensity as follows: brain = kidney > pancreas = lung > placenta = liver > heart > muscle. A major band at 3.6 kb was labeled in each case, except in skeletal muscle, where a strong band at 1.6 kb was also labeled (Fig. 6B).

Chromosomal assignment in the human genome and genetic mapping in the mouse genome. Southern blots of a hybrid panel of DNA digested with *Eco*RI identified three specific fragments (8.2, 5.8, and 3.0 kb) in human DNA, two cross-hybridizing fragments (10.2 and 5.8 kb) in mouse DNA, and one cross-hybridizing fragment (12.3 kb) in Chinese hamster DNA. Because a 5.8-kb fragment was detected in both human and mouse DNA, it was not scored in mouse-human hybrids. The discordance analysis of the human 8.2- and 3.0-kb fragments in this hybrid panel is shown in Table 3. Analysis of metaphase chromosome spreads showed that, of 143 grains over 70 cells scored, 26 (15.7%) were found at chromosome 1p31.3-p32 (Fig. 7). No other site was labeled over background.

The chromosomal location of the glycine transporter gene *Glyt1* in the mouse genome was determined with a panel of DNA samples from an interspecific cross that has been characterized for >600 genetic markers throughout the genome.

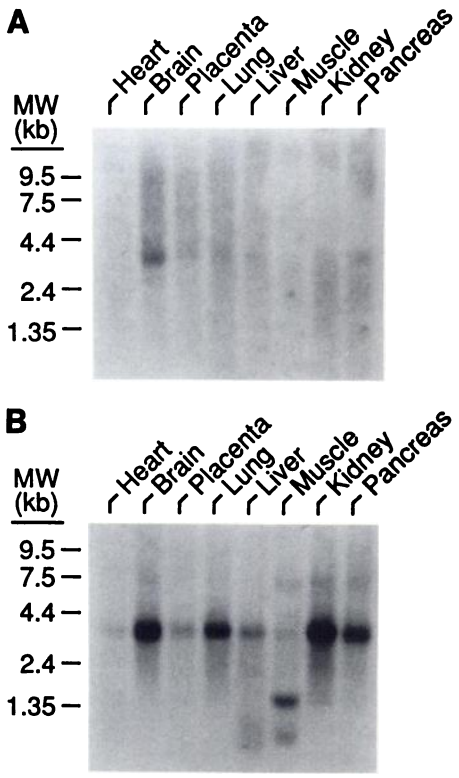

Fig. 6. A, Localization of GlyT-1c mRNA in various human tissues. The cDNA probe used is specific for the 162-bp alternatively spliced exon of GlyT-1c. B, Localization of GlyT-1 mRNA in various human tissues. The cDNA probe used is common to the three isoforms GlyT-1a, -1b, and -1c.

The *Glyt1* probe hybridized to 10.0-kb, 4.5-kb, 1.7-kb, and 1.5-kb *Bam*HI fragments in C3H/HeJ DNA and to 8.5-kb and 1.3-kb *Bam*HI fragments in *M. spretus* DNA. Reference loci used were the genes encoding avian myelocytomatosis virus (v-*myc*) oncogene homologue (L*myc*), avian sarcoma virus 17 (v-*jun*) oncogene homologue (*Jun*), and glucose transporter-1 (*Glut-1*), which have been previously localized to mouse chromosome 4 (24–28). One hundred-fourteen [(C3H/HeJ-*gld* × *M. spretus*)F_1 × C3H/HeJ-*gld*] backcross mice were typed by segregation analysis of unique *M. spretus* RFLV detected with these probes (30). At each locus, restricted DNA from the backcross mice displayed either a homozygous C3H or heterozygous F_1 pattern. The RFLV associated with *Glyt1* cosegregated with *Glut-1* on mouse chromosome 4. Haplotype analysis of the backcross mice is shown in Fig. 8. The best gene order (± standard deviation) (31) was determined to be as follows: centromere-*Jun*-11.4 ± 3.0 cM-(*Glyt1, Glut-1*)-2.6 ± 1.5 cM-L*myc*-telomere. No crossovers were detected between the genes grouped within parentheses. The chromosomal location of *Glyt1* on mouse chromosome 4 and human chromosome 1p31.3-p32 is in accord with the previous characterization of a linkage group conserved between these chromosomal regions (32). This genetic location of *Glyt1* on mouse chromosome 4 suggests it to be a good candidate gene for the mouse neurological mutation *clasper* (16). Mice homozygous for the recessive *clasper* mutation develop a syndrome of fine tremor and hobbling gait, with the feet clasped together (16).

Discussion

In this paper, we report the cloning and pharmacological characterization of a human glycine transporter. The human

TABLE 3

Segregation of human *Glyt1* cDNA human chromosomes in human × rodent somatic cell hybrids

Hybridization/chromosome	Human chromosomes																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
Concordant hybrids																								
+/+	4	3	3	4	2	4	4	4	0	2	0	2	3	4	4	1	4	4	4	4	1	2	0	1
−/−	15	12	8	7	8	7	8	6	14	9	8	7	11	5	7	14	4	11	10	7	7	8	11	11
Discordant hybrids																								
+/−	0	1	1	0	1	0	0	0	4	1	3	1	0	0	0	2	0	0	0	0	2	1	3	3
−/+	0	3	6	6	7	8	6	6	1	2	5	8	2	9	5	1	11	4	2	7	8	5	1	2
Total discordant hybrids	0	4	7	6	8	8	6	6	5	3	8	9	2	9	5	3	11	4	2	7	10	6	4	5
Total informative hybrids[a]	19	19	18	17	18	19	18	16	19	14	16	18	16	18	16	18	19	19	16	18	18	16	15	17
Discordancy (%)	0	21	39	35	44	42	33	38	26	21	50	50	13	50	31	17	58	21	13	39	56	38	27	29

[a] Human chromosomes present at a frequency of 0.1 or less were excluded from the analysis.

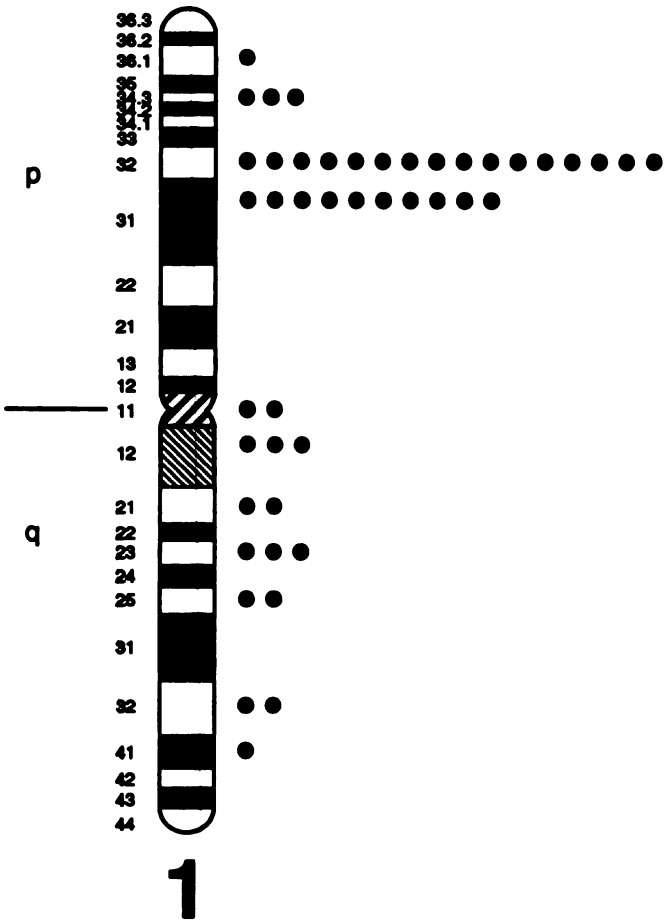

Fig. 7. *In situ* chromosomal assignment of the human glycine transporter. Silver grain distribution along chromosome 1 after *in situ* hybridization with human GlyT-1 probes is shown, illustrating the localization of a positive signal within the region 1p31.3-p32.

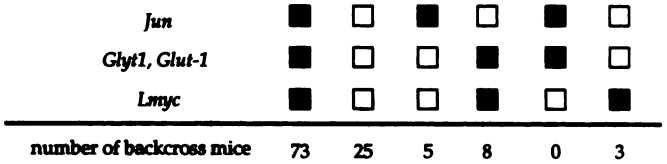

Fig. 8. Segregation of *Glut-1*, *Glyt1*, *Jun*, and *Lmyc* on mouse chromosome 4 in 114 [(C3H/HeJ-*gld* × *Mus spretus*)F_1 × C3H/HeJ-*gld*] interspecific backcross mice. ■, homozygous C3H pattern; □, F_1 pattern.

cDNA characterized here represents the human homologue of the rat (11, 14) and mouse (12) GlyT-1b subtype. In addition, we show that, in the human genome, an additional isoform of the glycine transporter exists. This novel isoform, which is referred to as the GlyT-1c subtype, is distinct from the GlyT-1a isoform that has been found in the rat and mouse genomes (13, 14). GlyT-1c arises by the alternative splicing of a 162-bp exon within the region of the cDNA coding for the amino-terminal part of the human glycine transporter. By PCR amplification of cDNA from human substantia nigra mRNA, using primers complementary to a common exon of the GlyT-1 subtype and the first exon of the GlyT-1a isoform, we have demonstrated that the homologue of rat GlyT-1a also exists in the human genome. Therefore, in the human genome at least three distinct forms of the glycine transporter appear to be present, GlyT-1a, GlyT-1b, and GlyT-1c. Interestingly, the 54-amino acid insert in GlyT-1c introduces two consensus phosphorylation sites for casein kinase type II, thus possibly imparting differential regulatory properties.

As shown in Fig. 2B, the characterization of intron-exon organization of the 5′ part of GlyT-1c demonstrates that the 162-bp (54 amino acid) insert in GlyT-1c arises from an alternatively spliced exon. Recently, the genomic organization of the 5′ part of the mouse GlyT-1 gene has been published, and the two specific exons for GlyT-1b and GlyT-1a have been localized (18). Therefore, it is now possible to establish the complete organization of the first four exons of the GlyT-1 gene (Fig. 2C). This gene, which gives rise to at least three distinct GlyT-1 isoforms, is different from the gene for GlyT-2 (18), which encodes a sarcosine-insensitive glycine transporter.

In the course of the characterization of human GlyT-1b and -1c, several other apparently aberrant isoforms of the glycine transporter were obtained from PCR amplification or screening of a cDNA library (Fig. 3). These nonfunctional variants appeared to fall into two distinct classes. First, the three clones indicated as clones 3, 5, and 8 (Fig. 3) probably represent "splicing accidents" in which an exon was incorrectly spliced. These splicing mistakes might have occurred at random during this delicate process and were probably detected because of the extreme sensitivity of the PCR amplification. These forms are unlikely to possess any physiological role, insofar as the function of the protein appears to be lost. The second case, clone 7, is more difficult to rationalize. In this case, very localized point mutations were found at four different codons and appear to impair totally the transporter synthesis or function. We believe

these mutations are unlikely to represent random PCR artifacts because, among 15 clones analyzed after PCR amplification, not a single point mutation was detected in the other 14 clones, whereas four specific changes were found in clone 7. The possibility still remains that some peculiar secondary structure in the mRNA or the cDNA renders it more prone to mutations at these particular loci. Morever, the possibility that these changes could result from an editing mechanism has not been explored. Whether a protein derived from the clone 7 transcript plays a physiological role cannot be assessed at this point. It remains very puzzling that the single changes of asparagine to serine and valine to methionine in the amino-terminal tail could totally impair either the folding processes or the uptake activity of the protein.

As we document in this paper, the two forms of the human glycine transporter (GlyT-1b and -1c) characterized here appear to take up glycine with very similar properties (K_m values of 80–90 μM) when expressed in host cells. These kinetic properties are very similar to those of the rat GlyT-1b (13) and the rat and mouse GlyT-1a subtypes (11–12). Although slight differences in K_m and K_i values for glycine and sarcosine among these transporter isoforms are evident, it is likely that these small differences are due to the various expression systems used (oocytes versus eukaryotic cell lines). Thus, the kinetics of all of these transporter isoforms appear to be essentially indistinguishable.

Borowsky *et al.* (14) have shown that the rat GlyT-1a and -1b isoforms differ in their tissue expression pattern. Whereas GlyT-1b is specifically expressed in the central nervous system (glial cells and neurons), where it shows specific patterns of expression, GlyT-1a is expressed both in the central nervous system and in peripheral tissues. This latter form of the glycine transporter appears to be localized within macrophages in the spleen, lung, liver, and thymus and in mast cells. In humans, when a probe common to all GlyT-1 isoforms is used, a somewhat different distribution is observed, compared with results found for the rat. The most striking difference is seen in kidney, where a signal as strong as that present in the brain is found (Fig. 6B), whereas virtually no mRNA for GlyT-1 is found in rat kidney (14). This difference is not likely to be produced by the use of different probes. It may be that in humans GlyT-1 plays an important role in the uptake of glycine in the kidney, where the uptake system ASC is only poorly expressed (33, 34). Interestingly, GlyT-1c seems to be expressed only in the brain (Fig. 6A), as are GlyT-1b (14) and GlyT-2 (18), whereas GlyT-1a would be the only subtype of Na^+/Cl^--dependent glycine transporter expressed in peripheral tissues.

Chromosomal localization of the *Glyt1* gene revealed mapping within a conserved linkage group on human chromosome 1p31–32 and mouse chromosome 4, close to the locus for the mouse neurological disorder *clasper* (16). Unfortunately, only the gross phenotype of *clasper* has been described, and few studies have been devoted to the molecular understanding of this disorder. Although no more direct evidence than the genetic mapping presented exists for the possible role of the glycine transporter in the etiology of *clasper*, it is interesting to note that another neurological mutation in the mouse, *spastic*, is associated with an alteration in the glycine system. The phenotypes of *spastic* and *clasper* are strikingly similar and have similar onsets of appearance, at 2–4 weeks after birth. In *spastic*, a dramatic decrease of strychnine binding to glycine receptors has been documented (17). The fact that these two mutations eventually impair the glycine neurotransmission system, either on its postsynaptic side (receptors in the case of *spastic*) or on its presynaptic side (a potential uptake alteration in the case of *clasper*), is fascinating and definitely deserves more attention.

Three different Na^+-dependent systems capable of handling the transport of glycine have been described in mammalian systems, i.e., system Gly (glycine), system A (alanine), and system ASC (35, 36). Among these three systems, system Gly is the most specific for glycine. The system A transporter and system ASC transporter have recently been cloned (33, 34, 37), and both belong to transporter families other than that of GlyT-1. Although systems A and ASC have much broader specificity than does system Gly and are likely composed of more than one molecular entity each, it is unlikely that GlyT represents one of them. As depicted in Table 2 for human GlyT-1b, and as already noted for rat GlyT-1b (13) and GlyT-1a (11), glycine uptake is poorly competed for by most of the other amino acids, including alanine, which is the preferred substrate for system ASC and system A. Also, sarcosine, which is an *N*-methylated derivative of glycine, behaves as a competitive blocker of glycine uptake by GlyT-1b, -1b, and -1c but is a poor blocker of the other systems, including GlyT-2 (18). These pharmacological characteristics, together with the Na^+ and Cl^- dependence of glycine uptake (Fig. 3), definitively suggest that GlyT-1, -2, and -3 are responsible for the "system Gly" uptake that was physiologically described previously (35, 36, 38, 39). One difference found between the cloned human glycine transporter and the glycine transport studied in slice preparations of rat spinal cord (38) is the lower affinity for sarcosine (here about 1 mM, compared with 100 μM in slice preparations). This lower affinity could be a peculiarity of the human glycine transporter or a consequence of the expression system we used.

When glycine transport is compared with the transport of other biologically active molecules such as the neurotransmitters dopamine, norepinephrine, and serotonin (8–10), it is striking that few pharmacological tools such as ligands and inhibitors are available to study and characterize the glycine transporter. One probable contributing factor is that several ubiquitous transport systems (see above) exist for glycine, and these have made it much more difficult to characterize the "neurotransmitter uptake" process of glycine. Glycine represents a major inhibitory system in the central nervous system and is a main component of the excitatory amino acid pathways, through its binding site regulating the glutamate-NMDA receptor. Thus, modifying the glycine concentration at the synapse by enhancing or inhibiting its reuptake should lead to noticeable effects on central nervous system functions such as the state of arousal and the control of motor functions. The rational design of specific inhibitors of the human glycine transporters should now be much easier to develop, with the availability of cell systems expressing a homogeneous population of this transporter at high levels.

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References

1. Aprison, M. H. The discovery of the neurotransmitter role of glycine, in *Glycine Neurotransmission* (O. P. Ottersen and J. Storm-Mathisen, eds.). John Wiley and Sons, Ltd., New York, 1–23 (1990).
2. Werman, R., R. A. Davidoff, and M. H. Aprison. Inhibitory action of glycine on spinal neurons in the cat. *J. Neurophysiol.* **31**:81–95 (1968).

3. Langosch, D., C. Becker, and H. Betz. The inhibitory glycine receptor: a ligand-gated chloride channel of the central nervous system. *Eur. J. Biochem.* **194**:1–8 (1990).
4. Johnson, J. W., and P. Ascher. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature (Lond.)* **325**:529–533 (1987).
5. Kleckner, N. W., and R. D. Kingledine. Requirement for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes. *Science (Washington D. C.)* **241**:835–837 (1988).
6. Iversen, L. L. Role of transmitter uptake mechanisms in synaptic neurotransmissions. *Br. J. Pharmacol.* **41**:571–591 (1971).
7. Bennett, J. P., Jr., A. H. Mulder, and S. Snyder. Neurochemical correlates of synaptically active amino acids. *Life Sci.* **15**:1045–1056 (1974)
8. Uhl, G. R., and P. R. Hartig. Transporter explosion: update on uptake. *Trends Pharmacol. Sci.* **13**:421–425 (1992).
9. Amara, S. G., and M. J. Kuhar. Neurotransmitter transporters: recent progress. *Annu. Rev. Neurosci.* **16**:73–93 (1993).
10. Giros, B., and M. G. Caron. Molecular characterization of the dopamine transporter. *Trends Pharmacol. Sci.* **14**:43–49 (1993).
11. Guastella, J., N. Brecha, C. Weigmann, H. A. Lester, and N. Davidson. Cloning, expression, and localization of a rat brain high-affinity glycine transporter. *Proc. Natl. Acad. Sci. USA* **89**:7189–7193 (1992).
12. Liu, Q., H. Nelson, S. Mandiyan, B. Lopez-Corcuera, and N. Nelson. Cloning and expression of a glycine transporter from mouse brain. *FEBS Lett.* **305**:110–114 (1992).
13. Smith, K. E., L. A. Borden, P. R. Hartig, T. Branchek, and R. L. Weinshank. Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. *Neuron* **8**:927–935 (1992).
14. Borowsky, B., E. Mezey, and B. J. Hoffman. Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. *Neuron* **10**:851–863 (1993).
15. Giros, B., S. El Mestikawy, L. Bertrand, and M. G. Caron. Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS Lett.* **295**:149–154 (1991).
16. Sweet, H. O. Clasper (*cla*). *Mouse News Lett.* **73**:18 (1985).
17. White, W. F., and A. H. Heller. Glycine receptor alteration in the mutant mouse *spastic*. *Nature (Lond.)* **298**:655–657 (1982).
18. Liu, Q.-R., B. López-Corcuera, S. Mandiyan, H. Nelson, and N. Nelson. Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. *J. Biol. Chem.* **268**:22802–22808 (1993).
19. Giros, B., S. El Mestikawy, N. Godinot, K. Zheng, H. Han, T. Yang-Feng, and M. G. Caron. Cloning, pharmacological characterization and chromosomal assignment of the human dopamine transporter. *Mol. Pharmacol.* **42**:383–390 (1992).
20. Giros, B., P. Solokoff, M.-P. Martres, J.-F. Riou, J. L. Emorine, and J.-C. Schwartz. Alternative splicing directs the expression of two D2 dopamine receptor isoforms. *Nature (Lond.)* **342**:923–926 (1989).
21. Frohman, M. A., M. K. Dush, and G. R. Martin. Rapid production of full-length cDNAs from rare transcripts: amplication using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**:8998–9002 (1988).
22. Yang-Feng, T. L., G. Floyd-Smith, M. Nemer, J. Drouin, and U. Francke. The pronatriodilatin gene is located on the distal short arm of human chromosome 1 and on mouse chromosome 4. *Am. J. Hum. Genet.* **37**:1117–1128 (1985).
23. Seldin, M. F., H. C. Morse III, J. P. Reeves, C. L. Scribner, R. C. LeBoeuf, and A. D. Steinberg. Genetic analysis of autoimmune *gld* mice. 1. Identification of a restriction fragment length polymorphism closely linked to the *gld* mutation within a conserved linkage group. *J. Exp. Med.* **167**:688–693 (1988).
24. Ceci, J. D., L. D. Siracusa, N. A. Jenkins, and N. G. Copeland. A molecular genetic linkage map of mouse chromosome 4 including the localization of several proto-oncognenes. *Genomics* **5**:699–709 (1989).
25. Bahary, N., G. Zorich, J. E. Pachter, R. L. Leibel, and J. M. Friedman. Molecular mapping of mouse chromosomes 4 and 6. *Genomics* **11**:33–47 (1991).
26. Cutting, G. R., S. Curristin, H. Zoghbi, B. O'Hara, M. F. Seldin, and G. R. Uhl. Identification of a putative γ-aminobutyric acid (GABA) receptor subunit ρ2 cDNA and colocalization of the genes encoding ρ2 (GABRR2) and ρ1 (GABRR1) to human chromosome 6q14-q21 and mouse chromosome 4. *Genomics* **12**:801–806 (1992).
27. Gregor, P., R. H. Reeves, E. W. Jabs, X. Yang, W. Dackowski, J. M. Rochelle, R. H. Brown, Jr., J. L. Haynes, B. F. O'Hara, G. R. Uhl, and M. F. Seldin. Chromosomal localization of glutamate receptor genes: relationship to familial amyotrophic lateral sclerosis and other neurological disorders of mice and humans. *Proc. Natl. Acad. Sci. USA* **90**:3053–3057 (1993).
28. Shows, T. B., R. L. Eddy, M. G. Byers, Y. Fukushima, C. R. Dehaven, J. C. Murray, and G. I. Bell. Polymorphic human glucose transporter gene (*GLUT*) is on chromosome 1p31.3-p35. *Diabetes* **36**:546–549 (1987).
29. Liu, Q.-R., S. Mandyan, H. Nelson, and N. Nelson. A family of genes encoding neurotransmitter transporters. *Proc. Natl. Acad. Sci. USA* **89**:6639–6643 (1992).
30. Green, E. L. Linkage, recombination and mapping, in *Genetics and Probability in Animal Breeding Experiments* (E. Green, ed.). Macmillan, New York, 77–113 (1981).
31. Bishop, D. T. The information content of phase-known matings for ordering genetic loci. *Genet. Epidemiol.* **2**:349–361 (1985).
32. Abbott, C. M., R. Blank, J. T. Eppig, J. M. Friedman, K. E. Huppi, I. Jackson, B. A. Mock, J. Stoye, and R. Wiseman. Mouse chromosome 4. *Mamm. Genome* **3**:S55–S64 (1992).
33. Arriza, J. L., M. P. Kavanaugh, W. A. Fairman, Y. N. Wu, G. H. Murdoch, R. A. North, and S. G. Amara. Cloning and expression of a human neutral amino-acid transporter with structural similarity to the glutamate transporter gene family. *J. Biol. Chem.* **268**:15329–15332 (1993).
34. Shafqat, S., B. K. Tamarappoo, M. S. Kilberg, R. S. Puranam, J. O. McNamara, A. Guadano-Ferraz, and R. T. Fremeau, Jr. Cloning and expression of a novel Na^+-dependent neutral amino acid transporter structurally related to mammalian Na^+/glutamate cotransporters. *J. Biol. Chem.* **268**:15351–15355 (1993).
35. Christensen, H. N. Recognition sites for material transport and information transfer. *Curr. Top. Membr. Transp.* **6**:227–258 (1975).
36. Christensen, H. N. Organic ion transport during seven decades: the amino-acids. *Biochim. Biophys. Acta* **779**:255–269 (1984).
37. Kong, C. T., S. F. Yet, and J. E. Lever. Cloning and expression of a mammalian Na^+/amino acid cotransporter with sequence similarity to Na^+/glucose cotransporters. *J. Biol. Chem.* **268**:1509–1512 (1993).
38. Johnston, G. A. R., and L. L. Iversen. Glycine uptake in rat central nervous system slices and homogenates: evidence for different uptake systems in spinal cord and cerebral cortex. *J. Neurochem.* **18**:1951–1961 (1971).
39. Zafra, F., and C. Gimenez. Characteristics and adaptative regulation of glycine transport in cultured glial cells. *Biochem. J.* **258**:403–408 (1989).

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