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Molecular cloning and functional expression of the human glycine transporter GlyT2 and chromosomal localisation of the gene in the human genome

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Abstract **Neurotransmitter transport systems are major targets for therapeutic alterations in synaptic function. We have cloned and sequenced a cDNA encoding the human type 2 glycine transporter GlyT2 from human brain and spinal cord. An open reading frame of 2391 nucleotides encodes a 797 amino acid protein that transports glycine in a Na^+/Cl^--dependent manner. When stably expressed in CHO cells, human GlyT2 displays a dose-dependent uptake of glycine with an apparent K_m of 108 μM. This uptake is not affected by sarcosine at concentrations up to 1 mM. Radiation hybrid analysis mapped the GlyT2 gene to D11S1308 (LOD = 8.988) on human chromosome 11p15.1–15.2.**

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Key words: Glycine transporter; cDNA cloning; Genomic localization; Neurotransmitter; Human spinal cord; Brain stem; Cerebellum

1. Introduction

The amino acid glycine is thought to have two major roles in mammalian central neurotransmission. Firstly, glycine acts as the major inhibitory neurotransmitter in the caudal brain and spinal cord [1] where its action is mediated by the strychnine-sensitive glycine receptor to produce inhibitory post-synaptic potentials [2]. In addition to this inhibitory role, glycine also modulates excitatory neurotransmission by potentiating the action of glutamate at *N*-methyl-D-aspartate (NMDA) receptors [3,4].

The termination of action of most neurotransmitters is mediated by rapid re-uptake into the pre-synaptic terminal or surrounding glial cells [5,6]. The active transport of neurotransmitters across the plasma membrane is driven by the transmembrane Na^+ gradient generated by the plasma membrane Na^+/K^+-ATPase [6]. To date, molecular cloning studies have lead to the identification of two distinctive gene families encoding neurotransmitter transporter proteins. These include the Na^+/K^+-dependent (but Cl^--independent) excitatory amino acid transporter family GLAST [7], EAAC-1 [8], GLT1 [9], and the more recently discovered EAAT4 [10]. The second, Na^+/Cl^--dependent family includes transporters for γ-aminobutyric acid (GABA) [11–15], norepinephrine/epinephrine [16], dopamine [17–22], serotonin [23,24], proline [25], choline [26], betaine [27], taurine [28–30], creatine [31] and glycine [32–37].

High-affinity glycine transporters have so far been shown to be encoded by two separate genes. GlyT1 [32–34,36,37] occurs in three separate isoforms (GlyT1a, -1b and -1c) encoded by a single gene which differ only in their amino-terminal region [32]. GlyT1a and -1b originate from transcription directed from alternate promoters whereas GlyT1c is a splice variant of the -1b transcript [38]. GlyT1 is expressed predominantly in glial cells [38,39] of brain areas such as hippocampus, cortex and cerebellum, and also in the brain stem and spinal cord [33,37–41]. Based on its tissue distribution, GlyT1 has been suggested to co-localise with the NMDA receptor [37] where it may modulate the concentration of glycine at its co-agonist binding site [3]. Indeed, a recent study has demonstrated that GlyT1b has the ability to control the response of the NMDA receptor when co-expressed in *Xenopus* oocytes [42].

More recently, a second glycine transporter (GlyT2) was reported [35] and shown to be encoded by a separate gene. GlyT2 is distinguishable from GlyT1 in terms of primary structure (48% amino acid sequence identity), tissue distribution and pharmacological properties [35]. In contrast to GlyT1, GlyT2 is predominantly neuronal in nature [39], and restricted to the spinal cord, brain stem, and to a lesser extent the cerebellum [39,41,43,44] where it co-localises with strychnine binding sites [44]. This correlation with the distribution of inhibitory, strychnine-sensitive glycine receptors suggests that GlyT2 is involved in the termination of inhibitory neurotransmission at strychnine-sensitive glycinergic synapses.

Abnormalities of neurotransmitter transport can contribute to neuropathological processes. For example, blockage of and/or reversal of the glutamate transporters during ischaemia or anoxia elevates the extracellular concentration of L-glutamate to neurotoxic levels resulting in nerve cell damage [45,46]. Neurotransmitter transporters are also major targets for therapeutic and pathological alterations in synaptic function. This is particularly apparent by the ability of monoamine reuptake inhibitors such as tricyclic antidepressants, amphetamines and cocaine to produce dramatic behavioural changes [47]. Also, selective GABA reuptake inhibitors (e.g. tiagabine) are being developed as anticonvulsant and anxiolytic agents [48]. Similarly, the development of selective GlyT2 inhibitors could prove to be useful agents for modulating glycinergic neurotransmission in the caudal brain and spinal cord.

In this study, we report the isolation of a cDNA from human brain and spinal cord which is homologous to the

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Abbreviations: GlyT, glycine transporter; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; dNTP, deoxyribonucleotide triphosphate; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; HBSS, Hanks' balanced salts solution

The nucleotide sequence presented here has been submitted to the GenBank database under accession number AF085412.

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rat GlyT2 sequence. We show that it encodes a Na^+- and Cl^--dependent type 2 glycine transporter with similar pharmacology reported for rat GlyT2, and that this transcript is similarly located in spinal cord, brain stem and cerebellum.

2. Materials and methods

2.1. Cloning of human GlyT2

A human GlyT2 probe was prepared by performing PCR on a human whole brain cDNA library (λgt11, Clontech) using a pair of primers (sense: 5′-CAGTGGGGCTGGGTAATGTTTGG-3′ and antisense: 5′-AAAGATGATGGCCCAGAATGGAGA-3′) corresponding to conserved regions in the first and eighth transmembrane domains of rat GlyT2. Reactions (100 μl) contained 30 pmol of each primer, 2.5 U Taq DNA polymerase (Gibco BRL) in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, and subjected to 40 cycles (1 min, 94°C; 1 min, 60°C; 1 min 72°C). This gave rise a product of expected size (1045 bp) which was purified using the Geneclean II kit (Bio 101) and ligated into the TA plasmid pCRII (Invitrogen). All plasmids were transformed into competent *Escherichia coli* TOP10F′ cells (Invitrogen) and propagated using standard conditions [49]. The DNA sequence of the PCR product was confirmed by dideoxy sequencing using the Pharmacia autoread sequencing kit and ALF automated DNA sequencer. This product was then used to screen human whole brain, spinal cord (both λgt11) and brainstem-medulla (λgt10) cDNA libraries (Clontech). Phage (40 000 per 150 mm diameter plate) were transferred to nitrocellulose filters (Costar) as described in Sambrook et al. [49]. Filters were prehybridised at 42°C for 2 h with 50% formamide, 6×SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 5×Denhardt's (0.1% w/v polyvinylpyrrolidone, 0.1% w/v Ficoll type 400, 0.1% bovine serum albumin), and 100 μg/ml sheared, denatured salmon sperm DNA. Hybridisation was performed overnight at 42°C in the same solution containing [^{32}P]dCTP-labeled probe (DNA probes were prepared using the Pharmacia Ready-To-Go DNA labeling kit). The filters were washed twice with 2×SSC, 0.1% SDS (room temperature), then twice with 0.5×SSC, 0.1% SDS (50°C) and exposed to Hyperfilm MP (Amersham).

Positives were picked and purified by further rounds of screening. Phage DNA was prepared using the Qiagen lambda maxi kit, with cDNA inserts being excised by *Eco*RI digestion and subcloned into pUC18 for further analysis.

The 5′ end of the hGlyT2 cDNA was isolated using the cerebellum 5′-RACE Ready cDNA kit (Clontech). Primary PCR was carried out using the supplied anchor primer (AP1) together with a gene-specific primer (antisense: 5′-TGCCCTTGCCCACACTCACGTTCGCAT-3′). The reaction was carried out with Amplitaq Gold (Perkin-Elmer) in the manufacturer supplied buffer with the addition of 5% DMSO (94°C denaturation/activation followed by 30 cycles of 30 s, 94°C; 30 s 65°C; 2 min 72°C). This was followed up by a secondary PCR using the supplied nested anchor primer (AP2) together with a nested gene-specific primer (antisense: 5′-CCGCGGGCTACTGAGTTTGCAA-GACC-3′). Cycling parameters were the same as for the primary PCR reaction. Four products were obtained of approximately 600 bp, 500 bp, 350 bp and 300 bp and termed RACE clones 1–4 respectively. These were purified and cloned into pCR2.1 (Invitrogen) and their identity confirmed by dideoxy sequencing.

The full-length hGlyT2 cDNA was constructed using the 5′ RACE PCR products together with the three overlapping cDNA clones (clones 1, 10 and 11) isolated from the cDNA library screens (Fig. 1A). The 5′ end was reconstructed by ligating *Hin*dIII/*Eco*72I fragment from pCR2.1/RACE clone 1 into *Hin*dIII/*Eco*72I digested pUC/clone 10 DNA. This gave rise to a 5′ end clone containing 300 bp of untranslated 5′ leader sequence. The *Hin*dIII/*Nco*I fragment from this 5′ end containing clone was ligated together with the *Nco*I/*Nae*I fragment from clone 11 and *Nae*I/*Eco*RI fragment from clone 1 into *Hin*dIII/*Eco*RI digested pcDNA3 (Invitrogen) and denoted pcDNA3/hGlyT2. In this construct, expression of the hGlyT2 cDNA is under control of the CMV IE gene promoter.

2.2. Generation of the genomic/cDNA fusion construct

An earlier attempt at isolating the 5′ end of the hGlyT2 sequence from a genomic clone (isolated from a genomic DNA library in the λ Dash II vector (Stratagene)) revealed a canonical 3′ splice acceptor site at the position where the translational initiation codon was expected (Fig. 4). In an attempt to increase expression from the pcDNA3/hGlyT2 construct, a genomic/cDNA fusion was created by including this intron in the expression construct. The intron was cloned by PCR using primers directed against exon sequence on either side of the intron (sense: 5′-CCAGTCTTGTCAATAGCGGGTTT-CAC-3′; antisense: 5′-TGCCCTTGCCCACACTCACGTTCGCAT-3′) using human genomic DNA as a template. PCR was performed using the Clontech Advantage Genomic PCR kit (95°C 1 min, then 35 cycles of: 95°C, 15 s; 68°C, 12 min). This gave rise to a single fragment of approximately 1800 bp that was purified and cloned into pCR2.1 (Invitrogen). Dideoxy sequencing confirmed that this fragment contained the intron flanked by exon sequence. The genomic/cDNA fusion construct was then generated by purifying the *Eco*RI/*Eco*72I fragment from the pCR2.1/genomic sequence and ligating this with the *Eco*72I/*Xho*I fragment from pcDNA3/hGlyT2 into *Eco*RI/*Xho*I-digested pcDNA3.

2.3. Northern blot analysis

The cDNA insert from clone 10 was radiolabeled using the Pharmacia Ready-To-Go DNA labeling kit. Human multiple tissue Northern blots (Clontech) were hybridised for 2 h at 68°C in ExpressHyb hybridisation solution (Clontech) and washed in 2×SSC, 0.05% SDS at room temperature, twice for 40 min with 0.1×SSC, 0.05% SDS at 50°C, then exposed to Hyperfilm MP (Amersham) with one intensifying screen at −80°C for up to 6 weeks.

2.4. Genomic localisation

The genetic locus of hGlyT2, was determined by PCR assay of the Genebridge-4 radiation-reduced somatic cell DNA panel consisting of 84 hybrids each containing a previously characterised complement of human DNA [50] (UK MRC HGMP Resource Centre, Cambridge). This was performed using primers (sense: 5′-GGCTATTTCCA-TTCCCCCTTTACCCTTAC-3′ and antisense: 5′-TGTTGGCTGG-CGTTTATTCATTTCCTT-3′) directed against hGlyT2 genomic sequence (unpublished data) encompassing an intron/exon boundary with sub-chromosomal mapping of the 334 bp PCR product being analysed via the Whitehead Institute radiation hybrid mapper: http://www.genome.wi.mit.edu/ftp/pub/software/rhmapper. Each PCR reaction was carried out with 100 ng template DNA using Amplitaq Gold in the manufacturer-supplied buffer and subjected to 30 cycles (30 s, 94°C; 30 s 56°C; 40 s, 68°C). PCR reactions were subsequently electrophoresed on 1% agarose gels and analysed for the presence of the hGlyT2 product.

2.5. Cell culture and transfection

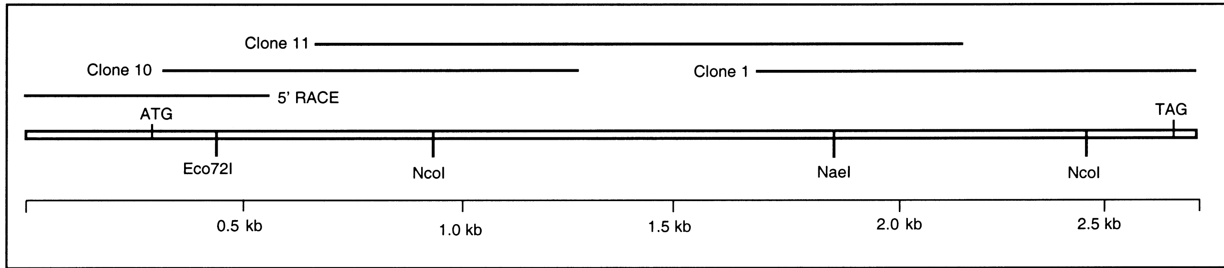
CHO cells were propagated in culture medium containing Dulbecco's modified Eagle's medium (DMEM/Nut mix 1:1 with GlutaMAX1, Gibco BRL) supplemented with 10% fetalclone II (Hyclone) and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells for transfection were trypsinised the day before the experiment and seeded at 1.5×10^6 per 10 cm dish.

For transfection, cells were washed twice with OpitMEM (Gibco BRL) before exposure to transfection medium for 5 h. Transfection medium consisted of 16 μg of DNA and 100 μl LipofectAMINE (Gibco BRL). After 5 h, 5 ml of growth medium plus 20% FBS was added, the cells incubated overnight, and the medium changed again. Stably transfected cells were selected for 1 week in growth medium containing 2 mg/ml Geneticin (Gibco BRL), and the remaining cells transferred to cloning plates in growth medium containing 1 mg/ml Geneticin. Individual clones were picked for further analysis and positives passaged routinely in growth medium containing 0.5 mg/ml Geneticin. Cells were seeded in either 24 well plates (200 000 cells/well) or 96 well plates (30 000 cells/well) 24 h before assaying.

2.6. Glycine uptake assay

To measure glycine transport, cells were washed twice with Hanks' balanced salts solution (HBSS). The plates were then equilibrated to 37°C with HBSS for 5 min (400 μl in 24 well plates; 200 μl in 96 well plates). For uptake inhibition studies, glycine and sarcosine were included at this stage. In 24 well plates, 100 μl of [^{3}H]glycine (150 μM, 248 Bq/nmol, NEN) was added to each well with a further 100 μl aliquot taken for scintillation counting to determine total activity added. Incubation was continued for 5 min and uptake terminated by washing the cells three times with ice-cold HBSS. After removal of

A



B

1 CGCCCGGGCAGGTCAAACACGCAGCCCCCTCCCGCTGGAGCGACAACTGGCCAGCATACTCTAGGCTGTTGTCCCTTTAAAACTTGAATCCAAGGGGGTA 100

101 ATGATTTATCAAACTTGTATTATCAAGAAAATGTCAAACCAAGGGCACCTTGCTTTGCACTGACGCAAACCCGGCCTTTCCCAAGGAGATATAGAAAGCG 200

201 CCTCTCCTGCCTGAGCCAAACCCAGTCTTGTCAATAGCGGGTTTCACCCTCCACCAGTTCAGTCTGTTGCCTGTGTCAGACATGGATTGCAGTGCTCCCA 300
M D C S A P K

301 AGGAAATGAATAAACTGCCAGCCAACAGCCCGGAGGCGGCGGCGGCGCAGGGCCACCCGGATGGCCCATGCGCTCCCAGGACGAGCCCGGAGCAGGAGCT 400
E M N K L P A N S P E A A A A Q G H P D G P C A P R T S P E Q E L

401 TCCCGCGGCTGCCGCcCCGCCGCCGCCACGTGTGCCCAGGTCCGCTTCCACCGGCGCCCAAACTTTCCAGTCAGCGGACGCGCGAGCCTGCGAGGCTGAG 500
P A A A A P P P P R V P R S A S T G A Q T F Q S A D A R A C E A E

501 CGGCCAGGAGTGGGGTCTTGCAAACTCAGTAGCCCGCGGGCGCAGGCGGCCTCTGCAGCTCTGCGGGACTTGAGAGAGGCGCAAGGCGCGCAGGCCTCGC 600
R P G V G **S** C K L S **S** P R A Q A A S A A L R D L R E A Q G A Q A S P

601 CCCCTCCCGGGAGCTCCGGGCCCGGCAACGCGTTGCACTGTAAGATCCCTTCTCTGCGAGGCCCGGAGGGGGATGCGAACGTGAGTGTGGGCAAGGGCAC 700
P P G S S G P G N A L H C K I P **S** L R G P E G D A N V S V G K G T

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L E R N N T P V V G W V N M S Q S T V V L G T D G I T S V L P G S

801 GTGGCCACCGTTGCCACCCAGGAGGACGAGCAAGGGGATGAGAATAAGGCCCGAGGGAACTGGTCCAGCAAACTGGACTTCATCCTGTCCATGGTGGGGT 900
V A T V A T Q E D E Q G D E N K A R G N W **S** S K L D F I L S M V G Y

901 ACGCAGTGGGGCTGGGCAATGTCTGGAGGTTTCCCTACCTGGCCTTCCAGAACGGGGGAGGTGCTTTCCTCATCCCTTACCTGATGATGCTGGCTCTGGC 1000
A V G L G N V W R F P Y L A F Q N G G G A F L I P Y L M M L A L A

1001 TGGATTACCCATCTTCTTCTTGGAGGTGTCGCTGGGCCAGTTTGCCAGCCAGGGACCAGTGTCTGTGTGGAAGGCCATCCCAGCTCTACAAGGCTGTGGC 1100
G L P I F F L E V S L G Q F A S Q G P V S V W K A I P A L Q G C G

1101 ATCGCGATGCTGATCATCTCTGTCCTAATAGCCATATACTACAATGTGATTATTTGCTATACACTTTTCTACCTGTTTGCCTCCTTTGTGTCTGTACTAC 1200
I A M L I I S V L I A I Y Y N V I I C Y T L F Y L F A S F V S V L P

1201 CCTGGGGCTCCTGCAACAACCCTTGGAATACACCAGAATGCAAAGATAAAACCAAACTTTTATTAGATTCCTGTGTTATCAGTGACCATCCCAAAATACA 1300
W G S C N N P W N T P E C K D K T K L L L D S C V I S D H P K I Q

1301 GATCAAGAACTCGACTTTCTGCATGACCGCTTATCCCAACGTGACAATGGTTAATTTCACCAGCCAGGCCAATAAGACATTTGTCAGTGGAAGTGAAGAG 1400
I K N S T F C M T A Y P N V T M V N F T S Q A N K T F V S G S E E
* * * *

1401 TACTTCAAGTACTTTGTGCTGAAGATTTCTGCAGGGATTGAATATCCTGGCGAGATCAGGTGGCCACTAGCTCTCTGCCTCTTCCTGGCTTGGGTCATTG 1500
Y F K Y F V L K I S A G I E Y P G E I R W P L A L C L F L A W V I V

1501 TGTATGCATCGTTGGCTAAAGGAATCAAGACTTCAGGAAAAGTGGTGTACTTCACGGCCACGTTCCCGTATGTCGTACTCGTGATCCTCCTCATCCGAGG 1600
Y A S L A K G I K T **S** G K V V Y F T A T F P Y V V L V I L L I R G

1601 AGTCACCCTGCCTGGAGCTGGAGCTGGGATCTGGTACTTCATCACACCCAAGTGGGAGAAACTCACGGATGCCACGGTGTGGAAAGATGCTGCCACTCAG 1700
V T L P G A G A G I W Y F I T P K W E K L T D A T V W K D A A T Q

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I F F S L S A A W G G L I T L S S Y N K F H N N C Y R D T L I V T C

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T N S A T S I F A G F V I F S V I G F M A N E R K V N I E N V A D

1901 CCAAGGGCCAGGCATTGCATTTGTGGTTTACCCGGAAGCCTTAACCAGGCTGCCTCTCTCTCCGTTCTGGGCCATCATCTTTTTCCTGATGCTCCTCACT 2000
Q G P G I A F V V Y P E A L T R L P L S P F W A I I F F L M L L T

2001 CTTGGACTTGACACTATGTTTGCCACCATCGAGACCATAGTGACCTCCATCTCAGACGAGTTTCCCAAGTACCTACGCACACACAAGCCAGTGTTTACTC 2100
L G L D T M F A T I E T I V T S I S D E F P K Y L R **T** H K P V F T L

2101 TGGGCTGCTGCATTTGTTTCTTCATCATGGGTTTTCCAATGATCACTCAGGGTGGAATTTACATGTTTCAGCTTGTGGACACCTATGCTGCCTCCTATGC 2200
G C C I C F F I M G F P M I T Q G G I Y M F Q L V D T Y A A S Y A

2201 CCTTGTCATCATTGCCATTTTTGAGCTCGTGGGGATCTCTTATGTGTATGGCTTGCAAAGATTCTGTGAAGATATAGAGATGATGATTGGATTCCAGCCT 2300
L V I I A I F E L V G I S Y V Y G L Q R F C E D I E M M I G F Q P

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N I F W K V C W A F V T P T I L T F I L C F S F Y Q W E P M T Y G S

2401 CTTACCGCTATCCTAACTGGTCCATGGTGCTCGGATGGCTAATGCTCGCCTGTTCCGTCATCTGGATCCCAATTATGTTTGTGATAAAAATGCATCTGGC 2500
Y R Y P N W S M V L G W L M L A C S V I W I P I M F V I K M H L A
*

2501 CCCTGGAAGATTTATTGAGAGGCTGAAGTTGGTGTGCTCGCCACAGCCGGACTGGGGCCCATTCTTAGCTCAACACCGCGGGGAGCGTTACAAGAACATG 2600
P G R F I E R L K L V C S P Q P D W G P F L A Q H R G E R Y K N M

2601 ATCGACCCCTTGGGAACCTCTTCCTTGGGACTCAAACTGCCAGTGAAGGATTTGGAACTGGGCACTCAGTGCTAGTCCAGTGGTGTGGGATGGTCCAGAC 2700
I D P L G T S S L G L K L P V K D L E L G T Q C *

2701 TTGATCCTGTTTTTCCTCTCTGCCCCGGA 2729

Fig. 1. A: Reconstruction of the full-length human GlyT-2 cDNA. The full-length cDNA was constructed using the three overlapping cDNA clones (clones 1, 10 and 11) isolated from the human brain stem (medulla) and spinal cord cDNA libraries, together with the RACE PCR clone containing the 5′ end. The relative positions of these clones are indicated in the figure together with the restriction enzyme sites used to fuse the fragments together as described in Section 2. The positions of the translational initiation and stop codons are also shown, and the bottom line represents a scale bar marked off in kb. B: Nucleotide and deduced amino acid sequence of the cDNA encoding the human glycine transporter GlyT-2. The cDNA was cloned and sequenced as described in Section 2. Putative *N*-linked glycosylation sites are indicated by a star and protein kinase C phosphorylation sites are underlined in bold.
←

excess liquid, 250 μl of 1% sodium dodecyl sulphate was added to each well and the plates left for 1 h. A 150 μl aliquot was removed for scintillation counting and placed in a 6 ml scintillation vial with 2 ml Ultima Gold scintillation cocktail (Canberra Packard). For 96 well plates, 50 μl of [^{3}H]glycine (50 μM, 248 Bq/nmol) was added to each well with a further 50 μl taken for scintillation counting to determine total activity added. Incubation was continued for 20 min and uptake terminated by washing the cells with three aliquots of ice-cold HBSS. After removal of excess liquid, 200 μl of Microscint 20 cocktail (Canberra Packard) was added. The plates were then sealed with Topseal film and shaken to ensure samples were homogenous before scintillation counting in a plate counter (1450 MicroBeta Trilux, Wallac). Data were analysed using the GraphPad Prism analysis package and the curve fitting option sigmoid dose response (variable slope) to produce pIC_{50} (the negative logarithm of the concentration of test compound causing 50% inhibition of uptake) values where appropriate.

3. Results

3.1. Cloning and analysis of hGlyT2 cDNA

In order to clone the human type 2 glycine transporter, PCR was performed on a human whole-brain cDNA library using oligonucleotides based on the rat GlyT2 sequence and directed against regions of high homology (transmembrane domains 1 and 8 respectively) in the Na^+/Cl^--dependent transporter family. This produced a fragment of expected size (1045 bp) which, when cloned and sequenced, was found to be highly homologous (91%) to the corresponding rat GlyT2 sequence. The polypeptide encoded by this fragment is 98.8% homologous to the corresponding rat GlyT2 sequence with all four amino acid residue substitutions being conservative. These differences are consistent with the PCR product being the likely human homologue of the rat GlyT2 sequence. This PCR product was then used to screen cDNA libraries derived from human spinal cord and brain stem (medulla), areas which, in the rat, have been demonstrated to be rich in GlyT2 mRNA [39,41,43,44]. This gave rise to three overlapping positive cDNA clones (Fig. 1A). The first (clone 1), isolated from the medulla library (from a total of 2.4 million clones screened), was 1008 bp long and included the 3′ end of the human GlyT2 reading frame. Screening a similar number of clones from the spinal cord library gave rise to another two clones (clone 10, 968 bp and clone 11, 1490 bp). Analysis of these clones indicated that clone 10, which extended furthest 5′ of the three clones, terminated 26 bp short of the expected translational initiation codon when compared to the rat GlyT2 sequence. The remaining 5′ sequence of the hGlyT2 cDNA was finally isolated using 5′ RACE PCR. This generated four products with the longest including 300 bp of 5′ untranslated leader sequence. The full-length hGlyT2 sequence was generated using the overlapping RACE and cDNA clones as described in Section 2.

Analysis of the full-length cDNA (Fig. 1B) revealed a 2391 bp reading frame encoding a 797 amino acid polypeptide with a calculated molecular mass of 87 370 Da. This is 88% homologous with the rat GlyT2 sequence at the DNA level and 94% at the amino acid level (Fig. 2) which is consistent with this cDNA encoding the human homologue of the rat GlyT2 sequence. Interestingly, most (42 from 47) of the differences at the amino acid level occur within the extended hydrophilic

Fig. 2. Alignment of the amino acid sequences of human GlyT1b, GlyT2 and rat GlyT2. The deduced amino acid sequences of human GlyT1b, GlyT2 and rat GlyT2 were aligned using a DNAman program. Conserved amino acid residues are indicated with a star, while conservative changes are marked with a dot. Putative transmembrane domains are marked by solid lines above the sequence.

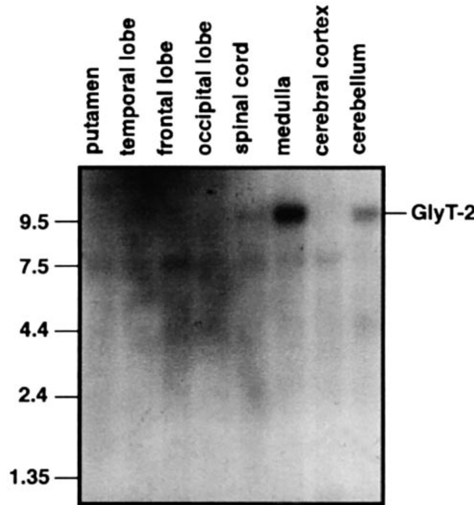

Fig. 3. Localisation of hGlyT2 in the nervous system. Northern hybridisation was performed as described in Section 2. The size of RNA standards is indicated in kb. A single specific band of approximately 9.5 kb is indicated in medulla, cerebellum and less clearly in spinal cord. No signal is observed in any other brain area.

amino-terminal domain. The deduced protein contains five potential *N*-linked glycosylation sites; four in the large extracellular loop between the putative third and fourth transmembrane domains (Asn-343, 353, 358, 364) and one (Asn-712) between the 11th and 12th transmembrane domains. All are conserved in the rat sequence. There are also six consensus sites for protein kinase C-dependent phosphorylation: Ser-79, 84, 124 and 195 in the long amino-terminal domain (84 and 195 are conserved in the rat sequence); Ser-417 between transmembrane domains 4 and 5 where similar sites are found in several other members of this transporter family [32,51], and a threonine at position 600 between transmembrane domains 8 and 9.

3.2. Northern blot analysis

To determine the tissue distribution of the mRNA encoding hGlyT2, Northern analysis was performed with blots containing mRNA isolated from different parts of the human CNS using clone 10 as a hGlyT2-specific cDNA probe. This probe hybridised weakly to a single band of approximately 9.5 kb in medulla, and to a lesser extent in spinal cord and cerebellum (Fig. 3). No signal was observed in any other brain area, including whole brain mRNA. This is consistent with the known tissue distribution of GlyT2 mRNA in the rat nervous system [39,41,43,44].

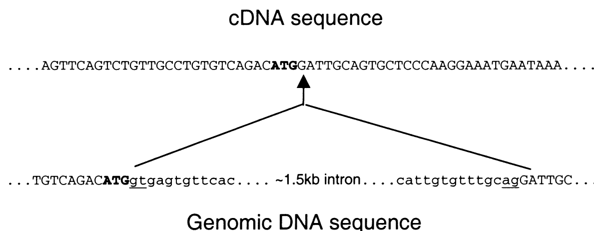

Fig. 4. Generation of the genomic/cDNA fusion construct. The cDNA sequence is shown on the top line with the translational initiation codon highlighted in bold. The position of the single endogenous intron cloned and incorporated into the cDNA sequence as described in Section 2 is indicated by the arrow. The lower line shows the genomic sequence with the intron sequence in lower case. The GT/AG dinucleotides typically found at 5′ and 3′ splice junctions respectively are underlined.

3.3. Genomic localisation

PCR analysis of the Genebridge-4 radiation-reduced somatic cell DNA panel [50] for a hGlyT2 specific product (data not shown) indicated that the GlyT2 gene maps to locus D11S1308 on chromosome 11 (LOD = 8.988). This corresponds to the cytogenetic band 15.1–15.2 on the short arm of chromosome 11 (11p15.1–15.2).

3.4. Stable expression of hGlyT2 in CHO cells

In order to determine the substrate specificity and kinetics of this transporter, the putative hGlyT2 cDNA was transfected into CHO cells. Initial studies (data not shown) demonstrated that this cDNA exhibited very low levels of expression, however, a genomic/cDNA fusion construct containing a single endogenous intron found at the 5′ end of the human GlyT2 gene (Fig. 4; generated as described in Section 2) exhibited somewhat higher levels. Stable cell lines were then established using this construct with one, clone #91, being employed in all subsequent analysis.

To determine the specificity of the transporter, the CHO/hGlyT2 cell line was assayed for its ability to accumulate a variety of potential substrates. As observed in Fig. 5A, this cell line accumulated up to 15-fold more [^{3}H]glycine than the

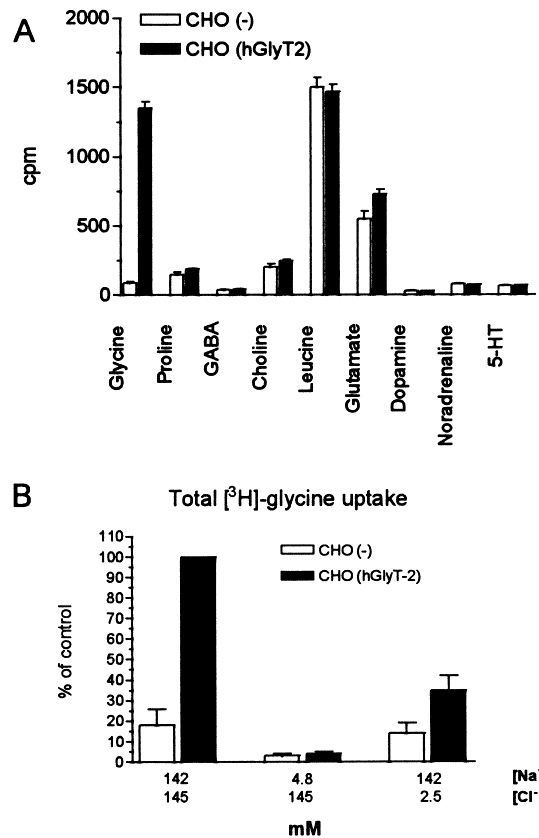

Fig. 5. A: Uptake of a range of ^{3}H-labeled substrates (5 nM) in CHO/hGlyT-2 clone 91 and in the untransfected parental cell line (CHO(−)). B: Ionic dependence of glycine uptake in hGlyT2 clone and in the untransfected parental cell line (CHO(−)). Data are expressed as % of the control conditions ([Na$^+$] = 142 mM, [Cl$^-$] = 145 mM) for CHO (hGlyT-2). Error bars indicate standard deviation (n = 3).

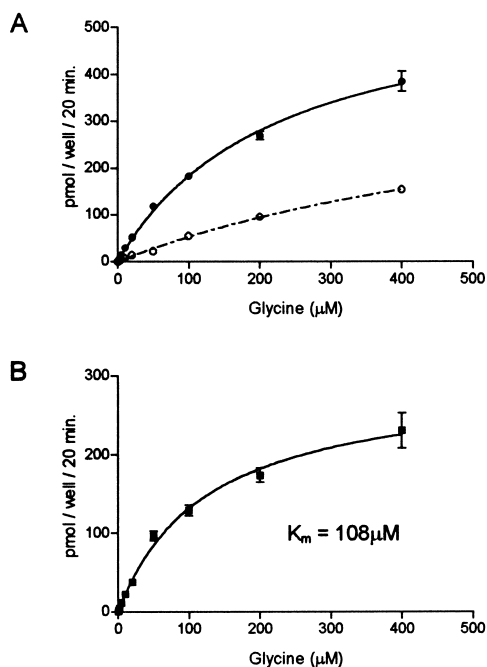

Fig. 6. Uptake of glycine during a 20 min period as a function of increasing substrate concentration. A: Data for clone 91 (●) and untransfected parental cell line (○). B: Difference between transfected and untransfected cell lines (■).

parental untransfected CHO cell line. Furthermore, there was no significant difference in uptake of a number of other substrates including radiolabeled proline, GABA, choline, leucine, glutamate, dopamine, norepinephrine and serotonin. Glycine transport exhibited by this cell line was also demonstrated to be Na^+- and Cl^-dependent by the substitution of these ions by Li^+ and acetate (Fig. 5B).

To determine the kinetics of the transporter, the CHO/hGlyT2 cell line was incubated with varying concentrations of [^{3}H]glycine, and the specific accumulation of radioactivity determined. A representative experiment illustrated in Fig. 6 demonstrates that GlyT2 confers saturable uptake with a Michaelis constant (K_m) of 108 μM. This figure was calculated by subtracting the low-affinity glycine uptake endogenous to CHO cells from the total uptake exhibited by the CHO/hGlyT2 cell line (Fig. 6A), to yield the apparent high-affinity component expressed by GlyT2 (Fig. 6B). Glycine inhibited the accumulation of [^{3}H]glycine with a pIC_{50} value of 4, whereas sarcosine (*N*-methylglycine), which has been shown to inhibit GlyT1 in a dose-dependent manner [35,37], had no effect on glycine uptake by hGlyT2 at concentrations up to 1 mM (Fig. 7). This observation is similar to that reported for the cloned rat GlyT2 cDNA [35], and accordingly, we conclude that our cDNA encodes the human type 2 glycine transporter.

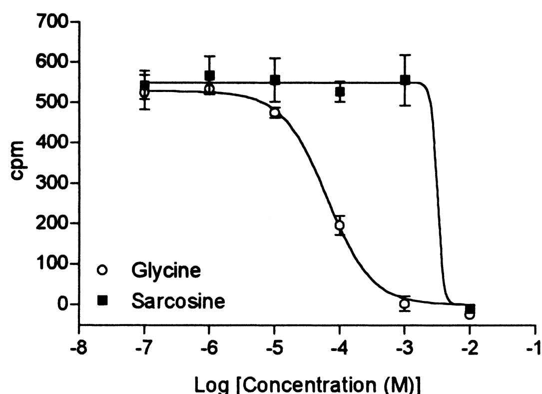

Fig. 7. Inhibition of [^{3}H]glycine uptake in CHO/hGlyT-2 clone 91. Data obtained in the untransfected cell line (CHO) has been subtracted from the corresponding values for clone 91 to produce the above curves. Data taken from a representative experiment ($n = 3$).

4. Discussion

In this study, we report the isolation of a cDNA encoding a high-affinity Na^+/Cl^--dependent type 2 glycine transporter from human brain and spinal cord. The protein encoded by this cDNA shares many of the features common to members of the Na^+/Cl^--dependent transporter family and is highly homologous to the rat GlyT2 sequence. Expression of this cDNA in stably transfected CHO cells resulted in high affinity glycine uptake with a K_m of 108 μM. This is higher than that reported for rat GlyT2 and probably reflects the nature of the expression systems used (oocytes for rat vs eukaryotic cell line (CHO) for human). Indeed, this is reminiscent of the discrepancies noted for GlyT1 with K_ms of 94 μM (CV-1 cells) [33], 123 μM (COS-7 cells) [37] compared 32 μM [34] and 25 μM [36] when expressed in oocytes. Also, the lack of inhibition by sarcosine that distinguishes GlyT1 from GlyT2 leads us to the conclusion that our cDNA encodes the human homologue of the rat GlyT2 sequence.

Glycine is transported by three classically defined systems distinguishable by substrate affinities and ionic dependence [52,53]. The type 2 glycine transporters reported by ourselves and Lui et al. [35], together with GlyT1, most likely constitute at least part of system Gly that is most specific for glycine and its *N*-methyl derivative, sarcosine [54]. The high selectivity of these transporters for glycine clearly distinguishes them from the other two classically defined amino acid transporter systems, system A and system ASC, which possess much broader specificity. Indeed, molecular cloning studies have demonstrated that at least one component of system ASC is encoded by a novel transporter, ASCT-1 [55] whose structure is very similar to that of the glutamate transporter gene family. Another, SAAT-1 [56], encodes a neutral amino acid transporter similar to Na^+/glucose cotransporters with specificity characteristics of system A.

Chromosomal localisation mapped the GlyT2 gene on human chromosome 11p15.1–15.2, however, this does not correlate to any likely known disease locus. Localisation of the hGlyT2 mRNA by Northern analysis revealed a 9.5 kb transcript restricted to brain stem, spinal cord and cerebellum which correlates well with a number of more detailed studies of GlyT2 mRNA in rat [33,34,37,38,41,44]. A number of studies have examined in detail the distribution of GlyT1 and GlyT2 by in situ hybridisation [33,34,37,38,41,44], and more recently by immunocytochemistry with antibodies raised against peptides corresponding to parts of the amino acid sequences of these molecules [39,57]. These studies have demonstrated that GlyT2 is predominantly neuronal with the exception of the cerebellum where it is also found in glial elements of the molecular layer [39]. Its localisation in brain stem and spinal cord correlates well with the distribution of the

strychnine-sensitive glycine receptor [2], and indeed, there is a high level of correspondence between GlyT2 levels and strychnine binding sites in the CNS [43]. Ultrastructural analysis of immunocytochemical staining using antisera directed against GlyT2 has indicated the presence of the protein in axons and axonal boutons enriched with glycine-like immunoreactivity [57]. Conversely, with the exception of the retina, areas devoid of strychnine-sensitive glycine receptors are also low in GlyT2. This evidence strongly suggests that GlyT2 is responsible for the termination of neurotransmission at strychnine-sensitive glycinergic synapses. While GlyT1 is also abundantly expressed in brain stem and spinal cord, it is also found in more rostral regions such as hypothalamus, thalamus and hippocampus [37,39]. Its presence in areas devoid of inhibitory glycinergic neurons and rich in NMDA receptors has lead to speculation that GlyT1 has a role in regulation of glycine levels in NMDA receptor-mediated neurotransmission, however, this has yet to be established unambiguously. Indeed, there is some speculation that GlyT1 may have a role in the regulation of glycine levels at the strychnine- (and non-strychnine-) sensitive inhibitory glycine receptors [39,41]. The relationship between GlyT1, GlyT2 and the NMDA/strychnine-sensitive glycine receptors, especially in areas rich in all four molecules such as spinal cord, is particularly intriguing and may only be resolved following the development of selective inhibitors of these transporters.

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