The open reading frame of the Na^+-dependent glutamate transporter GLAST-1 is expressed in bone and a splice variant of this molecule is expressed in bone and brain

Jim Huggett, Anne Vaughan-Thomas, Deborah Mason*

School of Bioscience, Cardiff University, Museum Avenue, P.O. Box 911, Cardiff CF10 3US, UK

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Abstract Previously we have reported expression of an mRNA with homology to the Na^+-dependent glutamate transporter, GLAST-1, in bone. Here we demonstrate that the complete open reading frame of GLAST-1 mRNA and corresponding 69 kDa protein are expressed in rat bone in vivo. We have also discovered a novel splice variant (GLAST-1a), lacking exon 3, expressed in rat bone and brain. A 55 kDa protein detected by anti-GLAST antibody in rat cerebellum corresponds to the molecular weight of unglycosylated GLAST-1a. This has led us to propose that GLAST-1a has an opposite orientation in the cell membrane to GLAST-1.

Key words: GLAST-1; Bone; Glutamate; Transporter; Splice variant

1. Introduction

Maintenance of optimal bone mass is controlled by systemic hormones and local factors such as cytokines, growth factors and the mechanical environment of bone cells. An imbalance of these factors may disrupt bone turnover and result in pathological bone loss. Glutamate signalling, more commonly associated with the central nervous system (CNS), has recently been implicated as a mechanism by which bone cells might communicate in response to their mechanical environment. This was first suggested by the discovery that an mRNA with homology to GLAST-1 is down-regulated by mechanical loading of osteocytes in vivo [1].

In the CNS, GLAST-1 functions as a Na^+-dependent symport of glutamate and aspartate that transports free excitatory amino acids (EAA) subsequent to neurotransmission [2]. The family of neurotransmitters to which GLAST-1 belongs also act as ligand-gated chloride channels that can regulate excitatory neurotransmission [3]. The protein structure of the high affinity Na^+-dependent EAA transporters has been studied extensively (reviewed by Slotboom et al. [4]). While it is agreed that GLAST-1 has six transmembrane (TM) α-helices at the N-terminal end, the C-terminal structure is less clear. Wahle and Stoffel proposed that the C-terminal is composed of four TM β-sheets [5] but Seal et al. propose a TM α-helix N-terminal of a re-entrant loop pore, followed by two hydrophobic regions that do not span the membrane and a final TM α-helix [2]. Further evidence for the re-entrant loop hypothesis is also provided by cysteine scanning mutagenesis studies in homologous regions of other members of the glutamate transporter family: rat Glt-1 [6] and GltT expressed by *Bacillus stearothermophilus* [7].

Our previous study suggested that GLAST-1 is expressed as a number of splice variants. Firstly, the differential display clone isolated as a mechanically regulated gene in osteocytes showed 81% identity with the 3′ untranslated region (UTR) of GLAST-1 over 33 bases, although spanning sequences were not similar. Secondly, Northern blot analysis using a probe to a translated region of GLAST-1 (bases 310–655, accession no. S49018) revealed expression of 2.6 kb and 4.5 kb mRNAs in brain and a single 2.6 kb transcript in bone [1].

Here we have used reverse transcription (RT)-PCR to show all 10 exons of GLAST-1 are expressed in bone in vivo. Sequencing of cloned RT-PCR products revealed the expression of two mRNA transcripts, one containing the complete open reading frame (ORF) (GLAST-1) and a novel variant where exon 3 is deleted (GLAST-1a). Western blot analysis revealed immunoreactive protein species corresponding to the molecular weight of both GLAST-1 and GLAST-1a. Recent data showing that glutamate uptake by astrocytes is mediated by increased transporter activity rather than receptor activation [8] suggest that transporters such as GLAST may control EAA signalling. If GLAST-1a represents a functional transporter, it is critical that it is considered when unraveling mechanisms of glutamate signalling both in brain and bone.

2. Materials and methods

2.1. Tissue preparation and RNA extraction

Rat tibiae were dissected from Wistar rats and epiphyses removed. Diaphyses were placed into 1.5 ml centrifuge tubes and the bone marrow flushed from the cavity by centrifugation at 1000 rpm for 30 s. Bones were snap frozen in liquid nitrogen and powdered (B. Braun Biotech Dismembrator, 2000 rpm for 3 min at −120°C) in 1 ml Trizol® reagent (Gibco, BRL). Total RNA was extracted following the manufacturer's instructions, precipitated with 0.5% (v/v) isopropanol and purified with 0.05% (v/v) Tack resin (Biogenesis). RNA was extracted from 100 mg of rat cerebellum using the same method. Contaminating genomic DNA was removed from all RNA samples by digestion with 2 U of RQ1 DNase (Promega) in the presence of 40 U RNasin® ribonuclease inhibitor (Promega) at 37°C for 15 min. DNase was removed by re-extraction of RNA in 500 μl Trizol®, and concentration and purity of RNA were estimated by measuring absorbance at 260 nm and 280 nm.

*Corresponding author. Fax: (44)-2920-874594.
E-mail: masondj@cardiff.ac.uk

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2.2. RT-PCR of GLAST mRNA

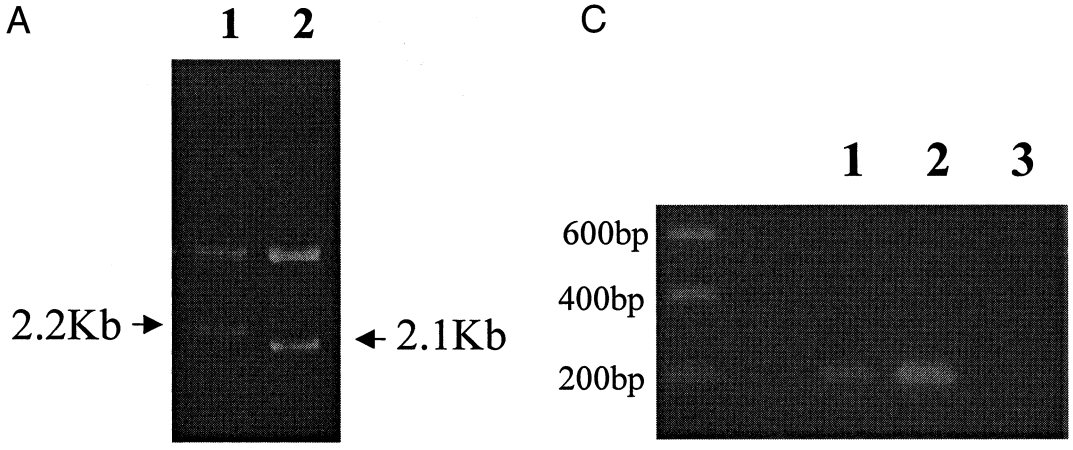
2.5 μg of RNA, primed with 0.5 μg of Oligo $dT_{(15)}$, was reverse-transcribed using Superscript™ II (Gibco, BRL) according to the manufacturer's instructions. cDNA samples were made up to 100 μl with dH_2O. PCR primers designed to sequences in exon 1 (forward 5′-TCCACCAGTCACAGAATCAGA) and exon 10 (reverse 5′-GAGTCAGAAGAAAGGGCAAAC) of the published GLAST-1 sequence (accession no. S49018 [9]) were used to amplify bases 66–2267 of the GLAST-1 cDNA. 1 μl of bone cDNA was amplified in 25 μl (3 mM $MgCl_2$, 150 nM of each primer, 1 U of Advantage DNA polymerase in 1× reaction buffer (Clontech)). Thermocycling parameters were 40 cycles of 95°C for 1 min, 63°C for 1 min and 72°C for 2.5 min. Amplicons were incubated at 95°C for 20 min to inactivate proofreading enzyme.

2.3. Cloning of GLAST cDNAs

Adenosine overhangs were added to the 3′ ends of the GLAST-1 RT-PCR products by a further 20 min incubation at 72°C in the presence of 5 U of *Taq* polymerase (AGS gold: Hybaid). Amplicons were TA-cloned into pCR®-XL-TOPO and transformed into TOPO 10 chemically competent cells following the manufacturer's instructions (Invitrogen). Plasmids were purified (Wizard® SV Plus miniprep kit, Promega) and 5 μl restricted with 5 U of *Eco*RI (37°C, 1 h, 1× buffer containing 10 μg bovine serum albumin (Promega)) to confirm the presence of the insert. Clones were sequenced using M13 vector primers and forward and reverse sequencing primers designed to GLAST-1 (forward: 762 5′-CCAGCTATGAGAAGAGAAGCTT and reverse: 1630 5′-GTGGTTCGGAGGCGGTCCAG accession no. S49018).

2.4. Confirmation of expression of GLAST-1a splice variant in bone and brain

Primers were designed to specifically amplify the GLAST-1a splice variant. The forward primer (5′-CAGCGCTGTCATTGTGGGAATGGC) was designed to prime across the exon 2–4 boundary and the reverse primer (5′-AGGAAGGCATCTGCGGCAGTCACC) was designed to the 3′ end of exon 4 of GLAST-1 (bases 285–495, accession no. AF265360). 1 μl of bone cDNA was amplified in a 25 μl reaction (3 mM $MgCl_2$, 150 nM of each primer, 1 U of AGS DNA polymerase in 1× reaction buffer (Hybaid)). Thermocycling parameters were 40 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 2 min.



B

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G-1a  :241 atgtgaagagctacctgtttcggaatgcctttgtgctactcaccgtcagcgctgtcattg 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G-1   :241 atgtgaagagctacctgtttcggaatgcctttgtgctactcaccgtcagcgctgtcattg 300

G-1a  :300 tgg------------------------------------------------------------ 302
           |||
G-1   :301 tgggtacaatccttggatttgccctccgaccgtataaaatgagctaccgggaggtcaagt 360

G-1a  :302 ------------------------------------------------------------ 302

G-1   :361 acttctcctttcctggggagcttctgatgcggatgctgcagatgttggtcttacccctga 420

G-1a  :302 ----------------------gaatggcggccctagatagtaaggcatctgggaagatgg 342
                                 ||||||||||||||||||||||||||||||||||||||
G-1   :421 tcatctccagtcttgtcacaggaatggcggccctagatagtaaggcatctgggaagatgg 480

G-1a  :343 ggatgcgagctgtggtctattacatgactaccaccatcattgctgtggtgatcggcataa 402
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G-1   :481 ggatgcgagctgtggtctattacatgactaccaccatcattgctgtggtgatcggcataa 540
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Fig. 1. (A) *Eco*RI digest of cloned products from exon 1–10 PCR revealed two different sized inserts. (B) BLAST2 comparison (www.ncbi.nlm.nih.gov/gorf/b12.html) of the inserts derived from exon 1–10 PCR of bone cDNA. Bases 241–540 of GLAST-1 (G-1) and 241–402 of GLAST-1a (G-1a) illustrate the absence of exon 3 in GLAST-1a. Flanking sequences were identical. (C) RT-PCR using GLAST-1a specific primers revealed the expected 210 bp product in rat tibia (1) and brain (2), lane 3 = water control.

2.5. Analysis of hydrophobicity

Hydrophobicity plots were performed on amino acids 1–172 of GLAST-1 and 1–126 of GLAST-1a using TMPRED (www.embnet.org/software/TMPRED_form.html).

2.6. Protein extraction

Rat long bone and cerebellum samples were homogenised using a dismembrator (B. Braun Biotech) for 3 min at 2000 rpm at −120°C in 1 ml 10 mM Tris–HCl (pH 7.5), 1 μg/ml soya bean trypsin inhibitor, 1.2 mg/ml *N*-ethylmaleimide, 10 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminotetraacetic acid and 2 mM dithiothreitol (DTT). The tissue homogenates were fractionated sequentially by centrifugation at $10\,000\times g$ for 1 h at 4°C (pellet taken as crude extract) and $100\,000\times g$ at 4°C for 1 h (pellet taken as membrane-enriched extract). Pellets were resuspended in 100 μl 10 mM Tris–HCl (pH 7.5), 2 mM DTT and incubated at room temperature for 1 h. Both fractions were alkylated by incubating for 15 min with 5 mM iodoacetamide at room temperature. Fractions were freeze-dried overnight.

2.7. Western blot analysis using anti-GLAST-1 antibody

Immunoblot analysis [10] was used to confirm the presence of GLAST-1 protein expression in long bones and to identify GLAST isoforms present in rat cerebellum. Lyophilised fractions were dissolved in sample buffer (8 M urea, 2 M thiourea, 5% (w/v) sodium dodecyl sulfate (SDS), 25 mM Tris–HCl (pH 7.5), 1% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol) to a final concentration of 10 mg/ml and incubated at 60°C for 15 min. 50 μg of each extract was resolved on 7.5% or 10% SDS–polyacrylamide gels and subsequently transferred to polyvinyldifluoride membrane (Immobilon-PVDF, Millipore). 5 μl of prestained SDS–PAGE protein standards (Bio-Rad Laboratories) was also resolved on each gel and the mobilities of these standards (molecular weights 28.5–113 kDa) were used to determine the molecular weight of GLAST isoforms.

Non-specific binding sites on the membrane were blocked by incubating in 1% (w/v) skimmed milk powder in TBS (0.05 M Tris–HCl, pH 8.0, 0.15 M NaCl) for 30 min. Membranes were incubated sequentially with an antibody preparation that recognises amino acids 24–40 of the rat GLAST-1 protein (kindly provided by Wilhelm Stoffel, University of Cologne [5]), diluted 1:1000 in TBS containing 0.2% (v/v) Tween 20 (TBS-Tween) and horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:10 000 with TBS-Tween. An additional blot was incubated without primary antibody to control for non-specific binding of secondary antibody. Membranes were washed extensively in between incubations with TBS-Tween. Specific binding of the anti-GLAST-1 antibody was detected by enhanced chemiluminescence on Hyperfilm-ECL (Amersham, UK).

3. Results

3.1. GLAST-1 cDNA from bone

RT-PCR of bone RNA using primers to exons 1 and 10 of the published GLAST-1 sequence [9] yielded an amplicon of the expected 2201 bp for this molecule. Sequence analysis confirmed that this bone-derived PCR product contained the complete ORF of GLAST-1 mRNA previously thought to be exclusively expressed in the CNS of both rats and humans [9]. The bone cDNA sequence differs from the published GLAST-1 sequence [9] by a G to C transversion resulting in a valine to leucine polymorphism at amino acid number 302 as observed in more recent brain-derived GLAST-1 cDNA by other workers [11].

3.2. A splice variant that excises exon 3

*Eco*RI restriction digest of cloned exon 1–10 PCR products yielded an insert of approximately 2 kb as well as the expected 2.2 kb (Fig. 1A). Comparison of DNA sequence data revealed a novel variant of GLAST-1 mRNA that does not possess exon 3 (Fig. 1B). We have called this variant GLAST-1a and it has been assigned DDBJ/EMBL/GenBank accession number AF265360. RT-PCR, using an upstream primer to the exon 2–4 boundary and a downstream primer to exon 4 to specifically amplify GLAST-1a, demonstrated that it is expressed in brain as well as bone in vivo (Fig. 1C).

3.3. TM modelling

Hydrophobicity predictions of the first four exons of GLAST-1 reveal that they encode three hydrophobic regions that correspond to potential TM domains (Fig. 2A). Interestingly TM prediction of the hypothetical protein without exon 3 reveals that there are only two hydrophobic regions, which would correspond to just two TM domains (Fig. 2B). Thus loss of exon 3 alters the N-terminal region from the three potential TM domains of GLAST-1 to two in GLAST-1a.

3.4. GLAST proteins are expressed in bone and brain

Western blot analysis revealed a number of proteins expressed in bone and brain with reactivity to the anti-GLAST antibody (Fig. 3). Molecular weights ± S.D. are given from

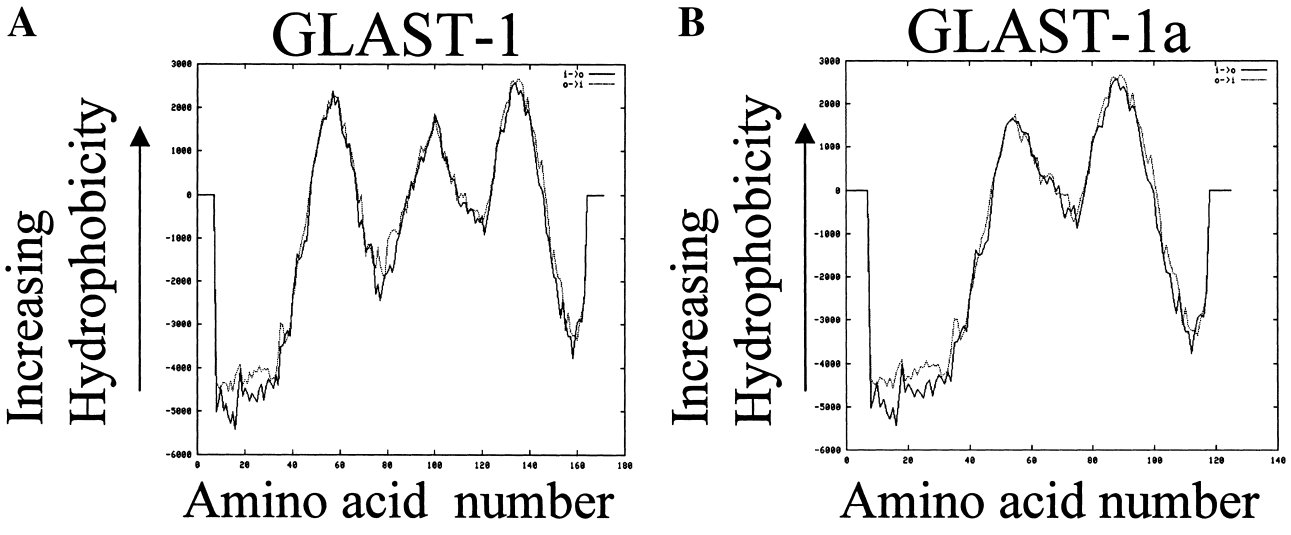

Fig. 2. Hydrophobicity plots of the amino acids (A) 1–172 of GLAST-1, (B) 1–126 of GLAST-1a (TM prediction: www.ch.embnet.org/software/TMPRED_form.html). Showing three TM domains in GLAST-1 and two in GLAST-1a.

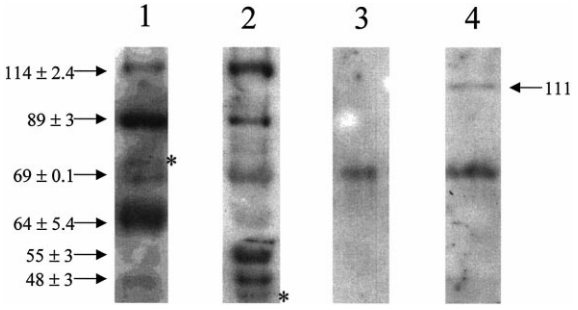

Fig. 3. Western blot analysis using anti-GLAST antibody. Lanes 1 and 2 = cerebellum crude and membrane-enriched fractions, respectively. Lanes 3 and 4 = bone crude and membrane-enriched fractions, respectively. Band sizes shown in kDa ± S.D. derived from three independent determinations. Bands marked with * are due to non-specific binding of secondary antibody.

three independent determinations. Both brain and bone express a 69 ± 0.1 kDa protein and a 64 ± 5.4 kDa protein was also observed in brain. The molecular weights of these proteins suggest they correspond to the two glycosylation states of the GLAST-1 glycoprotein reported by Condradt et al. [12]. Three larger proteins (114 ± 2.4 kDa and 89 ± 3.0 kDa in brain; 111 kDa in bone) probably represent multimers of GLAST that have been described previously [13]. We also detect 55 ± 3.0 kDa and 48 ± 3.0 kDa immunoreactive proteins in the brain membrane-enriched fraction that have not been described previously. The 55 kDa protein corresponds closely to the calculated molecular weight of unglycosylated GLAST-1a (54.4 kDa).

4. Discussion

The role of GLAST-1, a Na^+-dependent transporter of EAAs, has been extensively studied in the rat CNS. GLAST-1 is a member of the family of high affinity glutamate transporters responsible for terminating the excitatory signal across the nerve synapse.

Previously, we identified a molecule with homology to bases 2574–2606 of the 3′ UTR of GLAST-1 as a candidate gene for involvement in osteogenesis in vivo [1]. RT-PCR confirmed that an mRNA possessing exons 2, 3 and 4 of GLAST-1 is expressed in rat bone, suggesting a potential role for EAAs in bone cell signalling. In support of this, other components of the glutamate signalling system have since been identified in bone and bone cell lines. The metabotropic glutamate receptor mGluR1b mRNA has been detected in rat primary osteoblasts [14] and the ionotropic *N*-methyl-D-aspartic acid receptor (NMDAR) subunits 1, 2 and 3 are present in clonal (MG-63, SaOS-2) and primary osteoblasts [15,16]. NMDAR 1 protein is expressed by osteoclasts and osteoblasts in vivo [15,17]. The glutamate vesicle-associated protein (Doc2) and synaptic proteins (syntaxin 1,4,6, SNAP-25, rSec8, synaptophysin) have been shown to colocalise with glutamate in clonal (MG-63, SaOS-2, TE85) and primary rat osteoblasts [18]. These data reveal that the molecular machinery for glutamate signalling is present in osteoblasts and electrophysiological analysis suggests bone cells have functional NMDAR [19] and mGluR activity [14]. The restriction of EAA signalling to the CNS has been questioned by reports of GLAST-1 mRNA expression in other tissues [11] and there is now good evidence for this signalling mechanism in bone.

4.1. The complete ORF of GLAST-1 is expressed in bone

We have used RT-PCR to demonstrate that an mRNA molecule that possesses exons 1–10 of GLAST-1 is expressed in rat bone. This first report of the expression of the entire ORF of GLAST-1 in bone suggests that functional GLAST-1 protein is expressed in this tissue. The differential display clone originally isolated as a mechanically regulated gene in osteocytes represented the 220 bp of cDNA adjacent to the poly A tail. 33 bases of this clone showed 81% identity with the 3′ UTR of GLAST-1 but flanking sequences were not similar [1]. The sequence described in this paper includes 416 bases of the 5′ end of the 3′ UTR which is identical in bone-derived GLAST-1, GLAST-1a and the published brain-derived cDNA sequence (accession no. S49018). Since Northern blot analysis using a probe to a translated region of GLAST-1 (bases 310–655, accession no. S49018) revealed 2.6 and 4.5 kb mRNAs in brain and a single 2.6 kb transcript in bone [1], we predict that further splicing in the UTR may occur. The 3′ UTR of GLAST-1 has a number of potential splice sites (Huggett and Mason, unpublished data) which may alternatively splice the GLAST-1 and/or GLAST-1a transcripts in bone explaining the differences in size observed by Northern blot analysis.

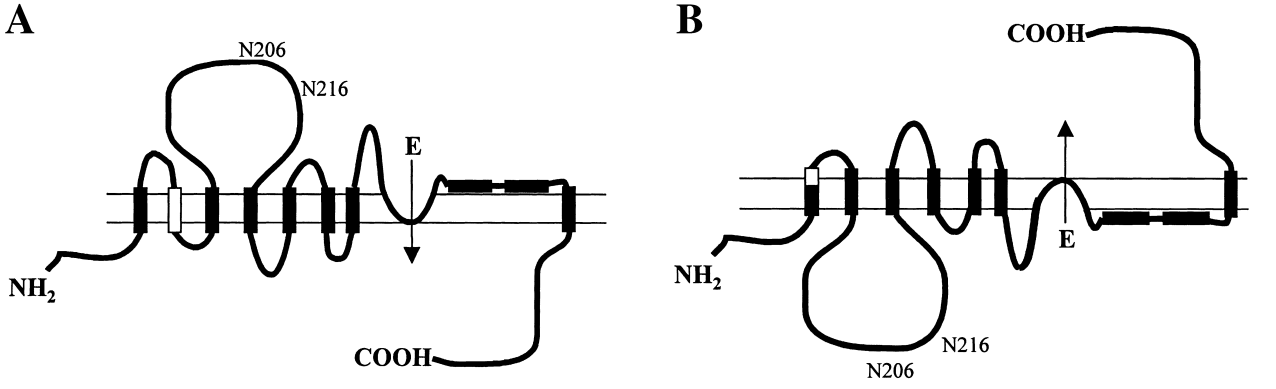

Fig. 4. Topological model of (A) GLAST-1 [2] and our hypothetical model of (B) GLAST-1a. The loss of exon 3 transforms the first two TM domains into one, which we predict will flip the C-terminal and reverse glutamate (E) transport. Note extracellular asparagine residues N^{206} and N^{216} become intracellular in GLAST-1a.

4.2. A novel splice variant of the GLAST gene

We detected the expression of a novel variant of GLAST-1 with exon 3 excised in rat bone and brain. Exon 3 is in frame at phase 1 in rats and humans. Splicing of exons 2 and 4 generates a novel but conservative codon (GGA) to that at the exon 2 to 3 boundary (GGT) which both encode a glycine residue. The loss of the 46 amino acid residues encoded by exon 3 would considerably alter the structure of the protein that might be translated from this transcript. Previous predictions of GLAST-1 protein structure agree that there are six TM α-helices in the N-terminal [2,5,20]. There is some disagreement as to the structure of the remaining C-terminal; Seal et al. predict that this region has a re-entrant loop [2] (Fig. 4A) while Wahle et al. favour the formation of four β-sheets [5]. The first three TM α-helices (amino acids 48–68, 91–111 and 121–145) are encoded by exons 2, 3 and 4 in GLAST-1 (Fig. 2A), however, the loss of exon 3 in GLAST-1a removes amino acids 61–107 (Fig. 2B). Consequently GLAST-1a protein will lose the first extracellular domain and a portion of the first and second TM domains of GLAST-1 such that the first and second hydrophobic regions fuse to generate a single predicted TM domain (Fig. 4B).

Hydrophobicity analysis indicates that if GLAST-1a encodes a functional transporter, its orientation within the plasma membrane may be reversed resulting in an extracellular C-terminal and intracellular localisation of *N*-glycosylation sites 206 and 216 [12].

4.3. Expression of GLAST proteins in bone and brain

The detection of a ~69 kDa protein with an anti-GLAST antibody (Fig. 3) within the bone and brain samples reveals that bone is expressing the GLAST-1 protein described by Storck et al. [9]. Since the predicted molecular weight of unglycosylated GLAST-1a is 54.4 kDa, the detection of a ~55 kDa protein in brain (Fig. 3) supports the notion that GLAST-1a is also expressed at the protein level. If GLAST-1a was glycosylated in the same manner as GLAST-1, then a much larger protein molecular weight would be expected; unglycosylated GLAST-1 has a molecular weight of 59.6 kDa, however the glycoprotein has been detected as isomers of 64 kDa and 70 kDa [12].

4.4. Potential assembly of GLAST-1a

The assembly of TM proteins in eukaryotes is not fully understood, the most simple model is sequential start stop transfer where hydrophobic domains insert into the plasma membrane one after the other in an orientation governed by the most N-terminal sequence [21]. This model was questioned as the only mechanism of membrane protein assembly by Gafvelin et al. who demonstrated that the presence or absence of positively charged residues in the most N-terminal non-hydrophobic region orientates it within the cytoplasm or endoplasmic reticulum (ER) lumen, respectively [22]. Interestingly, unlike the prokaryotic system, they found that charged residues on subsequent non-hydrophobic regions have less of a cytoplasmic influence on orientation, with highly charged loops capable of being translocated into the ER lumen. A recent review of structure and function of known Na^+-dependent EAA transporters predicts that the N-terminal of GLAST-1, which has five arginyl and eight lysyl residues, would be cytoplasmic [20]. We therefore predict that the N-terminal of GLAST-1a translated product would also be cytoplasmic as it is composed of the same amino acid sequence. Since subsequent non-hydrophobic regions have less of an orientational influence in eukaryotes, the loss of exon 3, converting three hydrophobic regions into two, could flip the remaining C-terminal of the protein from intra- to extracellular (Fig. 4B). If this were the case then the second large extracellular domain of GLAST-1, that is glycosylated at asparagines 206 and 216 [12], would become cytoplasmic and therefore not presented for glycosylation within the ER lumen. Assuming no further post-translational modification, the unglycosylated GLAST-1a would have a molecular weight of 54.4 kDa. We believe that the ~55 kDa immunoreactive protein expressed in brain (Fig. 3) represents unglycosylated GLAST-1a, supporting our reversed orientation theory. Our proposed model for the reversed orientation of the GLAST-1a molecule, C-terminal of TM domain 1, presents us with the interesting possibility that the orientation of the predicted pore structure [2] and possibly the direction of glutamate transport may also be reversed (Fig. 4). Whole cell clamping experiments have shown that under appropriate conditions glutamate transporters will operate in reverse, transporting glutamate from inside to outside of the cell [23]. This has recently been demonstrated in brain slices, where neurotoxic release of glutamate due to anoxia was found to be largely due to reversed operation of glutamate transporters [24]. While membrane transporters can transport in both directions under appropriate ionic gradients [25], the novel splice variant described in this paper encodes an ideal candidate protein for an alternative mechanism of glutamate release that may influence neurotoxic accumulation of glutamate.

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