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Perspective Article

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Glutamate transporters in bone

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Abstract

In the central nervous system Na^+-dependent glutamate transporters bind extracellular glutamate and transport it into cells surrounding the synapse, terminating excitatory signals. These glutamate transporters also function as ion channels. The glutamate transporter, GLAST-1, is expressed in the plasma membrane of osteoblasts and osteocytes and is the same molecular weight as in brain. Thus in bone cells GLAST-1 may transport glutamate or operate as a glutamate gated ion channel. A splice variant, GLAST-1a, is also expressed in bone. Hydropathy and Western blot analysis suggest GLAST-1a adopts a reversed orientation within the cell membrane. Sodium and potassium ion gradients drive glutamate transport but glycosylation, oxidation and phosphorylation modulate transporter activity. Reversal of GLAST-1a would alter these modifications varying its transport activity under the same ionic gradients. The significance of GLAST-1/1a in bone *in vivo* is unknown. GLAST-1 knockout mice show no major disruption of skeletal development but have not been investigated in detail. Glutamate affects both osteoclast and osteoblast biology and the regulation of GLAST-1 by mechanical loading in bone suggests a role for glutamate transporters in osteogenesis. Differential regulation and modification of GLAST variants may provide an intricate mechanism controlling extracellular glutamate levels and thus its downstream signalling effects in bone.

Keywords: GLAST-1, GLAST-1a, EAAT-1, Glutamate Transporter, Bone

Introduction

This paper reviews the role of glutamate transporters in bone. After a very brief overview of the sodium-dependent glutamate transporter family, we will focus on one transporter, glutamate/aspartate transporter 1 (GLAST-1), which is the only glutamate transporter to be investigated in bone in any detail. The feasibility of GLAST-1 operating as a functional transporter in bone is discussed as is the potential function of the recently discovered splice variant, GLAST-1a. Finally, evidence for a significant role for these transporters in bone biology is considered in the light of *in vitro* experiments investigating glutamate signalling and *in vivo* phenotypes associated with mutations in the GLAST-1/1a gene.

Background

The first indication of a role for glutamate signalling in bone was the discovery of GLAST-1 as a potential candidate for mediating mechanically-induced osteogenesis *in vivo*[1]. This experiment used a previously characterized model of mechanical loading in which rats were subjected to a non-invasive loading regime that caused the actively resorbing medial surface of the rat ulna to be converted to a bone forming surface[2,3]. We had demonstrated that mRNA isolated from cryosections of cortical bone immediately underlying the midshaft medial periosteum was uncontaminated with markers of mature osteoblasts or osteoclasts and therefore represented a cell population of predominantly osteocytes[4]. The mRNA derived from these cortical bone samples was compared between loaded and control ulnae from individual animals using the RNA fingerprinting technique of differential RNA display (DRD). Since loaded and control RNA fingerprints were derived from the same animal, the cell population was relatively pure and the bone sample was accurately defined by cryosectioning, the possibility of false positive DRD data was minimised. Reamplification and cloning of a cDNA down-regulated in loaded bone revealed a 220 bp clone, which showed homology to the 3' untranslated region of rat GLAST-1. PCR primers designed to amplify a short translated region of the GLAST-1 cDNA between exons 2 and 4 revealed an identical RT-PCR product in both brain and bone. A multiple tissue northern blot probed with exon 2-4 of the GLAST-1 cDNA revealed a 2.6 Kb mRNA in

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rat bone and brain as well as the previously reported 4.5 Kb mRNA[5] in brain. These data led to the conclusion that glutamate may represent a novel signalling molecule in bone and that the glutamate transporter GLAST-1 may be important in mechanically-induced osteogenesis.

Overview of the glutamate transporter family

The amino acid L-glutamate is the major mediator of excitatory signals in the mammalian central nervous system (CNS) where it is released into the extracellular fluid to activate various classes of glutamate receptors. Termination of the excitatory signal and prevention of excessive activation of glutamate receptors is achieved by glutamate transporters located within the membranes of cells in the CNS. These proteins bind extracellular glutamate and transport it to the inside of the cells where it is then used in metabolic processes or recycled for subsequent neurotransmission. Studies investigating glutamate uptake within the CNS have revealed that it is electrogenic and driven by ion gradients of K^+, Na^+ and H^+ [6-8]. These transporters also operate as ion channels where glutamate binding triggers anion conductance[9]. In addition, GLAST-1 has a constitutive cation permeability dependent on Cl^- [10].

There are a number of proteins expressed within the brain capable of transporting glutamate. GLAST-1 is a member of the sodium and potassium coupled glutamate transporter family, which are also referred to as excitatory amino acid transporters (EAATs) or 'high affinity' glutamate transporters. This family of transporters represent the only known mechanism for termination of excitatory signals that is fast enough to allow synaptic function and their role in this process has been extensively investigated (recently reviewed in 11).

Nomenclature of these transporters has been inconsistent in the literature as genes were cloned from different species by various research groups. The alternative names for each transporter are given below. Five different transporters of this family have been identified and cloned to date:- EAAT1/GLAST-1/ GluT-1/SLC1A3[5,12], EAAT2/GLT-1/ SLC1A2[13], EAAT3/EAAC-1/SLC1A1[14], EAAT4[15] and EAAT5[16]. This review focuses on the first member of the transporter family to be identified, which we will refer to as GLAST-1. These transporters share 50-60 % amino acid sequence identity with each other and show some homology with the neutral amino acid transporters ASCT1 and ASCT2 and bacterial glutamate and dicarboxylate transporters[17]. The five glutamate transporters of the EAAT family have different properties, are susceptible to different glutamate uptake inhibitors and are expressed by different cell types in different locations within the brain[11]. This has led to the conclusion that they provide an intricate and finely tuned system for control of extracellular glutamate concentration.

GLAST-1 (EAAT1) was the first glutamate/aspartate transporter to be isolated from rat brain[5]. The 66 kDa GLAST-1 glycoprotein was purified and a short peptide sequence used to generate an oligonucleotide probe that revealed the GLAST-1 cDNA in a rat brain cDNA library. Northern blot analysis of multiple tissues (not including bone or cartilage) revealed that mRNA expression appeared limited to the CNS[5]. Since then GLAST-1 protein expression has been reported in various rat tissues (bone-[1,18], mammary gland[19], placenta[20], retina[21,22]) and canine erythrocytes[23]. In addition, Northern blot analysis has revealed GLAST-1 mRNA expression in rat lung, spleen, skeletal muscle, testis[12], rat bone[1] and human heart, lung, skeletal muscle, placenta, liver and kidney[24]. Furthermore, we have detected GLAST-1 mRNA expression in human primary chondrocytes, primary osteoblasts, the osteoblast-like cell line SaOS-2 and the osteocyte-like cell line MLO-Y4 by reverse transcriptase linked PCR (RT-PCR) (Huggett and Mason, unpublished data).

Glutamate transporter expression in bone

To date three glutamate transporters have been investigated in bone, GLAST-1(EAAT-1), GLT-1 (EAAT-2) and EAAC-1 (EAAT-3). EAAC-1 protein expression was not detected in neonatal rat femur and rat ulna. However, GLT-1 immunolocalised to mononuclear cells of the bone marrow and both GLAST-1 mRNA and protein were constitutively expressed in osteocytes *in vivo*[1]. All of these transporters were detected using previously characterized antibodies against the C-terminal peptide sequences of each transporter[25]. The constitutive osteocyte expression of GLAST-1 protein became undetectable after 5 daily periods of mechanical loading and the limited GLAST-1 expression on quiescent bone surfaces became more abundant on surfaces where loading had induced bone formation[1].

Northern blot analysis using a probe to exons 2-4 of GLAST-1 revealed that a ~2.6 Kb mRNA transcript is expressed in bone but the ~4.5 Kb mRNA that had been originally described in brain was not detected. This led us to determine whether all of the exons of GLAST-1 are expressed in bone and also to attempt to clone the bone-derived GLAST-1 mRNA transcript. The rat GLAST-1 gene has not yet been described but the human[26] and mouse[27] genes each consist of 10 exons. The rat cDNA was aligned to the mouse cDNA to determine likely exon boundaries and forward primers to GLAST-1 exons 1, 2 and 4 and reverse primers to exons 6, 7, 8, 9 and 10 were designed. RT-PCR of cDNA derived from adult rat femur (minus bone marrow) revealed that each exon was expressed in bone. Cloning and DNA sequencing of the exon 1-10 RT-PCR product revealed a 2.2 Kb cDNA[18]. The bone derived cDNA sequence encoded the entire open reading frame of the previously published GLAST-1 cDNA[5] and contained a single valine to leucine amino acid substitution at amino acid 302, a polymorphism previously reported by other workers[12]. This proved that the mRNA expressed in bone, if translated, could encode a GLAST-1 transporter identical to that found in the brain. Further corroboration was provided by Western blot analysis using an anti-GLAST antibody[28] which con-

firmed that the ~69 kDa protein detected in membrane proteins extracted from brain was also found in bone[18]. A ~64 kDa protein was also detected in brain extracts consistent with previous reports of the 2 glycosylation states of GLAST-1[29]. Five other immunoreactive species were detected of ~48, 55, 89, 111 and 114 kDa. Other workers have also noted the expression of higher molecular weight multimeric forms of GLAST-1 that correspond closely to these sizes (90 - 110kDa)[29,30]. These multimeric forms appear to result from both covalent and irreversible crosslinking although it is unclear whether interacting molecules are monomers of GLAST-1 or consist of other attachment proteins[11]. The lower molecular weight proteins had not previously been reported.

The GLAST-1a splice variant

Cloning of PCR products derived from GLAST-1 exon 1 – 10 RT-PCR of bone cDNA also revealed the expression of a splice variant in which exon 3 is removed. We have called this variant GLAST-1a (AF265360[18]). Exon 3 comprises 138 bases encoding 46 amino acids and its removal does not disrupt the open reading frame of the gene (Figure 1a). This has led us to investigate GLAST-1a expression in other tissues and determine whether the GLAST-1a mRNA could encode a functional protein.

A PCR forward primer designed to span the exon 2-4 junction was used alongside a downstream exon 4 reverse primer to specifically amplify cDNA derived from GLAST-1a mRNA (bases 285-495, AF265360,[18]). PCR of cDNA derived from rat bone and cerebellum clearly reveals the expected 210 bp product (Figure 1b[18]). However, this variant is expressed at lower levels than GLAST-1 since it is not normally visible after RT-PCR with primers that span exons 2 to 4. To quantify the ratio of GLAST-1a: GLAST-1 mRNA we have increased the sensitivity of this RT-PCR assay by Southern blotting PCR products and hybridising with digoxigenin-labelled GLAST-1 probes which are then detected by enhanced chemiluminescence. Our results show that this ratio varies between bone (minus marrow) and brain *in vivo*, as well as in the human osteoblast-like cell line SaOS-2 and the osteocyte cell line MLO-Y4 (Huggett, O'Neal, Mustafa and Mason, unpublished data). The preliminary data in Figure 2 reveal that the GLAST-1a: GLAST-1 mRNA ratio is much higher in MLO-Y4 cells. In order to determine the significance of the variable GLAST-1: GLAST-1a mRNA ratio, it is necessary to establish whether GLAST-1a is translated.

Does GLAST-1a mRNA encode an expressed ***protein?*** The calculated molecular weight for GLAST-1a is 54.4 kDa based upon the 497 amino acids that are encoded by this mRNA. Western blot analysis of proteins extracted from membrane-enriched fractions of cerebellum using an anti-GLAST antibody does reveal an immunoreactive protein at approximately 55 kDa, corresponding closely to the predicted size for GLAST-1a (Figure 3[18]). This blot was immunodetected with a polyclonal antibody (kindly provided by

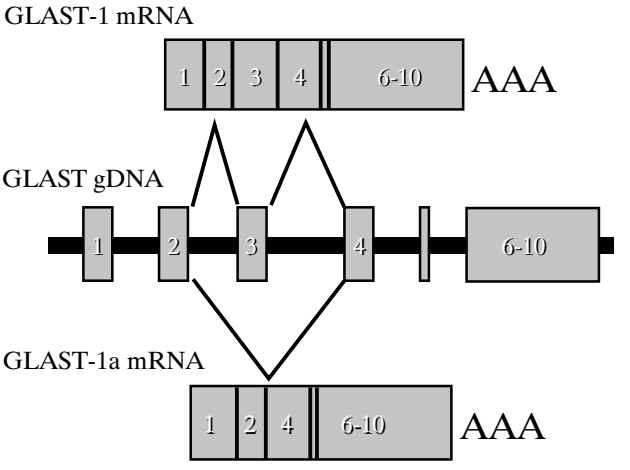

Figure 1a. Exon structure and alternative splicing of the GLAST gene. Inclusion or removal of exon 3 yields GLAST-1 or GLAST-1a respectively.

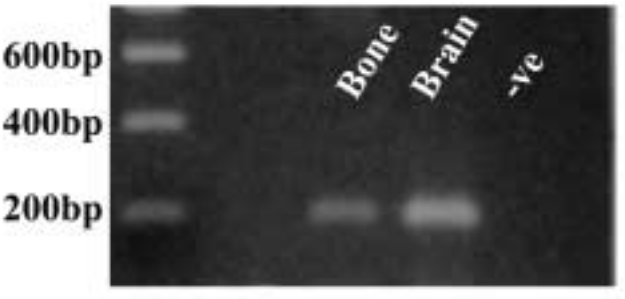

Figure 1b. GLAST-1a specific RT-PCR. A forward primer to the exon 2/4 boundary and reverse primer to exon 4 reveals a 210 bp GLAST-1a RT-PCR product in bone and brain[18].

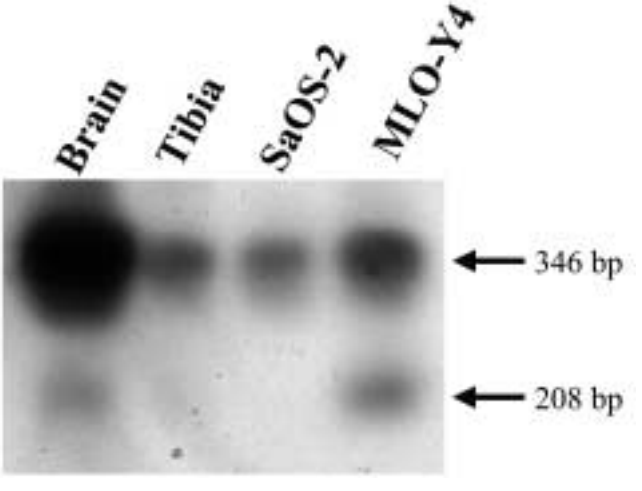

Figure 2. Southern blot of GLAST exon 2-4 RT-PCR. Southern blotting of exon 2-4 RT PCR products reveal that the relative expression of GLAST-1a (208 bp amplicon) to GLAST-1 (346 bp amplicon) is greater in MLO-Y4 cells.

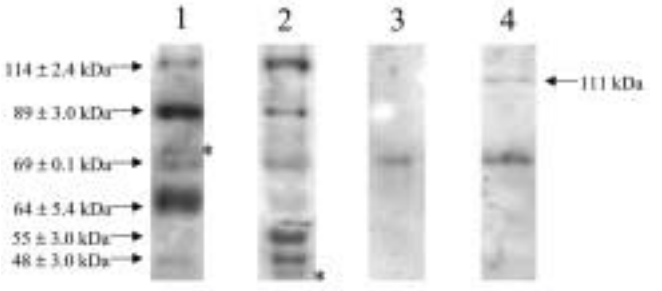

Figure 3. Western blot analysis using anti-GLAST antibody. Cerebellum crude (Lane 1) and membrane enriched (Lane 2) fractions, and bone crude (Lane 3) and membrane enriched (Lane 4) fractions. Band sizes shown as kDa ± S.D. derived from three independent determinations. Bands marked with * are due to non-specific binding of the secondary antibody[18].

Professor Stoffel, University of Cologne[28]) against a synthetic peptide comprising amino acids 24-40 at the N-terminus of GLAST-1. This sequence is upstream of exon 3 and therefore present in both GLAST-1 and GLAST-1a. If the ~55 kDa protein expressed in cerebellum does represent GLAST-1a it is likely that this protein is unglycosylated as glycosylation of GLAST-1 in brain has been reported to increase its molecular weight by ~10 kDa[29]. We have not detected expression of this ~55 kDa protein in bone but the much lower expression levels of both GLAST-1 and GLAST-1a mRNA in bone (200x less abundant than in brain[31]) may have prevented its detection. If, as we hypothesise, the ~55 kDa protein represents GLAST-1a, the consequences of the variants' protein structure should be considered in order to propose a functional role.

GLAST-1 and GLAST-1a structure

Investigations into the topology of Na^+-dependent glutamate transporters have revealed that although the N-terminal part of the protein consists of 6 transmembrane (TM) alpha helices with a long extracellular region between TM domains 3 and 4, the C-terminal structure is far less clear[9,17,28,32-35]. The most recent structure reported for GLAST-1 is shown in Figure 4A[35] although there is still much debate over the topology between TM domains 6 and 8[33,36,37] with more recent analysis of GLT-1 indicating 2 reentrant loops downstream of TM domain 6 prior to the final TM domain and cytoplasmic C-terminus[38]. While there is some divergence in amino acid sequences across this family of transporters, hydropathy profiles of the sequences are highly conserved, representing another level of homology that indicates structural similarity[39]. A comparison of the hydropathy profiles of GLAST-1 and GLAST-1a reveals that the first 2 TM domains of GLAST-1 become truncated into 1 potential TM domain in GLAST-1a (Figure 5)[18] with the remainder of the profile being unaltered. Since the N-terminus of GLAST-1a is identical to that of GLAST-1, we predict it will also be cytoplasmic and hypothesise that the loss of a TM domain in GLAST-1a would cause it to orientate in reverse in the plasma membrane, C-terminal of the novel TM domain α (Figure 4B). This would mean that the long extracellular loop between TM domains 3 and 4 of GLAST-1 that contains two N-linked glycosylation sites (Arg-206 and Arg-216) in GLAST-1[29] would become intracellular and thus not be presented for glycosylation in the endoplasmic reticulum. This hypothetical model is consistent with the ~55 kDa protein detected on Western blots that corresponds to the predicted molecular weight of unglycosylated GLAST-1a (Figure 3)[18].

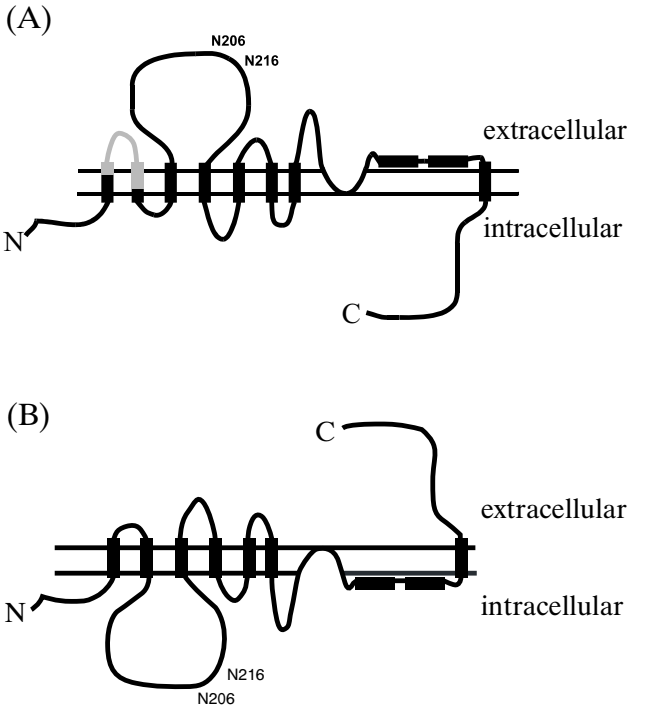

Figure 4. Topological model of (A) GLAST-1[35] and (B) GLAST-1a[18]. The loss of exon 3 removes the grey region of GLAST-1 generating a novel TM domain from the remnants of TM domains 1 and 2. Note that in our model of GLAST-1a, asparagines N206 and N216 are intracellular.

GLAST-1 and GLAST-1a glutamate transport function

For GLAST-1 and GLAST-1a to transport glutamate in bone they would have to retain the appropriate amino acids for transport function in a suitable conformation and location to allow exposure to the ionic gradients that drive glutamate uptake. Each of these issues is discussed briefly below.

Ionic gradients drive glutamate transport. The Na^+-dependent glutamate transporters utilise ionic gradients of sodium, potassium and H^+ to drive glutamate transport against the concentration gradient. Stoichiometry may vary

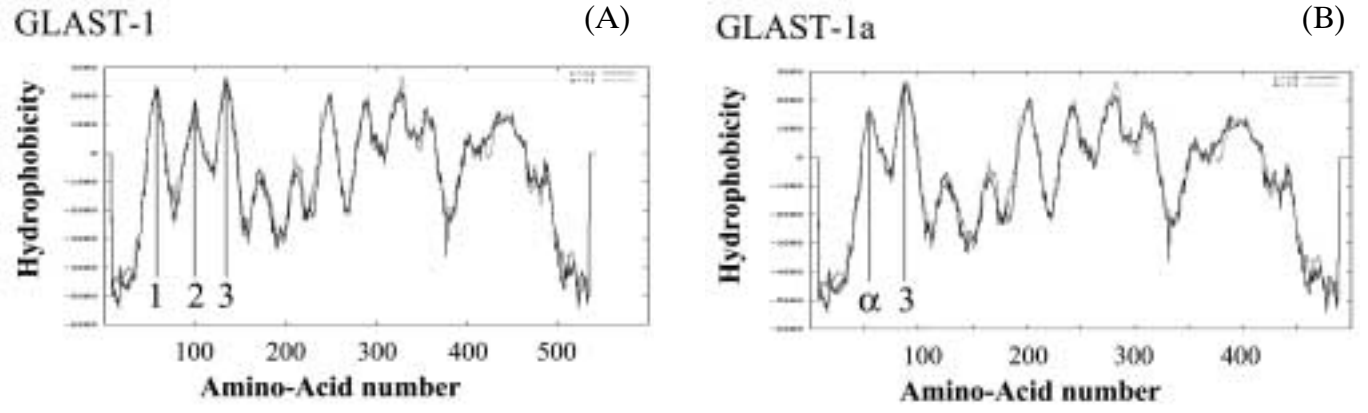

Figure 5. Hydropathy profiles of GLAST-1 (A) and GLAST-1a (B). GLAST-1a has a novel hydrophobic peak α in place of peaks 1 and 2 of GLAST-1 indicating the loss of one TM domain.

for individual transporters of this family. GLAST–1 transports one glutamate molecule together with three Na^+ ions in exchange for one K^+ ion[40] resulting in a net positive charge moving inwards into the cell that can be measured using electrophysiological procedures. Since the ionic gradients are maintained by the sodium pump, they are dependent upon ATP production. When ATP levels drop (e.g. ischaemia) increased extracellular K^+ and reduced extracellular Na^+ results in a neurotoxic release of glutamate in the CNS that has been attributed to reversed operation of glutamate transporters[41]. Thus GLAST-1 may transport glutamate in either direction dependent upon the ionic gradient across the plasma membrane. It is unclear whether such gradients occur in bone *in vivo* but GLAST-1a may represent an alternative isoform of GLAST-1 displaying different transport properties under the same ionic gradients.

Amino acid residues required for glutamate transport. Amino acid residues 392 – 415 of GLAST-1 are highly conserved throughout the glutamate transporter family and amino acid residues Arg-122, Arg-280, Tyr-405 and Arg- 479 are present in all eukaryotic and prokaryotic glutamate transporters indicative of a critical role in transporter function[37]. Site-directed mutatagenesis of GLAST-1 and subsequent expression in Xenopus oocytes has revealed that Tyr-405 and Arg- 479 are essential for transporter function[42]. Mutation of Arg-122 in the 1st intracellular loop and Arg-280 in the 2nd intracellular loop of GLAST-1 does not affect glutamate transport properties but alters the Km values of aspartate suggesting a subtle role for these residues in substrate recognition. All of these amino acids are retained in GLAST-1a although their intra/extracellular localization would be reversed if our topology hypothesis is correct (Figure 4). The effect of a reversed orientation of GLAST-1a on transporter function is difficult to predict since the topology of the apparently critical regions of the transporters is still controversial. However, since glutamate transporters can operate in reverse it is clear that glutamate binding and subsequent transport can be mediated by either side of the transporter. Interestingly, consensus sites for N-linked glycosylation occur in a large extracellular loop between TM domains 3 and 4 in all of the glutamate transporters that have been cloned so far[9]. Although site-directed mutagenesis of these residues indicated that they were not essential for glutamate transport function, absence of glycoslylation at these residues did appear to prevent dimerisation of GLAST-1[29]. The conservation of these glycosylated residues in glutamate transporters is indicative of a potential function *in vivo*, possibly involved with multimeric assembly, that would clearly be absent in GLAST-1a, if reversed.

Modification of transporter function. Both phosphorylation and oxidation of GLAST-1 have been reported to modify its function. Protein kinase C (PKC) has been shown to directly phosphorylate GLAST-1 causing transport activity to reduce to 25 %[43]. PKC phosphorylation of glutamate transporters has been shown to regulate trafficking of the transporters from intracellular stores to the plasma membrane[44]. Rapid increases in surface expression of GLAST-1 have been detected in primary astrocytes in response to extracellular glutamate[45] indicative of substrate-induced transporter trafficking. Transfection of MLO-Y4 cells with recombinant green fluorescent protein (GFP) -tagged GLAST-1 reveals intracellular pools of protein extending from the endoplasmic reticulum which may represent transporter storage vesicles (Figure 6C, Huggett and Mason, unpublished data). PKC modification of GLAST-1 transport activity is not mediated by PKC consensus sites (Ser-116, Thr-341, Thr-372)[43] and the site is not yet defined. If this site becomes extracellular in GLAST-1a then this modification of function would clearly be lost.

Oxidation is also important in modulating GLAST-1 operation. Glutamate transport activity in liposomes containing GLAST-1 can be reversibly decreased or increased by alternate treatment with a thiol oxidising agent (5,5'-dithio-bis(2-nitrobenzoic) acid) and a disulphide reducing agent (dithiothreitol)[46,47]. Maximal transport activity occurs in the reduced state and minimal in the oxidised state where

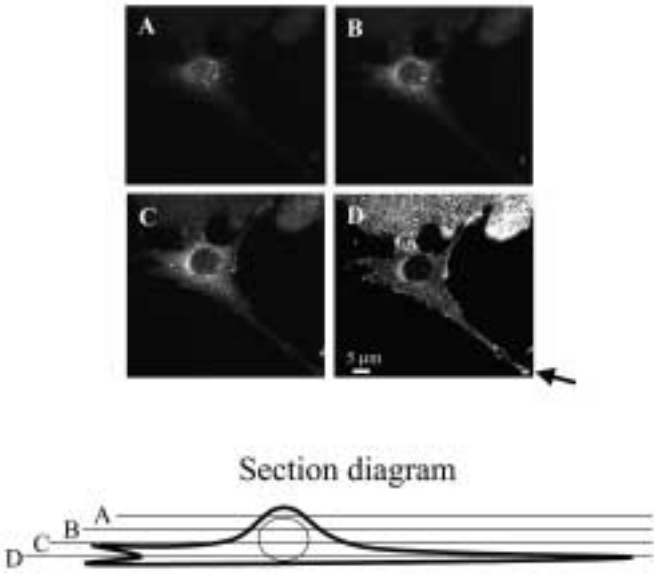

Figure 6. Scanning laser confocal imaging of MLO-Y4 osteocyte-like cell expressing GLAST-1 GFP chimeric protein. The section diagram at the bottom depicts approximate section positions through cell. Image A reveals high expression in the endoplasmic reticulum (ER). Images B and C reveal GLAST-1 expression in the ER surrounding the nucleus is absent from the nucleus but extends from this location possibly as extracellular trafficking or internal storage vesicles. Image D reveals GLAST-1 protein expression in the plasma membrane of the cell with a higher expression at the ends of the cellular processes (arrow).

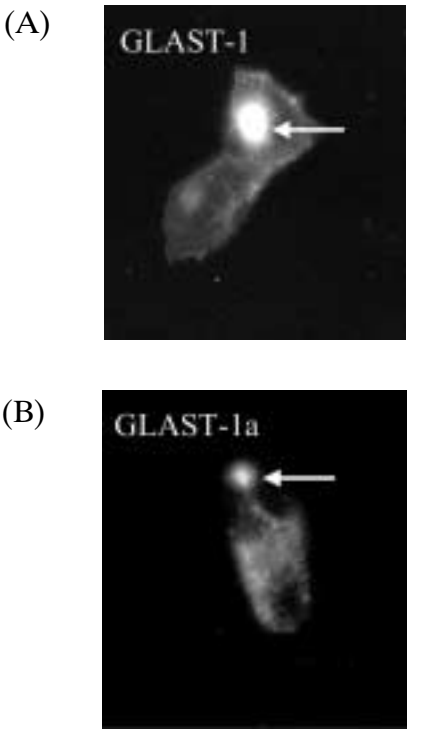

Figure 7. Fluorescence imaging (250x magnification) of SaOS-2 osteoblast like cells expressing (A) GLAST-1 GFP and (B) GLAST-1a GFP chimeric proteins. GLAST-1 expression appears throughout the cell plasma membrane with a non-nuclear focus of expression (arrow) possibly representing an internal reservoir. GLAST-1a expression appears to be less evenly distributed but also shows a focus of expression (arrow).

disulphide bond formation occurs between cysteine residues. The disulphide bonds may form within or between transporter monomers or link them to regulatory proteins. The redox-sensing elements in GLAST-1 are not yet identified. GLAST-1 contains three cysteine residues (positions 186, 252, 375), two of which occur on extracellular domains and one within TM domain 4. If the orientation of the two extracellular cysteine residues of GLAST-1 become reversed in GLAST-1a, intracellular versus extracellular accumulation of oxidants may differentially regulate glutamate transport by each variant.

Subcellular localization. Previous reports of GLAST-1 expression and localization mostly rely upon antibodies or oligonucleotide probes that are unlikely to discriminate between GLAST-1 and GLAST-1a. Therefore the current literature may represent expression patterns and subcellular localization of either or both variants. GLAST-1 is best detected using nucleic acid probes or antibodies to peptide sequences encoded by exon 3 alone and GLAST-1a using the novel exon 2-4 boundary sequence. We have started to address this problem by transfecting primary osteoblasts and bone cell lines (SaOS-2 and MLO-Y4) with GFP-tagged GLAST-1 and GLAST-1a (Huggett and Mason, unpublished data). Scanning laser confocal microscopy of transfected cells clearly shows plasma membrane localization of GLAST-1 in all of these cell types indicative of a similar glutamate transport function in bone as in the brain (Figure 6). Our preliminary data also indicate that GLAST-1 may accumulate within intracellular storage vesicles as well as at the ends of osteocytic processes. GLAST-1a has not yet been investigated in primary osteoblasts or MLO-Y4 cells but transfected SaOS-2 indicate a more patchy distribution of this recombinant protein in the plasma membrane (Figure 7). These data indicate that both GLAST-1 and GLAST-1a can be expressed in the plasma membrane of osteoblast-like bone cells in an appropriate position for glutamate transport.

GLAST-1 and GLAST-1a ion channel function

In addition to glutamate transporter activity, GLAST-1 possesses both substrate dependent and independent ion channel activity. All five transporters in this family exhibit Na^+-dependent glutamate gated chloride-channel activity that is independent of glutamate transport[36]. GLAST-1 also exhibits a glutamate independent cation flux that requires chloride but which is distinctive from the chloride channel activity described above[10]. In the CNS the chloride channel may compensate for the membrane potential changes due to

glutamate uptake or modulate excitatory responses. It may perform a similar function in bone or operate more like a receptor whereby glutamate binding opens the chloride channel activating intracellular signalling cascades. This would represent a novel mechanism through which bone cells could respond to extracellular glutamate levels.

Recently it has been shown that EAAT3 expressed in Xenopus oocytes arrange as homo-pentamers within the plasma membrane[48]. The authors predict that while each monomer may be capable of catalysing glutamate transport, formation of a pentameric structure containing a central pore may facilitate the chloride channel activity. This is consistent with the crystal structure of other channels (bacterial potassium channel, aquaporin family) which also contain reentrant loop pores through which substrates flow and which form tetrameric or pentameric structures[49-51]. It is interesting to note that the higher molecular weight oligomer in bone (111 kDa) is a different molecular weight to those in brain (89 and 114 kDa) suggesting that the transporter in bone may form different oligomers or interact with different proteins (Figure 3).

Evidence for a role for GLAST-1/1a in bone

In vivo evidence for a functional significance of GLAST-1/1a in bone biology is limited as there are very few studies. Whilst glutamate transporters have been implicated in a wide range of diseases where glutamate levels are disrupted (epilepsy, Alzeimers, Parkinsons, stroke, dementia, glaucoma, inflammation), we are not aware of any reports linking mutations in GLAST-1 to a human disease. This means that human phenotypes associated with GLAST-1 mutations have not yet been described. A GLAST-1 knockout mouse has been generated by deletion of exon 6 which would prevent expression of both splice variants of the gene[52]. Only one study to date has investigated the bone phenotype of these animals[53]. In this, the limb bones, calvaria and mandibles were examined by scanning electron microscopy to determine trabecular structure, resorption lacunae and collagen fibril orientation and digital radiography was used to assess total bone mineral content. At 6 months of age, no significant differences were observed in tibia, femur or mandible lengths or mandible heights between 6 knockout and 6 wildtype males. The authors were unable to observe morphological differences by eye but, apart from the bone length measurements above, quantitative analysis of morphological characteristics were not presented. High-resolution X-ray microtomography did not reveal differences in trabecular structure and bone thickness but, since only 2 knockout animals were compared with a single wildtype, it is possible that subtle differences were missed. This data, combined with *in vitro* bone formation and bone resorption assay data (also discussed in 54-56), led the authors to conclude that glutamate does not play a major role in bone growth. More detailed morphological analysis of these knockouts may reveal differences but since the transporter has been implicated in mechanical signal transduction, it would be more informative to test responses of these bones to load or other stimuli known to affect bone remodelling. Until such studies are performed, the functional significance of GLAST-1 and GLAST-1a in bone cannot be assessed. Since the open reading frames of bone-derived and brain-derived GLAST-1 mRNAs are the same and the resultant proteins have the same molecular weight in both tissues *in vivo*[18], it appears likely that GLAST-1 also performs a glutamate transport and/or ion channel function in bone. The *in vivo* experiments demonstrating mechanically-induced down-regulation of GLAST-1 in osteocytes and up-regulation in bone forming osteoblasts[1] are indicative of a role for this transporter in osteogenesis.

GLAST-1 mediated glutamate transport has not yet been demonstrated in bone cells but GFP-tagged recombinant GLAST-1 and GLAST-1a proteins are expressed in the plasma membrane of all osteoblast and osteocyte cells tested (Huggett and Mason, unpublished data). The higher expression of GLAST-1a mRNA relative to GLAST-1 in MLO-Y4 cells may indicate a functional significance of this isoform in osteocytes. Osteoblasts have been demonstrated to express key proteins involved in exocytotic glutamate release that are enhanced at intercellular contact sites and co-localize with immunoreactive glutamate[57]. In addition, constitutive glutamate release has been reported in osteoblasts (interestingly, not by osteocytic MLO-Y4 cells) and appears to be increased during osteoblastic differentiation[57,58]. Since both osteoblasts and osteoclasts express functional glutamate receptors which, when activated, can modify bone cell phenotype, a role for GLAST proteins in the modulation of receptor activation appears likely. In addition, the glutamate-gated ion channel activity of GLAST-1 may represent an alternative mechanism affecting bone cell responses to glutamate.

Concluding remarks

In the CNS glutamate transporters such as GLAST-1 control extracellular glutamate levels by rapidly binding glutamate and preventing over stimulation of glutamate receptors on post-synaptic neurons. In astrocytes, cell surface expression of GLAST-1 is rapidly up-regulated in response to extracellular glutamate. GLAST-1 may fulfil a similar role in bone acting as a regulator of glutamate molecules available to bind to receptors. Transcriptional regulation and mRNA splicing causing differential expression of GLAST-1 and GLAST-1a may affect glutamate transport and ion channel properties in osteocytes and osteoblasts. In addition, protein trafficking, phosphorylation, oxidation and multimeric assembly of GLAST isoforms may modulate both glutamate transport and ion channel function. Thus, transcriptional regulation and post-translational modification of GLAST isoforms may provide an intricate mechanism for closely regulating glutamate signalling in bone.

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