Glutamate transporters serve the important function of mediating removal of glutamate released at excitatory synapses and maintaining extracellular concentrations below excitotoxic levels. Excitatory amino acid transporter subtypes EAAT1 and EAAT2 have a high degree of sequence homology and similar predicted topology and yet display a number of functional differences. Several recombinant chimeric transporters were generated to identify domains that contribute to functional differences between EAAT1 and EAAT2. Wild-type transporters and chimeric transporters were expressed in *Xenopus laevis* oocytes, and electrogenic transport was studied under voltage clamp conditions. The differential sensitivity of EAAT1 and EAAT2 to transport blockers, kainate, *threo*-3-methylglutamate, and (2S,4R)‐4‐methylglutamate as well as *L*‐serine‐O‐sulfate transport and chloride permeability were employed to characterize chimeric transporters. One particular region, transmembrane domains 9 and 10, plays an important role in defining these functional differences. The intracellular carboxyl-terminal region may also play a minor role in conferring an effect on chloride permeability. This study provides important insight into the identification of functional domains that determine differences among glutamate transporter subtypes.

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Identification of Functional Domains of the Human Glutamate Transporters EAAT1 and EAAT2*

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transport by EAAT2 in a voltage-independent manner, suggesting that they block transport by competing with glutamate at an extracellular recognition site. Another compound, (2S,4R)-4-methylglutamate, shows some very distinctive differences in its mechanism of action on the transporters. (2S,4R)-4-Methylglutamate is a voltage-independent, competitive blocker of glutamate transport by EAAT2, but it is also a potent substrate of the EAAT1 transporter subtype. As such these compounds provide a set of selective molecular probes for the extracellular glutamate recognition site of the transporters.

In contrast to the competitive blockers of glutamate transporters, a number of other pharmacological agents have been shown to inhibit glutamate transport by competing with glutamate as a substrate for the transporters (8). One pharmacological substrate that shows some clear differences in transport parameters between EAAT1 and EAAT2 is L-serine-O-sulfate. L-Serine-O-sulfate is a substrate for both EAAT1 and EAAT2, but it is a less potent substrate on EAAT2 than EAAT1. In addition, the EC₅₀ values for L-serine-O-sulfate transport by EAAT2 are steeply voltage-dependent but relatively constant for transport by EAAT1 (16). This difference between EAAT1 and EAAT2 in L-serine-O-sulfate transport is likely to reflect a difference in the structure of the pore of the two transporters. Thus, L-serine-O-sulfate may be used as a molecular probe for the pore of the transporters.

In addition to the pharmacological differences, the different transporter subtypes allow a varying degree of a thermodynamically uncoupled chloride flux through the transporter (6, 7, 17–19). Different transporter substrates also appear to be coupled differentially to the activation of chloride fluxes (6, 17, 19). In particular, the chloride conductance activated by d-aspartate transport by EAAT1 is significantly greater than the chloride conductance activated by d-aspartate transport by EAAT2 (17). These differences in substrate-activated chloride conductance between transporter subtypes provide another independent molecular probe for identifying the domain responsible for forming the pore of the transporters.

We have constructed a series of chimeric glutamate transporters with the aim of investigating the importance of transmembrane domains 7–10 in determining functional differences between EAAT1 and EAAT2. The chimeric glutamate transporters were characterized in terms of electrogenic glutamate transport; sensitivity to the transport blockers kainate, threo-3-methylglutamate, and (2S,4R)-4-methylglutamate; L-serine-O-sulfate transport; and chloride permeability.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**L-Glutamate (Na⁺ salt), d-aspartic acid, L-serine-O-sulfate (K⁺ salt), kainic acid, and HEPES were obtained from Sigma, Sydney, Australia. (2S,4R)-4-Methylglutamate and threo-3-methylglutamate were obtained from Tocris Cookson, Bristol, U.K. All other chemicals used were of analytical grade.

**Construction of Chimeric Transporters**—The chimeric glutamate transporters were constructed using cDNAs encoding the EAAT1 and EAAT2 transporter subtypes. Two procedures were used to construct the various chimeras. First, an in vivo recombinant procedure was used to construct the chimeras based on the method described by Buck and Amara (20). EAAT1 and EAAT2 cDNAs were cloned in tandem into the pCMV plasmid (21) with a unique XbaI site between the cDNAs. The tandem construct was linearized with XbaI, and 100 ng of DNA was used to transform competent DH5α cells. Colonies were screened for recombinant plasmids that contained only a single cDNA and sequenced to determine the junction sites (Sequenase, U.S. Biochemical Corp.). A number of chimeras were isolated, and all of them had junction sites within the most highly conserved domain from serine 366 to glutamine 415 (EAAT1 numbering). One of these chimeras, termed EAAT1-EAAT2-Chimera-7 (E12C7), contained the EAAT1 sequence at the amino terminus and junctions with EAAT2 at leucine 404. The chimera was subcloned into pRUTV for synthesis of cRNA and expression in *Xenopus* oocytes. In all of the remaining chimeras unique restriction sites were engineered into the cDNAs, using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and then the cDNA fragments of one of the transporters were cut out of the plasmid and ligated into corresponding sites of the other transporter cDNA. More than 20 different chimeric glutamate transporters were constructed in this way, but only the chimeras in Fig. 1 were functional in terms of supporting electrogenic glutamate transport. All of the functional chimeras were sequenced using the BigDye terminator cycle sequencing method (Applied Biosystems, Foster City, CA). The functional chimeras were then used to transform competent DH5α cells.

**Functional Domains of Glutamate Transporters**

**RESULTS**

**Generation of Functional Chimeric Transporters**—A number of chimeric glutamate transporters (>20) were constructed using the EAAT1 and EAAT2 subtypes, but only the five chimeric transporters depicted in Fig. 1 showed high affinity electrogenic glutamate transport in oocytes expressing the corresponding cDNAs. The EC₅₀ values for glutamate transport by EAAT1 and EAAT2 are 14.7 ± 2.9 μM and 23.2 ± 1.2 μM, respectively, and the corresponding values for the five-
Functional Domains of Glutamate Transporters

For ease of interpretation the chimeric transporters are effectively dissected into four regions. Region 1 starts at the amino terminus and includes the first six transmembrane domains and part of transmembrane domain 7. Region 2 continues through the seventh transmembrane domain to the end of the eighth transmembrane domain. Region 3 includes transmembrane domains 9 and 10. Region 4 consists of the intracellular carboxyl-terminal region (Fig. 1).
Characterization of Chimeric Transporters—The wild-type and chimeric glutamate transporters have been characterized in terms of potency of L-serine-O-sulfate as a substrate, chloride permeability, sensitivity to the transport blockers kainate and threo-3-methylglutamate, and whether (2S,4R)-4-methylglutamate is a substrate or a blocker of transport. In each of these functional characteristics there are clear differences between EAAT1 and EAAT2 (Figs. 2–4 and Tables I–III).

Functional Domain Conferring Substrate Selectivity—The EC_{50} values for glutamate did not differ more than by a factor of 2 for the chimeras, with the exception of E121C7–8, EC_{50} 3.2 μM. To allow meaningful interpretation of EC_{50} data a selectivity ratio has been included for each chimera. This ratio represents the EC_{50} value for each substrate divided by the EC_{50} for glutamate for each chimera, allowing the comparison with wild-type transporters with standardization for the EC_{50} differences observed.

Wild-type transporters EAAT1 and EAAT2 display a 9-fold difference in L-serine-O-sulfate transport parameters (EAAT1; EC_{50} 14.7 ± 2.9, EC_{50R} 1.00 ± 0.03, EC_{50R} 1.3; EAAT2, EC_{50} 23.2 ± 1.2, EC_{50} 0.70 ± 0.03 7.6).
E12C10 (EC\textsubscript{50} 27.9 ± 7.1, I\textsubscript{max} 1.07 ± 0.03, EC\textsubscript{50R} 1.0), and E12C7–8 (8.4 ± 1.5, I\textsubscript{max} 1.3 ± 0.03, EC\textsubscript{50R} 2.6) (Fig. 2 and Table I). EAAT2 transport parameters were represented in chimeric transporters E21C10 (EC\textsubscript{50} 178 ± 26, I\textsubscript{max} 0.74 ± 0.03, EC\textsubscript{50R} 10.5) and E12C7 (EC\textsubscript{50} 213 ± 8, I\textsubscript{max} 0.62 ± 0.01, EC\textsubscript{50R} 5.7) (Fig. 2 and Table I).

The chimeric transporters define a functional domain (region 3) that influences the substrate selectivity of L-serine-O-sulfate observed with EAAT1 and EAAT2. Chimera E21C8, which contains EAAT1 sequence in region 3 and 4, has EAAT1-like L-serine-O-sulfate selectivity. Chimera E12C10 showed EAAT1-like L-serine-O-sulfate selectivity, and the reverse chimera E21C10 had EAAT2-like L-serine-O-sulfate selectivity, implying that the intracellular region 4 does not confer L-serine-O-sulfate selectivity. L-Serine-O-sulfate transport by chimera E12C7, which contains EAAT2 sequence in regions 2, 3, and 4, displayed EAAT2 selectivity, whereas E12C7–8, which contains EAAT1 sequence in regions 3 and 4, displayed EAAT1 selectivity, confirming the importance of region 3. In summary, these results suggest that region 3, which includes the 9th and 10th transmembrane domains, is a major determinant of differences in substrate selectivity between EAAT1 and EAAT2.

Functional Domain Determining Differences in Chloride Permeability—Chloride permeation of both wild-type and chimeric transporters was estimated by measuring voltage-dependent transport currents of D-aspartate and L-glutamate. Differences in chloride permeation between EAAT1 and EAAT2 are most obvious for D-aspartate transport (17) (Fig. 3 and Table II), and the membrane potential at which D-aspartate transport currents reverse direction may be used as an indicator of relative chloride permeability. The chimeric transporters for the most...
part clearly resembled either EAAT1 or EAAT2. Chimeric transporters E21C8 (E_{rev} D-aspartate +7.1 ± 1.5; E_{rev} L-glutamate > +50 mV) and E12C10 (E_{rev} D-aspartate +2.3 ± 1.2; E_{rev} L-glutamate +37.0 ± 3.8 mV) both have reversal potentials that are similar to EAAT1 (Fig. 3 and Table II). Chimera E12C7 (E_{rev} D-aspartate +50 mV; E_{rev} L-glutamate +50 mV) did not show a reversal of current in the membrane potential range up to +50 mV, indicative of EAAT2-like chloride permeability (Fig. 3 and Table II). Chimera E21C10 (E_{rev} D-aspartate +23.9 ± 2.5 mV; E_{rev} L-glutamate +50 mV) did not obviously correspond to either EAAT1 or EAAT2 classification (Fig. 3 and Table II).

The results from the reversal potential measurements of the chimeric transporters suggest that the third region, which is transmembrane domains 9 and 10, is a major determinant of the differences in chloride permeability observed between EAAT1 and EAAT2. Chimera E21C8, which contains EAAT1 sequence in regions 3 and 4, has chloride permeation parameters similar to those of EAAT1 wild-type, suggesting that the region conferring differences in chloride permeation is region 3 and 4, which contains transmembrane domains 9 and 10. The results for E12C7 are also consistent with this conclusion. The transport currents for chimera E12C7–8 showed some unusual characteristics. A comparison of the current-voltage relationships for L-glutamate and D-aspartate of chimera E12C7–8 displays relative maximal currents similar to those of EAAT1, especially at negative membrane potentials (Table II). However, at positive membrane potentials the D-aspartate current does not reverse direction, which is characteristic of EAAT2 (Fig. 3). In addition to the unusual current-voltage curves for L-glutamate and D-aspartate transport, the chimera E12C7–8 supports a leak current because of chloride-modulated fluxes of Na⁺ and K⁺ (23). Leak currents have not been detected in either EAAT1 (24) or EAAT2 (22), which suggests that the functional properties of the E12C7–8 chimera have been partially compromised. The lack of outward currents generated by D-aspartate transport at positive membrane potentials may be related to the compromised ion permeation properties of this chimera. As such, the reliability of any conclusions relating to ion permeation by E12C7–8 must be questioned.

The reversal potential measurements for the chimera E12C10 are similar to those of EAAT1, which is also consistent with region 3 conferring differences in chloride permeability. However, chimera E21C10 (which is the reverse chimera of E12C10 and contains only the carboxyl-terminal region 4 from EAAT1), displayed a lower reversal potential for D-aspartate than observed for EAAT2 (Table II). The current-voltage relationship for the chimera E21C10 resembled EAAT1-like characteristics more than EAAT2 (Fig. 3). The results from this chimera imply that the carboxyl terminus may play an additional role in determining differences in chloride permeation.

It appears that the major determinant of the differences in both chloride permeability and L-serine-O-sulfate substrate selectivity between EAAT1 and EAAT2 may be localized to the same region of the transporter, which includes transmembrane domains 9 and 10. In addition, the intracellular carboxyl-terminal region may also play a minor role in chloride permeation.

Domain Conferring Blocker Sensitivity—A defining feature of the differences between EAAT1 and EAAT2 transporters is the selective sensitivity of EAAT2 to the transport blockers kainate and threo-3-methylglutamate. EAAT2 glutamate transport currents are inhibited selectively by kainate (Kₜ 17.9 ± 2.2 μM) and threo-3-methylglutamate (Kₜ 23.2 ± 2.7 μM), whereas EAAT1 is relatively insensitive (Fig. 4 and Table III). The chimeric transporter that clearly displayed blocker sensitivity was E21C10 (kainate Kₜ 15.5 ± 0.6 μM; threo-3-methylglutamate Kₜ 20.5 ± 1.8 μM).

Although there are a number of similarities among the regions that confer the various functional differences between EAAT1 and EAAT2, there also appear to be some distinct differences in defining regions which confer inhibitor sensitivity compared with substrate selectivity and chloride permeability. Chimera E21C10 displays sensitivity to blockers, whereas chimera E12C10 is insensitive to them, indicating that region 4 does not contribute to the blocker sensitivity. Chimera E21C8, which contains the EAAT1 sequence in regions 3 and 4, is insensitive to kainate/threo-3-methylglutamate blockade, indicative of region 3 (transmembrane domains 9–10) playing a role in the blocker site.

Chimera E12C7 contains regions 2, 3, and 4, and EAAT2 sequence and is insensitive to kainate and threo-3-methylglutamate, indicating that region 3 alone does not account for all of the blocker site. The blocker sensitivity observed in chimera E21C10 is likely to be due to region 3 combined with other regions of the transporter forming the functional blocker site. Chimera E12C7–8 supported a leak current (23) that was blocked by kainate (IC₅₀ 95.1 ± 23.2 μM) and threo-3-methylglutamate (IC₅₀ 22.1 ± 2.9 μM) (Fig. 4 and Table III). However, the IC₅₀ values for inhibition of [³H]glutamate transport by kainate are considerably higher (IC₅₀ > 300 μM; data not shown). A possible explanation for this apparent discrepancy may be that kainate (and threo-3-methylglutamate) binds to separate sites on the transporter to block the leak current compared with block of transport.

The kainate/threo-3-methylglutamate-sensitive leak current was not observed in E21C10, the other kainate/threo-3-methylglutamate-sensitive chimeric transporter. The leak current observed in chimera E12C7–8 may have been generated as a consequence of the formation of the chimera and thus may cast doubt on the results obtained with this chimera in terms of defining a site that confers blocker sensitivity. It is possible that a new blocker site has been generated in this chimera through misfolding of the protein and hence may not be a true representation of a region conferring the blocker site.

In summary, the chimeric transporters indicate that region 3 (transmembrane domains 9–10) is important for blocker sensitivity, but it appears that other regions of the glutamate transporter are also required for blocker activity.

### Functional Domains of Glutamate Transporters

#### Table II

| Transporter | E_{rev} L-Glutamate | E_{rev} D-Aspartate |
|-------------|---------------------|---------------------|
| EAAT1       | >50 (n = 4)         | +6.9 ± 2.5 (n = 4)  |
| EAAT2       | >50 (n = 6)         | +50 (n = 6)         |
| E21C8       | >50 (n = 3)         | +7.1 ± 1.5 (n = 3)  |
| E12C10      | +37.0 ± 3.8 (n = 3) | +2.3 ± 1.2 (n = 3)  |
| E21C10      | >50 (n = 4)         | +23.9 ± 2.5 (n = 4) |
| E12C7       | >50 (n = 6)         | +50 (n = 6)         |
| E12C7–8     | >50 (n = 4)         | +50 (n = 4)         |

Results represent mean ± S.E. (n = 3–6).

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*Functional Characteristics Defined by (2S,4R)-4-Methylglutamate—(2S,4R)-4-Methylglutamate has proven to be a useful tool in discerning differences between EAAT1 and EAAT2 transporter subtypes. The chimeric transporters E21C8, E12C10, and E12C7, which are insensitive to kainate and threo-3-methylglutamate, transport (2S,4R)-4-methylglutamate (Fig. 4 and Table III). Chimera E21C10, which displayed sensitivity to kainate/threo-3-methylglutamate blockade, also displayed (2S,4R)-4-methylglutamate blocker sensitivity (Fig. 4 and Table III). Thus, the molecular determinants of whether (2S,4R)-4-methylglutamate is a blocker or substrate for the*
most part coincide with the determinants for kainate and threo-3-methylglutamate sensitivity.

Chimera E121C7–8, which displays a kainate/threo-3-methylglutamate-sensitive leak current, allows (2S,4R)-4-methylglutamate to be transported ($EC_{50}$ 1.5 ± 0.02 μM, $I_{\text{max}}$ 0.49 ± 0.02, $EC_{50R}$ 0.5) (Fig. 4 and Table III). The $EC_{50}$ for transport of (2S,4R)-4-methylglutamate for E121C7–8 is lower than that of EAAT1 $EC_{50}$ 16.8 ± 1.9 μM, but this may be a reflection of the lower $EC_{50}$ for glutamate for E121C7–8 ($EC_{50}$ 3.2 ± 0.4 μM) (Fig. 4 and Table III). The importance of region 3 playing a role

![Fig. 4](image-url)  
**A** Inhibitor and substrate profiles of glutamate transport by EAAT1, EAAT2, and chimeric transporters. Panel A, dose-dependent inhibition of glutamate (30 μM) transport induced by glutamate analogs kainate (○), threo-3-methylglutamate (●), and (2S,4R)-4-methylglutamate (■), applied to oocytes expressing EAAT2 and chimeric transporters. The responses were measured as described under “Experimental Procedures” and fitted to the equation $I = I_{\text{max}} - (I_{\text{max}}/[\text{blocker}])/(IC_{50} + [\text{blocker}])$, where $I_{\text{max}}$ is the current generated by 30 μM glutamate. The $K_i$ values for all compounds are presented in Table I. Data represent mean ± S.E. (n = 3–8). Panel B, dose-dependent transport currents induced by transport substrates (glutamate (○) and (2S,4R)-4-methylglutamate (■)) applied to oocytes expressing EAAT1 and chimeric transporters. The responses were measured as described under “Experimental Procedures” and fitted to the equation $I = I_{\text{max}}/[\text{substrate}]/(EC_{50} + [\text{substrate}])$. The $EC_{50}$ values for both compounds are presented in Table I. Data represent mean ± S.E. (n = 3–8).

**TABLE III**

| Transporter | Kainate $K_i/IC_{50}$ | T3MG $K_i/IC_{50}$ | (2S4R)-4-Methylglutamate |
|-------------|-----------------------|---------------------|--------------------------|
| EAAT1       | >1,000                | >1,000              | 16.8 ± 1.9               |
| EAAT2       | 17.9 ± 2.2            | 23.2 ± 2.7          | 24.0 ± 4.0               |
| E21C8       | >1,000                | >1,000              | 34.7 ± 4.8               |
| E21C10      | >1,000                | >1,000              | 5.6 ± 0.5                |
| E21C7       | 15.5 ± 0.6            | 20.5 ± 1.8          | 20.2 ± 3.4               |
| E21C7–8     | >1,000                | >1,000              | 1.5 ± 0.02               |
| E12C7       | 95.1 ± 23.2           | 22.1 ± 2.9          | 0.48 ± 0.03              |
| E12C7–8     | >1,000                | >1,000              | 0.49 ± 0.02              |

*These values represent IC$_{50}$ values for block of a leak current (see text).
in determining substrate versus blocker functionality of (2S,4R)-4-methylglutamate is highlighted by chimeras E21C8, E12C10, and E21C10. Chimera E12C7, on the other hand, supports the suggestion that other regions are also required for the blockade of glutamate transport functionality.

DISCUSSION

Various methods for identifying functional domains of glutamate transporters are possible. We have used the chimeric protein approach because it has a number of advantages over other methods such as site-directed point mutations. The principal advantage is that only fully functional proteins that show characteristics of one or both the parental transporters are used in the analysis. As such the conclusions are based on positive results rather than alteration of function or in some instances loss of function associated with the generation of site-directed mutations. One limitation of this approach is that only information concerning domains that confer functional differences will be obtained. These domains may not necessarily coincide with a region that forms the glutamate recognition site.

Although more than 20 different chimeric glutamate transporters were constructed, electrogenic glutamate transport was detected only in the five chimeras depicted in Fig. 1. This suggests that the formation of functional transporters requires a number of specific interactions among amino acid residues from multiple regions. Subtle differences in these specific interactions between EAAT1 and EAAT2 may explain the functional differences between these two glutamate transporters. In the formation of the chimeric transporters, some of these interactions may be altered, leading to loss of function.

The utility of the chimera approach is based on both the degree of the structural relatedness and the functional differences between the two proteins being studied. This study presents structure-function analyses of chimeric constructs of two subtypes of glutamate transporters, EAAT1 and EAAT2. These two glutamate transporters were chosen because they display a high degree of homology, 65%, and the greatest degree of difference in substrate selectivity, blocker sensitivity, and chloride permeability among transporter subtypes while transporting glutamate with a similar EC50 (8, 15). The pharmacological and electrophysiological differences were exploited to define domains of the transporters which confer particular characteristics.

Three different molecular probes were employed to characterize EAAT1, EAAT2, and chimeric transporters. The first molecular probe, L-serine-O-sulfate, is a substrate for transporters which displays a difference in affinity (8, 16, 23). Chloride permeability, the second molecular probe, is markedly different between EAAT1 transport of D-aspartate and EAAT2 transport of D-aspartate (17) and was employed to delineate further the differences in EAAT1 and EAAT2 related to the pore of the transporter. Kainate (8), threo-3-methylglutamate, and (2S,4R)-4-methylglutamate (15) provide probes for an extracellular glutamate recognition site on EAAT2. (2S,4R)-4-Methylglutamate provided added information in that it is transported by EAAT1 (15).

The chimeric transporters define a domain including transmembrane regions 9 and 10, which is of primary importance in determining functional differences between EAAT1 and EAAT2. This region consists of 57 amino acids of which 17 are not conserved between EAAT1 and EAAT2. Three of the 17 amino acids have been allocated to extracellular domains, 10 are located in the two transmembrane domains, and 4 are positioned intracellularly according to the model by Slotboom and colleagues (14). This particular region is highly conserved among the five different human glutamate transporters. In this region EAAT1 has 68% identity with EAAT2, 78% identity with EAAT3, 92% identity with EAAT4, and 81% with EAAT5. It is generally accepted that highly conserved amino acid sequences form functionally important structures, and it may be predicted that critical changes in amino acid sequence in such a region will alter the functional properties of the protein. One would predict that one or more of the nonconserved 17 amino acids in transmembrane domains 9 and 10 contribute to differences in L-serine-O-sulfate substrate selectivity, differences in chloride permeability, and differences in blocker sensitivity between EAAT1 and EAAT2.

Although this region is clearly involved in determining functional differences, other regions must also be involved. A recent report, using chimeras generated from GLT-1 (mouse equivalent of EAAT2) and EAAC1 (mouse equivalent of EAAT3) has suggested that an intracellular region between transmembrane domain 6 and transmembrane domain 7, from amino acid residues 301 to 338 of EAAC1, contributes to the formation of a dihydrokainate recognition site (25). The results of Kanai and co-workers (25) are consistent with the observation in this report, that other regions in addition to region 3 contribute to the formation of the kainate recognition site. These results pose an interesting conundrum as to how an intracellular site and two transmembrane domains form an extracellular recognition site for kainate or dihydrokainate. Two possibilities may be considered. First, the topological model is incorrect, and the 38-amino acid residue domain identified by Kanai et al. (25) is not intracellular but within the membrane or extracellular.

The second possibility is that the intracellular segment may associate with transmembrane domains 9 and 10 to alter the conformation of an extracellular site to that kainate or dihydrokainate binds. Clearly, finer resolution of the topological model is required before these alternate possibilities may be resolved.

The intracellular carboxyl-terminal domain may also contribute to differences between EAAT1 and EAAT2, defined by region 4. The intracellular carboxyl terminus has very low sequence homology between the two transporter subtypes, and predictions of which amino acid residues are conferring these minor effects is difficult. The intracellular carboxyl terminus appears to confer functional differences to EAAT1 and EAAT2 in conjunction with other regions. The effects on chloride permeability may result from an association of the intracellular carboxyl terminus with other intracellular domains, which in turn influence the conformation of the pore of the transporter.

It has been suggested that the highly conserved region from amino acid residues 383–415 (EAAT1 numbering) forms part of the pore of the transporter, based on point mutations (11, 13) and cysteine scanning mutagenesis.1 Mutation of glutamate 404 in transmembrane domain 7 of GLT-1 (rat equivalent to EAAT2) to aspartate disrupts glutamate transport without altering glutamate binding (13). The mutation of glutamate 404 to aspartate changes substrate selectivity and prevents both forward and reverse transport of potassium ions, whereas sodium transport and chloride ion fluxes are unaffected (11, 13). These results suggest that glutamate 404 does not disrupt substrate recognition but alters the permeation pathway and ion coupling of the transporter (11, 13). Thus, Kavanaugh and co-workers (11) concluded that this residue is closely associated with the pore of the transporter.

The importance of transmembrane domains 9 and 10 in determining differences in L-serine-O-sulfate substrate selectivity and chloride permeability has been identified in this study. This result implies either that transmembrane domains 9 and 10 form part of the pore region of the transporter or that they have an interactive role with the pore region, influencing
how substrates and chloride permeate the pore of the transporter. Hence, an interaction between transmembrane domains 9 and 10 and the highly conserved region which includes transmembrane domain 7 may form the basis of the differences observed in transport parameters between EAAT1 and EAAT2.

In this study we have identified an important region, transmembrane domains 9 and 10, which plays a role in defining functional differences in substrate selectivity, chloride permeability, and blocker sensitivity between EAAT1 and EAAT2. Other regions are also involved in defining the blocker sensitivity of EAAT2. A minor role is identified for the intracellular carboxyl-terminal region conferring an effect on chloride permeability. Further point mutations in these regions will assist in defining better the molecular basis for substrate recognition, substrate and ion translocation through the transporter, and release of substrates at the intracellular surface. As the topology of the excitatory amino acid transporters becomes more conclusively defined, the regions identified in this study will allow more directed predictions of the amino acid residues responsible for the differences in the transport process of EAAT1 and EAAT2.

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REFERENCES
1. Otis, T. S., Kavanaugh, M. P., and Jahr, C. E. (1997) Science 277, 1515–1518
2. Tong, G., and Jahr, C. E. (1994) Neuron 12, 1195–1203
3. Storck, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10955–10959
4. Pines, G., Danbolt, N. C., Borjas, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisens, Seeburg, E., and Kanner, B. I. (1992) Nature 360, 464–467
5. Kanai, Y., and Hediger, M. A. (1992) Nature 360, 467–471
6. Fairman, W. A., Vandenbarg, R. J., Arriza, J. L., Kavanaugh, M. P., and Amara, S. G. (1995) Nature 375, 599–603
7. Arriza, J. L., Eliasof, S., Kavanaugh, M. P., and Amara, S. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4155–4160
8. Arriza J. L., Fairman, W. A., Wediche, J. I., Murdoch, G. H., Kavanaugh, M. P., and Amara, S. G. (1994) J. Neurosci. 14, 5559–5569
9. Seal, B., Arriza, J. L., and Amara, S. G. (1995) Soc. Neurosci. Abstr. 21, 1861
10. Wahle, S., and Stoffel, W. (1996) J. Cell Biol. 135, 1867–1877
11. Kavanaugh, M. P., Bendahan, A., Zerangue, N., Zhang, Y., and Kanner, B. I. (1997) J. Biol. Chem. 272, 1703–1708
12. Conradt, M., and Stoffel, W. (1995) J. Biol. Chem. 270, 25207–25212
13. Pines, G., Zhang, Y., and Kanner, B. I. (1995) J. Biol. Chem. 270, 17083–17097
14. Slotboom, D. J., Lolkema, J. S., and Konings, W. N. (1996) J. Biol. Chem. 271, 31317–31321
15. Vandenber, R. J., Mitrovic, A. D., Cheh, M., Balc, V. J., and Johnston, G. A. R. (1997) Mol. Pharmacol. 51, 809–815
16. Vandenber, R. J., Mitrovic, A. D., and Johnston, G. A. R. (1998) Br. J. Pharmacol. 123, 1593–1600
17. Wediche, J. I., Amara, S. G., and Kavanaugh, M. P. (1995) Neuron 15, 721–728
18. Jahr, C. E., and Eliasof, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4153–4158
19. Billups, F., Rossi, D., and Attwell, D. (1996) J. Neurosci. 16, 6722–6731
20. Buck, K. J., and Amara, S. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12584–12588
21. Anderson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) J. Biol. Chem. 264, 8222–8229
22. Wediche, J. I., Arriza, J. L., Amara, S. G., and Kavanaugh, M. P. (1995) Neuron 14, 1019–1027
23. Vandenber, R. J., Arriza, J. L., Amara, S. G., and Kavanaugh, M. P. (1995) J. Biol. Chem. 270, 17668–17671
24. Lebrun, B., Sakaitani, M., Shimamoto, K., Yasuda-Kamatani, Y., and Nakajima, T. (1997) J. Biol. Chem. 272, 20336–20339
25. Kanai, Y., Utsunomiya-Tate, N., and Endou, H. (1997) Soc. Neurosci. Abstr. 23, 1485