An Extra Amino Acid Residue in Transmembrane Domain 10 of the γ-Aminobutyric Acid (GABA) Transporter GAT-1 Is Required for Efficient Ion-coupled Transport*

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The GABA transporter GAT-1 mediates electrogenic transport of its substrate together with sodium and chloride. It is a member of the neurotransmitter:sodium:symporters family, which are crucial for synaptic transmission. Compared with all other neurotransmitter:sodium:symporters, GAT-1 and other members of the GABA transporter subfamily all contain an extra amino acid residue at or near a conserved glycine in transmembrane segment 10. Therefore, we studied the functional impact of deletion and replacement mutants of Gly-457 and its two adjacent residues in GAT-1. The glycine replacement mutants were devoid of transport activity, as were mutants obtained by deleting positions on either side of Gly-457. However, the inward rectification of GABA-induced transport currents by all three deletion mutants was diminished, and the charge-to-flux ratio was increased by more than 2.5-fold, both of which indicate substantial uncoupled transport. These observations suggest that the deletions render the transporters less tightly packed. Consistent with this interpretation, the inactive G457A mutant was partially rescued by removing the adjacent serine residue. Moreover, the activity of several gating mutants was also partially rescued upon deletion of Gly-457. Structural modeling showed that the stretch surrounding Gly-457 is likely to form a π-helix. Our data indicate that the “extra” residue in transmembrane domain 10 of the GABA transporter GAT-1 provides extra bulk, probably in the form of a π-helix, which is required for stringent gating and tight coupling of ion and substrate fluxes in the GABA transporter family.

Neurotransmitter transporters play a crucial role in the process of neurotransmission. These transporters remove the neurotransmitters from the synaptic cleft and as a result the signaling by the neurotransmitter is terminated (1, 2). They utilize the energy from the electrochemical sodium gradient for the uphill translocation of neurotransmitters into the cells surrounding the synapse. With the exception of glutamate transporters, the transporters for other neurotransmitters, such as GABA (GAT1–4), serotonin (SERT), §3 dopamine (DAT), norepinephrine (NET), and glycine (GLYT1,2) belong to the family of neurotransmitter:sodium:symporters (NSS), which couple the flux of neurotransmitter not only to that of sodium, but also to that of chloride. The GABA transporter-1 (GAT-1) catalyzes electrogenic sodium-chloride-GABA transport with a stoichiometry of 2–3Na⁺:1Cl⁻:GABA (3–7). Because of this electrogenocity, transport can be monitored by electrophysiological measurements (5–8), in addition to tracer flux measurements.

The first structural information on the NSS transporters was obtained from a bacterial homologue LeuT, which has been crystallized in four conformations: outward-open with sodium, outward-occluded both with or without substrate, and inward-facing (9, 10). More recently, structures have been determined of thermostabilized versions of human SERT (11) and Drosophila melanogaster DAT (12) both in outward-facing conformations. All three proteins consist of 12 TMs with TM1–5 related to TM6–10 by a pseudo-2-fold axis in the membrane plane and its binding pocket is located at the symmetry axis. In the outward (extracellular)-facing conformations the binding pocket is occluded from the cytoplasm by ~20 Å of ordered protein including a charge pair (also known as the thin intracellular gate) composed of an arginine residue just “below” TM1 (Arg-5 in LeuT) and an aspartate located at the intracellular side of TM8 (Asp-369 in LeuT; Fig. 1A (9)). These residues are conserved in the NSS family, and in GAT-1 they are Arg-44 and Asp-410, respectively. Conversely, for the inward-facing structure of LeuT, the extracellular side is occluded by a “thin” extracellular gate, involving a pair of conserved aromatic amino acid residues, and a conserved charged pair composed of an aspar...
Therefore, we studied the functional impact of deletion and replacement mutants of the glycine residue, and of the two adjacent residues, and constructed a structural model of GAT-1 to examine the structural impact thereof. Our results indicate that the extra residue creates a $\pi$-helix in TM10, which is required for stringent gating and tight coupling of ion and substrate fluxes in GABA transport.

Results

Radioactive Transport of Deletion and Replacement Mutants—Mutation of Gly-457 to various residues resulted in defective [3H]GABA transport as monitored in the HeLa cell expression system (Fig. 2A). Remarkably, significant transport, measured at a subsaturating GABA concentration (see “Experimental Procedures”), was observed for the Gly-457 deletion mutant (Fig. 2A). The $K_m$ for the deletion mutant was 21.8 $\pm$ 3.1 $\mu$M, as compared with 3.6 $\pm$ 0.5 $\mu$M for GAT-1-WT and the $V_{max}$ of the mutant was 186.7 $\pm$ 21.9% of that of GAT-1-WT (Table 1). The lack of activity by the replacement mutants was not due to defective targeting to the plasma membrane as judged by surface biotinylation (Fig. 2B). All the bands are specific for the WT and mutant transporters, because they were not observed with cells transfected with the “empty” vector (Fig. 2, SK). The bands observed at around 50 and 100 kDa represent the full-length monomeric and dimeric N-glycosylated forms, and the band of around 45 kDa observed in the total samples represents the immature form of the transporter (17). Low levels of the latter species were also observed in the biotinylated WT samples (Fig. 2B), consistent with our earlier observations that some of the immature transporters in fact can reach the cell surface, at least in the case of the expression system used here (17). Thus the lack of activity of the replacement mutants is due to an intrinsic transport defect. On the other hand, the impact of mutation on the activity of the residues surrounding Gly-457 was less drastic. The activity of S456A was around 60% of that of GAT-1-WT and even though the transport activity of S456C was around 30% of that of GAT-1-WT and even though the transport activity of S456C was less drastic, it was still significant (Fig. 3). The activity of Met-458 replacement mutants was similar to that of GAT-1-WT (Fig. 3). Significant activity was also observed when Ser-456 or Met-458 was deleted (Fig. 3).

GABA-induced Currents by the Deletion Mutants—The GABA-induced steady-state currents by oocytes expressing GAT-1-WT exhibited the well known non-reversing inward currents (Fig. 4A), reflecting electrogenic Na$^+$- and Cl$^-$-coupled GABA influx (6). We shall refer to them as coupled or stoichiometric currents. In contrast to the situation for GAT-1-WT, GABA did induce outward currents at positive potentials (Fig. 4A). Transport currents were not seen when GABA was replaced by glycine (Fig. 5). The most obvious reason for the appearance of the GABA-induced outward currents is a leak current modulated by the substrate. On the other hand, because the deletion mutants exhibited substantial radioactive GABA transport in the HeLa cell expression system (Figs. 2A and 3), one would expect that at negative potentials, a component of the current would still be stoichiometric. To investigate...
if the GABA-induced currents also have a leak component at negative potentials, we measured charge/flux ratios (Fig. 4B). The charge/flux ratio of the deletion mutants at -100 mV was markedly increased relative to GAT-1-WT, namely 16.7 ± 3.6, 6.6 ± 1.0, and 6.9 ± 0.1 for ΔSer-456, ΔGly-457 and ΔMet-458, respectively, as compared with 2.5 ± 0.1 for GAT-WT (Fig. 4B). Thus, the GABA-induced currents by these mutants apparently reflect the sum of the inward rectifying coupled current and a bidirectional leak current. The reversal potential of the total GABA-induced current would then be determined by the relative contribution of each of these currents to the total. Because the transport of GABA is coupled to that of Na⁺ and Cl⁻, we investigated whether these ions were carrying the GABA-induced leak current. The reversal potential of the GABA-induced current was left shifted by 27.5 ± 3.15 mV when the extracellular Na⁺ concentration was lowered by a factor of 10 (n = 6) (Fig. 6). Lowering the extracellular Cl⁻ concentration by the same factor resulted in a right shift of 15.8 ± 1.7 mV (n = 5). Increasing the K⁺ concentration 15-fold also resulted in a right shift of the reversal potential of the currents induced by GABA, namely by 21.3 ± 0.2 mV (n = 4) (Fig. 6). Thus, each of these ions can permeate through the GABA-activated leak pathway, which appears to be non-selective with a slight preference for Na⁺. When 90% of the Cl⁻ was replaced by SCN⁻, the reversal potential of the GABA-induced currents was left shifted by 23.0 ± 6.7 mV (n = 5), indicating that the latter anion can also permeate through the mutant transporter.

**Constitutive Leak Currents by the Deletion Mutants**—The above data show that ΔGly-457 transporters exhibited leak currents in the presence of GABA, but does not speak to the mutant behavior in the absence of GABA, i.e. whether there is a constitutive leak current. We therefore used the non-transportable substrate analogue tiagabine to visualize the currents mediated by the mutant transporters in Na⁺-containing media. The constitutive leak currents for oocytes expressing ΔGly-457 are strongly outward-rectifying (Fig. 7A, right panel, and B). Similar outward-rectifying currents were also seen with ΔSer-456 and ΔMet-458 (Fig. 7B). In contrast, no such currents were seen by GAT-1-WT (Fig. 7A, left panel). The magnitude of the constitutive leak currents by ΔGly-457 was reduced by 41.1 ± 2.4% when 90% of the Na⁺ was replaced by choline and by

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**FIGURE 2. Transport activity of Gly-457 mutants and cell surface expression.** A, transport: GAT-1-WT (control) and the indicated mutants were transiently expressed in HeLa cells, and sodium-dependent [3H]GABA transport was measured at room temperature for 10 min, as described under “Experimental Procedures.” The percent of activity of GAT-1-WT data are given as mean ± S.E. (error bars) of at least three separate experiments performed in quadruplicate. B, cell surface biotinylation: HeLa cells expressing GAT-1-WT (WT) and the indicated mutants, as well as HeLa cells transfected with the vector alone (SK), were biotinylated and processed as described under Experimental Procedures. The bands were scanned after exposure of the films for 5 s, which was the time where the response was still linear. The markers shown were run in the lane to the left of SK and contain “Prestained Protein Marker, Broad Range,” catalog number P7708S from New England Biolabs.

**TABLE 1**

| Mutant        | Km (µM) | Vmax (% of WT) |
|---------------|---------|----------------|
| WT            | 3.6 ± 0.5 | 100            |
| ΔSer-456      | 1.3 ± 0.1 | 7.7 ± 2.3     |
| ΔGly-457      | 21.8 ± 3.1 | 186.7 ± 21.9  |
| ΔMet-458      | 16.4 ± 0.3 | 20.6 ± 1.7    |
| R44K          | 0.5 ± 0.1 | 2.4 ± 0.7     |
| R44K/ΔGly-457 | 5.4 ± 0.3 | 8.4 ± 1.6     |
| G457A/ΔSer-456| 64.6 ± 9.0 | 120.7 ± 24.2  |
FIGURE 4. GABA-induced steady-state currents and charge/flux ratios by GAT-1 WT and mutant transporters. A, GABA-induced currents: the membrane voltage of oocytes expressing the indicated mutants was stepped from a holding potential of $-25$ mV to voltages between $-140$ to $+60$ mV in 25-mV increments. Currents in ND96 were subtracted from those in the same medium supplemented with 1 mM GABA, at each potential from 420 to 480 ms were averaged and normalized to the GABA-induced current at $-140$ mV. These currents were then plotted against the corresponding potential. The data are the mean ± S.E. (error bars) of at least three repeats. Wherever error bars are not visible, the error was smaller than the size of the symbols. The currents at $-140$ mV induced by 1 mM GABA ranged from $-137$ to $-639$ nA in GAT-1-WT, from $-275$ to $-1401$ nA in ΔGly-457, from $-169$ to $-334$ nA in ΔSer-456, and from $-35$ to $-166$ nA in ΔMet-458. B, charge/flux ratios: oocytes expressing GAT-1-WT and the indicated mutants were voltage-clamped at $-100$ mV and the current induced by 20 μM radioactive GABA (0.4 Ci/mmol) was measured for 100 s. The charge moved during this time was obtained by integrating the current over time. The ratio was determined by calculation of the moles of charge and divided by the moles of radio-labeled substrate taken up as determined from scintillation counting after correction by the values for non-injected oocytes. The data are given in mean ± S.E. (error bars) of at least three separate experiments performed in quadruplicate.

FIGURE 5. GABA-induced steady-state currents by ΔGly-457. The membrane voltage of oocytes expressing ΔGly-457 was stepped from a holding potential of $-25$ mV to voltages between $-140$ to $+60$ mV in 25-mV increments as described in the legend to Fig. 4. The currents in ND96 were subtracted from those in the same medium supplemented with 1 mM GABA or glycine.

FIGURE 6. The substrate-induced currents from oocytes expressing ΔGly-457 were measured in the presence of two extracellular concentrations of sodium, chloride, and potassium. A, transport currents in the presence of the standard medium (open circle) or when 90% of the sodium was replaced by choline (filled circle). B, transport currents in the presence of the standard medium (open circle) or when 90% of the chloride was replaced by gluconate (filled circle). C, transport currents that measured in medium containing 20 mM NaCl, 2 mM KCl, and 80 mM ChCl (open circle) or 20 mM NaCl, 30 mM KCl and 50 mM ChCl (filled circle). The data are given in mean ± S.E. (error bars) of at least three oocytes.
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FIGURE 7. Tiagabine-sensitive currents by GAT-1-WT and deletion mutants. A, records of the sodium-dependent currents, using the voltage jump protocol described in the legend to Fig. 4. All traces show representative currents in ND96 minus those in ND96 plus 10 μM tiagabine. The dashed lines indicate zero current. The data are from representative oocytes, which are typical for at least three oocytes. B, current-voltage relationships of the tiagabine-sensitive currents by oocytes expressing deletion mutants. The currents in ND96 supplemented with 10 μM tiagabine were subtracted from those in ND96 alone. The currents were measured using the voltage jump protocol described in Fig. 4 and were plotted against the corresponding potential (mV). The tiagabine-sensitive currents were normalized to those at +60 mV. The data are the mean ± S.E. (error bars) of at least three repeats. Wherever error bars are not visible, the error was smaller than the size of the symbols. The currents at +60 mV ranged from 643 to 1438 nA in ∆Ser-456, from 304 to 1160 nA in ∆Gly-457 and from 32 to 247 nA in ∆Met-458.

43.1 ± 3.5% when 90% of the Cl⁻ was substituted by gluconate (n = 3).

Effect of Modifying Neighboring Amino Acids in Gly-457 Mutants on Radioactive GABA Transport—The data presented so far indicate that the deletion of single TM10 amino acid residues results in less tightly coupled transport than that by GAT-1-WT, although the molecular underpinnings of that effect were unclear. In structures of LeuT, the only NSS homolog for which the inward-open state has been reported, residues in this region (including Thr-409 and Ile-410) form contacts with TM6, especially Ala-246 and Ile-245 as well as with Arg-30 from TM1 (Fig. 8). This raises the possibility that the packing in this region is important for coupled transport, and therefore that the structure of the deletion mutants is poorly packed compared with that of GAT-1-WT. To address this possibility, we questioned whether the inactivity of Gly-457 replacement mutants (Fig. 2A) could be compensated by introducing modifications that reduce the protein volume nearby. As mentioned before, the G457A mutant did not exhibit any detectable transport activity in the WT background (Figs. 2A and 9). On the other hand, low but very significant radioactive GABA transport activity was observed when this mutation was placed in the background of ∆Ser-456 or ∆Met-458 (Fig. 9), suggesting that the additional bulk of the alanine at position 457 could be compensated by deleting a residue on either side. Deleting the larger Met-458 restored less of the activity than deleting Ser-456 in the G457A background (Fig. 9, G457A/∆Met-458 versus G457A/∆Ser-456). Interestingly, the reverse was true for modifications in the background of the larger G457L. That is, the deletion of Ser-456 did not restore activity lost in G457L, whereas deletion of the larger Met-458 did partially restore that activity (Fig. 9, G457L/∆Ser-456 versus G457L/∆Met-458).

Effect of Thin Gate Modifications of Gly-457 Deletion Mutant on Radioactive GABA Transport—in known structures of NSS transporters, the region near Gly-457 in TM10 is close to the extracellular gate residue Asp-451 (Figs. 1A and 10, A–C). To test if the postulated poorer packing of the deletion mutants results in less stringent gating requirements, we combined mutations of the thin extracellular or intracellular gates with the ∆Gly-457 deletion. The extracellular thin gate residue Arg-69 is critical for transport (14, 18, 19) and even its replacement by Lys was not tolerated (Fig. 11A). However, when the R69K mutation was introduced into the ∆Gly-457 background, small but significant radioactive GABA transport was observed (Fig. 11A). On the intracellular side, replacing the thin gate residue Arg-44 with Lys had a much smaller impact on transport activity than the R69K mutation (Fig. 11A). However, no currents induced by 1 mM GABA (monitoring I_{max}) were detectable in oocytes expressing this mutant (Fig. 11B, see legend). This is probably due to the fact that this mutant has a very low V_{max} (Table 1) and that therefore transport currents (I_{max}, measured with saturating GABA concentrations), which reflect the V_{max} of radioactive transport, cannot be detected. However, radioactive transport measured at...
concentrations far below $K_m$ is proportional to $V_{\text{max}} / K_m$. Because R44K has a very low $K_m$ (Table 1), radioactive transport by this mutant can be readily detected (17). Remarkably, robust GABA-induced currents were observed when the R44K mutation was introduced in the ΔGly-457 background (Fig. 11B), even though the $V_{\text{max}}$ of radioactive transport by this mutant is also very low (Table 1). This indicates that most of the transport currents by this double mutant are due to a GABA-induced leak current rather than the stoichiometric current. Indeed, the current/voltage relationship of the currents induced by GABA in oocytes expressing R44K/ΔGly-457 was similar to that of ΔGly-457 (Figs. 4A and 11B), showing outward currents at positive potentials. Thus the ΔGly-457 mutation results in uncoupling in both WT and R44K backgrounds. Such apparent discrepancies between $V_{\text{max}}$ and $I_{\text{max}}$ are also seen with other uncoupled mutants, such as ΔSer-456 (Table 1 and Fig. 4).

On the extracellular side, R69K alone also did not exhibit steady-state transport currents (legend to Fig. 11B, (19)), but again the introduction of ΔGly-457 restored significant activity (Fig. 11B). A similar restoration by introducing the ΔSer-456 mutation was also observed with the inactive G457A (Fig. 11B).

**Structural Model of rGAT-1**—In the absence of crystallographic data on a GABA transporter containing the extra residue in TM10, a structural interpretation of the above data is challenging. However, the recently reported structures of dmDAT (Fig. 10C) (12) and hSERT (Fig. 10A) (11) are closer homologues to rGAT-1 than the prokaryotic homolog, LeuT (Fig. 10A), particularly in TM10 (Fig. 1B), and so potentially provide relevant templates, at least for the outward-facing states. We therefore constructed homology models of rGAT-1 using either hSERT or dmDAT as a template. Of these, the models constructed using hSERT as a template had higher scores according to ProQM (see “Experimental Procedures”; Fig. 12). Because the insertion of a single residue can distort the segment around it, the region around Gly-457 (residues 453–459) in the hSERT-based models was further refined using a loop prediction approach (see “Experimental Procedures”). Clustering the output of the loop prediction identified a number of clusters with low scores (see “Experimental Procedures”), and we selected representative models from the largest three of those low-energy clusters to provide an indication of the variability among low-energy models (Fig. 10D). Analysis of these models suggests that the primary effect of the extra residue may be to elongate the wide helix turn (π-helix) found in related proteins (Fig. 10, E and F) and create additional protein bulk “beneath” (i.e. on the cytoplasmic side of) the extracellular gate (Fig. 10D). Although only found in <5% of structures (20, 21), π-helices have been found to result from functionally important, evolutionary insertions within α-helices (22), including in
transmembrane spanning segments (23, 24). In the case of rGAT-1, the additional volume of protein appears to be essential for closing off the pathway and thereby enabling tight coupling.

Discussion

Because the structural data on Mhp1, which belongs to a different family and yet shares the LeuT-fold, indicated that a glycine residue in TM10 could serve as a hinge involved in the occlusion of the substrate (16), we began our study with the idea that Gly-457 of GAT-1 would fulfill a similar role. Consistent with this notion, the impact of mutation of this residue was more dramatic than mutation of the neighboring residues, i.e. only the replacement mutants of Gly-457 were devoid of [3H]GABA transport (Figs. 2A and 9). Nevertheless, it is not clear if Gly-457 is the “extra” residue, because the single residue insertion in TM10 could be aligned to either Gly-457 (60.0% of the sequences) or Ser-456 (39.7% of the sequences). Because in...
the inward-open conformation of LeuT, a Thr at a similar position as Ser-456 participates in the closure of the thin extracellular gate (10), it is tempting to speculate that the Gly is the extra residue. Regardless of the evolutionary origins, the experimental data show that deletion of Ser-456 or Met-458 resulted in a similar phenotype as the mutant lacking Gly-457, namely inefficient ion-coupling of GABA transport (Fig. 4B).

The structural model we present here, explains all our data very well. We hypothesize that the wide, \( \pi \)-helical segment predicted to form in TM10 would be closely packed against the other amino acid residues in inward-facing conformations of GAT-1. A plausible consequence of this hypothesis is that all three deletion mutants would “loosen” this packing, increasing the probability for ions to slip through the transporter during the transport cycle, which is precisely what was observed (Fig. 7). This structural model suggests several predictions, which we experimentally verified. First, increasing the volume of the residues could result in steric clashes, thereby hampering the conformational changes required for the transport process. Most pronounced are the results obtained when replacing Gly-457 (Fig. 2A and 3). This presumably reflects the fact that glycine is the smallest of the 20 natural amino acids and therefore the relative change in the volume of the side chain at position 457 by mutation is the greatest. Moreover it is possible that Gly-457 could serve as a hinge in the context of the \( \pi \)-helical segment and the flexibility at this position may help to occlude the substrate, as proposed for Mhp1 (16). Importantly, the inactivity of Gly-457 replacement mutants (Fig. 2A) could be partially restored by introducing modifications that reduce the protein volume nearby (Fig. 9). Specifically, this result suggests that the additional bulk of an alanine at position 457 could be compensated by deleting a residue on either side. As described under “Results,” the partial restoration of activity of replacement mutants by a deletion mutant is quite subtle and seems to be a function of the sizes of the deleted and mutated residues, respectively (Fig. 9). Altogether the results suggest that the putative \( \pi \)-helix is optimally packed to enable efficient coupling between ion and substrate fluxes. A second prediction of our model was also experimentally satisfied, namely that the looser packing of the mutants at the putative \( \pi \)-helical segment destabilizes the inward-facing state, and thereby alters the contributions of the two thin gates. Indeed, our measurements suggest that the gating becomes less stringent (Fig. 11). It is possible that the less stringent gating contributes to the leak mode in the mutants, because this would increase the probability of both gates being open at the same time.

One of the pieces of evidence for the leak mode of the deletion mutants is the increased charge to flux ratio (Fig. 4B). In the case of GAT-1-WT we obtained a value of 2.5, which is somewhat higher than the value of 2 obtained in an earlier study (25). At the present time we have no explanation for this difference. Regardless, the significantly increased charge to flux ratio in the deletion mutants measured here appears to be due to a relatively nonselective GABA-gated ion permeation pathway (Fig. 6), which is compatible with the reversal potential of the GABA induced currents (Fig. 4A).

In addition to the GABA-dependent leak currents, robust outward-rectifying currents in each of the three deletion mutants were observed in the absence of the neurotransmitter substrate (Fig. 7). The partial Na\(^+\) and Cl\(^-\) dependence of these tiagabine-sensitive outward currents could be due to reduced block by the inhibitor when the external concentration of these ions is lowered. The difference in the characteristics of the leak conductance by the deletion mutants in the presence or absence of GABA could be a reflection of different transporter conformations. That is, in the presence of Na\(^+\) without the substrate the transporters predominantly reside in an outward-facing conformation while in the presence of Na\(^+\) with the substrate the transporters predominantly reside in an inward-facing conformation (13), suggesting that the strongly outward rectifying currents occur in this conformation. On the other hand, in the presence of GABA the proportion of inward-facing transporters is increased and it is possible that the almost non-rectifying currents reflect a leak pathway mediated by the inward-facing conformation of the deletion mutants.

Here, we elected to build models of rGAT-1 only using the closely related mammalian transporters as templates, to ensure that the regions surrounding TM10, including TM11 (Fig. 10, A–D), would be as reliable as possible, which we expected would be important for the loop prediction stage of the modeling. The resultant models predicted an extension of the \( \pi \)-helical segment in TM10 of hSERT (Fig. 10E), creating a wider and more bulky segment in GAT-1 than in the homologues. As mentioned above, this prediction fits well with the experimental findings. Moreover, in the structural models of outward-facing rGAT-1, the extracellular half of TM10 is exposed to the pathway, reflecting the environment of the equivalent residues in the hSERT template structure. Asp-451 and Tyr-452 line the extracellular pathway, consistent with the accessibility of Y452C to MTSET (13). Previous experiments showed Y453C is also highly sensitive to MTSET (13), but Tyr-453 is oriented toward the “back” of TM10 and therefore more buried than its neighbor. Nevertheless, the introduction of the smaller Cys at Tyr-453 would likely create an aqueous pocket, for which the major barrier to aqueous accessibility would be the long unstructured loop connecting TM11 and TM12, which is presumably rather flexible. Thus, it seems reasonable to expect that MTSET would react with Y453C, and that the resultant positively charged adduct could have a significant impact on transport. Put together, these data provide strong validation for the structural model of TM10 in rGAT-1 (Fig. 10D).

Aside from the internal structure of TM10, of course, it would also be useful to know how Gly-457 and Ser-456 pack against elements of the four-helix bundle (TM1 and TM6; Fig. 8) when the extracellular pathway is closed. Although it is possible to model this packing using inward-facing LeuT as a template, the uncertainty would be higher because LeuT differs in both TM6 and TM10; for example, TM6 of r-GAT-1 includes larger, more polar residues such as Asp-287 and Thr-290 (Fig. 10D). In future, structures of r-GAT-1 and other NSS transporters in both inward and outward conformations will be important to provide a more complete picture of the features of GABA transporters that confer this unique dependence.

Several other NSS transporters that do not contain the additional residue in TM10, such as DAT and SERT, have been reported to exhibit considerable uncoupling (26, 27). It is therefore tempting to assume that the absence of the additional residue results in uncoupling in all eukaryotic NSS transporters.
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However, the glycine transporters GlyT1 and GlyT2 also lack the additional residue (TM10 is the same length as in DAT and SERT) and these transporters, when expressed in Xenopus oocytes, do not exhibit uncoupled leak currents (28). It is possible that GlyT1 and GlyT2 are tightly coupled because unlike SERT and DAT they contain residues larger than glycine in the region of TM10 we have studied here. Thus, replacement of the appropriate residue with glycine in this area of GlyT1 and GlyT2 might render these transporters leaky, as in SERT and DAT. Moreover, the subsequent introduction of an extra amino acid residue, as in GAT-1, could potentially restore coupled transport in such leaky GlyT mutants. The importance of the putative wide π-helix turn in ion-coupling in the GABA subfamily of NSS transporters demonstrates that even though the general mechanism of transport is similar to that of the other eukaryotic and prokaryotic homologues (9, 10, 29–32), the precise mechanism can vary among family members.

Experimental Procedures

Generation and Subcloning of Mutants—Mutations were made by site-directed mutagenesis of GAT-1 in the vector pBluescript SK− (Stratagene) using single-stranded uracil-containing DNA as described previously (33, 34). Briefly, the GAT-1 containing plasmid was used to transform Escherichia coli C334 (dut-, ung-). From one of the transformants, single-stranded uracil-containing DNA was isolated upon growth in uridine-containing medium according to the standard protocol from Stratagene, using helper phage R408. This yields the sense strand, and consequently, mutagenic primers were designed to be antisense. The mutations were verified by sequencing the entire coding region of the cDNA.

Cell Growth and Expression—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 200 units/ml of penicillin, 200 μg/ml of streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus VTF7–3 (35) and subsequent transfection with plasmid DNA, as well as GABA transport, were done as published previously (36). The expression vector was pBluescript SK−. Radioactive transport, using the subsaturating GABA concentration of 22.3 mM, was performed usually for 10 min, unless indicated otherwise. Because this concentration is far below Km, radioactive transport is proportional to Vmax/Km. Thus, under our conditions, radioactive transport can be readily observed in mutants with a low Vmax provided that Km is also low for those mutants. Similarly, radioactive transport can be readily observed for the case that both parameters are increased in the mutants. The values for the mutants were normalized to those of GAT-1 WT, as indicated in the figure legends. In all of the experiments with HeLa cells, the expression vector was pBluescript SK−. Statistical evaluation of the activity of the different mutants utilized a one-way analysis of variance with a post-hoc Dunnett’s multiple comparison test, where p < 0.05 was taken as significant. Results were plotted using normalized data for each mutant, where the untreated activity levels are normalized to 100%.

Cell Surface Biotinylation—Labeling of wild type and mutant transporters at the cell surface, using Sulfo-NHS-SS-Biotin (Pierce), quenching the reaction, cell lysis, and isolation of the biotinylated proteins by streptavidin-agarose beads (Pierce) were done as described (37). After SDS-PAGE (10% gel) and transfer to nitrocellulose, the GAT-1 protein was detected with an affinity-purified antibody, directed against an epitope from the cytoplasmic C-terminal tail of GAT-1, at a 1:500 dilution, with horseradish peroxidase-conjugated secondary antibody at a 1:40,000 dilution, and with ECL. 1% of goat serum was present in all antibody, blocking, and washing solutions to minimize the appearance of nonspecific bands. The films were scanned using a standard scanner and quantitative densitometry was done using ImageJ 1.43u and statistical analysis was done with Origin 6.1 software (OriginLab Corporation).

Expression in Oocytes and Electrophysiology—cRNA was transcribed using mMESSAGE-mMACHINE (Ambion) and injected into Xenopus laevis oocytes, as described (19). Oocytes were placed in the recording chamber, penetrated with two agarose-cushioned micropipettes (1%/2 M KCl, resistance varied between 0.5 and 3 megaohms), voltage clamped using GeneClamp 500 (Axon Instruments), and digitized using Digitdata 1322 (Axon Instruments both controlled by the pClamp9.0 suite (Axon Instruments). Voltage jumping was performed using a conventional two-electrode voltage clamp as described previously (38). The standard buffer, termed ND96, was composed of 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Na-HEPES, pH 7.5). Wherever error bars are not visible, the error was smaller than the size of the symbols.

Modeling of rGAT-1—Homology models of rGAT-1 (SWISS-PROT identifier 128609) were constructed using the 3.1-Å resolution outward-open, paroxetine-bound structure of thermolabilized hSERT (PDB identifier 516X (11)), including one Na+ and one Cl as a template. AlignMe in PS mode (39) was used to generate the alignment, of which no adjustments were found to be necessary, and rGAT-1 models were generated using MODELLER version 9.15 (40); 1500 iterations of model building were sufficient for convergence of the MolPDF score, a measure of the agreement of the models with the restraints implied by the template and the Charmm27 force field. The 20 models with the lowest MolPDF score were further analyzed in PROCHECK (41) to confirm that their backbone dihedral angles fell within the allowed regions of the Ramachandran plot. From this set of 20, five models with the highest ProQM (42) scores (ranging from 0.841 to 0.848) were chosen to provide some diversity in the end points for loop refinement in the next step.

Loop Refinement—Using these five models, the region surrounding Gly-457 in TM10 (residues 453–459) was remodeled using the loop refinement function in MODELLER. For each of the five models, 2000 alternate conformations were built for this region, and the 1000 models with the lowest scores were selected for further comparison. The resultant 5000 models were pooled together and clustered based on the root mean square deviation of the backbone atoms of the TM10 segment using the average-linkage clustering program NMRCUCLUST (43). Of the top five largest clusters, we selected low-energy representatives from the three clusters with the lowest average MolPDF scores. These three representative models were steepest descents energy minimized using CHARMM version 34 and the Charmm36 force field (44, 45) for 300 steps with backbone
atoms constrained, and for another 300 steps with the constraints released.

**Structure Assessment**—The MolProbity (46) scores of the representative three models were \(-1.75\), and the ProQM scores were excellent (\(>0.84\)), and similar to that of the template, thermostabilized hSERT (0.80). The sequence identity between hSERT and rGAT-1 is 41%, and so the expected accuracy is \(\sim 1\) Å in the Ca positions (47). A structure of dmDAT (PDB identifier 4XP4 at 2.8-Å resolution bound to cocaine) was also tested as a possible template, because the sequence identity between dmDAT and rGAT1 is \(\sim 45\%\); however, the overall ProQM scores of the resultant models were consistently lower (0.835–0.841 for the 5 models with the lowest MolPDF scores) than for hSERT-based models (0.841–0.848; see above). Secondary structures of models and structures were assigned using SST (48) and DSSP version 2.2.1 (49). Figures of structures were made using PyMOL (Schrödinger, Inc.).

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