Oligomerization of the \( \gamma \)-Aminobutyric Acid Transporter-1 Is Driven by an Interplay of Polar and Hydrophobic Interactions in Transmembrane Helix II*

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The available evidence indicates that members of the neurotransmitter:sodium symporter family form constitutive oligomers. Their second transmembrane helix (TM2) contains a leucine heptad repeat proposed to be involved in oligomerization. In artificial transmembrane segments, interhelical interactions are stabilized by polar residues. We searched for these hydrogen bond donors in TM2 by mutating the five polar residues in TM2 of the \( \gamma \)-aminobutyric acid transporter-1 (GAT1). We tested the ability of the resulting mutants to oligomerize by fluorescence microscopy, Foerster resonance energy transfer, and \( \beta \)-lactamase fragment complementation. Of all generated mutants, only Y86A (but not Y86F-), E101A, E101Q-, and E101D-GAT1 were judged by these criteria to be deficient in oligomerization and were retained intracellularly. The observations are consistent with a model where the leucine heptad repeat in TM2 drives a homophilic association that is stabilized by Tyr\(^{86}\) and Glu\(^{101}\); Tyr\(^{86}\) participates in hydrophobic stacking. Glu\(^{101}\) is in the a-position of the leucine heptad repeat (where positions 1–7 are denoted a-g, and each leucine is in the central d-position). Thus, Glu\(^{101}\) is in the position predicted for the hydrogen bond donor (i.e. sandwiched between Leu\(^{87}\) and Leu\(^{84}\), which are one helical turn above and below Glu\(^{101}\)). These key residues, namely Tyr\(^{86}\) and Glu\(^{101}\), are conserved in related transporters from archaea to humans; they are therefore likely to support oligomeric assembly in transporter orthologs and possibly other proteins with multiple transmembrane segments.

\( \gamma \)-Aminobutyric acid (GABA)\(^1\)-mediated inhibitory synaptic transmission is terminated by clearance of GABA from the synaptic cleft by high affinity uptake proteins. The four known GABA transporters (GAT1, BGT1 (betaine/GABA transporter), GAT2, and GAT3) belong to the neurotransmitter:sodium symporter (NSS) (1) family, which also includes carriers for dopamine, serotonin, glycine, etc. (2). All members of the family share sequence similarity, membrane topology, and common functional features (e.g. ion channel-like properties, sodium, and chloride) as transported cosubstrates and bidirectional transport of substrates. The interest in transporters of this family is motivated in part by their clinical relevance; they are targets for drugs of abuse (e.g. cocaine, ecstasy, and metamphetamine) and for therapeutic agents including antidepressants (amitryptiline, desipramine, citalopram, and paroxetine) and anticonvulsants (tiagabine), etc. (reviewed in Ref. 3).

With the notable exception of the glycine transporter-1 (4), all members of the neurotransmitter transporter family that have so far been examined form oligomers (reviewed in Ref. 5). This is also true for GAT1, for which oligomers have been visualized in membranes of living cells by fluorescence resonance energy transfer (FRET) microscopy (6). The TM2 of GAT1 contains a leucine heptad repeat that seems to supports oligomer formation (7). The leucine heptad repeat in TM2 is a highly conserved feature throughout the NSS family. Consistent with this finding, TM2-mediated dimer formation was also suggested for dopamine transporter (8).

By analogy with leucine zippers in soluble proteins, the stretch of leucine residues in the TM2 of GAT1 lines one side of a hypothetical \( \alpha \)-helix. If this leucine heptad repeat is to serve as an interface between two transporter molecules, there must be additional forces that stabilize this arrangement. In aqueous solution, the hydrophobic leucine (or isoleucine) side chains are tightly packed to minimize the solvent exposed surface. It is, however, difficult to conceive how the hydrophobic environment in the membrane provides the force to drive the association of leucine residues. In fact, synthetic polyleucine segments show little propensity for self-association in the membrane (9, 10). Nevertheless, in phospholamban and in the erythropoietin receptor (11, 12), leucine and isoleucine-based packing interactions do occur between transmembrane domains. Similarly, artificial leucine-rich transmembrane segments can be forced to self-associate, provided that the interaction is stabilized by a polar hydrogen bond-donating residue (i.e. Ser, Thr, Gln, Glu, and Asp in the a-position sandwiched between two leucine residues in the d-positions of the heptad repeats) (9, 13). Based on these findings, we surmised that a polar residue must be essential to stabilize the homophilic interaction of TM2 segments in the GAT1 oligomer if the interaction is supported by packing of the leucine heptad repeat. We therefore focused on the polar or ionizable residues in TM2 of GAT1: Tyr\(^{86}\), Thr\(^{89}\), Glu\(^{101}\), Cys\(^{102}\), and Ser\(^{103}\). Our experiments identified the two residues Tyr\(^{86}\) and Glu\(^{101}\), both invariably present in the corresponding positions in all neurotransmitter:sodium symporters, as key players in TM2-mediated intramembrane interaction.

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tion. However, in Tyr⁶⁶ the hydrogen bond-donating hydroxyl group is dispensable, and it is the aromatic ring (and hence a hydrophobic interaction) that is (appears) essential for stabilizing the oligomer. In contrast, both, the size of the side chain and the hydroxyl group of Glu¹⁰¹ seem to be essential for supporting oligomer formation. Glu¹⁰¹ is in the α-position of the heptad repeat, sandwiched between Leu⁹⁷ and Leu¹⁰⁴, which, in a helical arrangement, are one turn above and below Glu¹⁰¹. Thus, Glu¹⁰¹ fulfills the criteria of the hydrogen bond donor capable of stabilizing the interhelical contact.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs and Mutagenesis—Plasmid DNA constructs carry GAT1, CFP-GAT1, YFP-GAT1, CFP-GATΔ37, CFP-GATΔ37, and CFP-GAT1-L97A were described elsewhere (7, 14). Mutated versions of GAT1, carrying single amino acid substitutions (Y86A, Y86F, T89A, T89F, G94A, E101A, E101D, E101Q, C102A, and S103A) were created by PCR. For amplification of DNA fragments encompassing nucleotides 1–1032 of mutant GAT1 DNA sequences, we used the pairs of primers listed in Table I.

The HindIII/Acl fragments excised from PCR products were cloned into a HindIII/Acl-cliged CFP-GAT1-L97A construct. At this step, the L97A mutation in the former was eliminated, and the desired ones were introduced. The derived transporters or truncated versions (amino acids 1–110) were cloned in frame with F1[1] and F2[2]. The derived transporters or truncated versions (amino acids 1–110) were cloned in frames with F1[1] and F2[2]. The derived transporters or truncated versions (amino acids 1–110) were cloned in frames with F1[1] and F2[2]. The derived transporters or truncated versions (amino acids 1–110) were cloned in frames with F1[1] and F2[2]. The derived transporters or truncated versions (amino acids 1–110) were cloned in frames with F1[1] and F2[2]. The derived transporters or truncated versions (amino acids 1–110) were cloned in frames with F1[1] and F2[2].

**Fluorescence Microscopy—**Fluorescence microscopy was performed using a Zeiss Axiosvert 200M inverted epifluorescence microscope equipped with a CoolSNAP fx cooled CCD camera (Photometrics, Roper Scientific, Tucson, AZ). The fluorescence filters were purchased from Chroma (Chroma Technology Corp., Brattleboro, VT; CFP filter set: excitation 436 nm, dichroic 455 nm, emission 480 nm; YFP filter set: excitation 500 nm, dichroic 515 nm, emission 535 nm; FRET filter set: excitation 436 nm, dichroic 455 nm, emission 535 nm). Coverslips with attached cells were mounted in the microscope chamber and put on the microscope stage. Images of cells with CFP- or YFP-tagged transporters were acquired through corresponding filter channles. For donor photobleaching, FRET cells expressing either CFP-tagged or CFP- and YFP-tagged transporters were continuously illuminated with a 100-watt mercury lamp and the CFP filter set for 1 min, a time interval sufficient to bleach the donor to an extent of less than 20% (with acquisition of one image every 3 s). Regions of interest were selected, and fluorescence emission intensities were quantified. The resulting fluorescence decay curves were fitted to the equation for a single exponential decay approaching a constant value: fluorescence intensity = A₀e⁻ᵏᵗ + offset, where A₀ denotes the starting value, offset is the final fluorescent signal, and K is the decay constant. The photobleaching lifetime constant τ is defined as 1/K. To measure donor recovery after acceptor photobleaching (DRA), we acquired a donor (CFP) image before (Ib) and after (Ia) photobleaching using the YFP setting for 90 s (excitation 500 nm, dichroic mirror 525 nm, emission 535 nm; CFP filter set: excitation 436 nm, dichroic 455 nm, emission 535 nm). Flourescence images were acquired and analyzed using the MetaMorph and MetaFluor of MetaSeries software package (release 4.8, Universal Imaging Corp., Downingtown, PA). For statistical comparisons of time constants of fluorescence efficiencies, ANOVA followed by Dunnett’s or Tukey’s test was used.

**β-Lactamase Protein Fragment Complementation Assay—**For the β-lactamase protein fragment complementation assay, we used plasmids pβDNA5.1-Zeo/F1[1]m182T and pCDNA3.1-Zeo/F2[2] to express proteins fused to β-lactamase fragments F1[1] and F2[2] (17). Constructs of wild type and mutant GAT1 were fused by PCR cloning. Cells were transfected with the indicated combinations of plasmids using the calcium phosphate precipitation method. After 24 h, cells were detached mechanically, lysed by a freeze-thaw cycle, and homogenized in phosphate-buffered saline. Membranes were sedimented by centrifugation at 50,000 × g for 20 min, and pellets were resuspended in phosphate-buffered saline. The membrane suspension (20-μl aliquot containing 50–100 μg of membrane protein) was incubated in the presence of 100 μM nitrocefin in phosphate-buffered saline in a total volume of 200 μl; the incubation times were as indicated for the indicated periods at 37 °C. Absorption at 492 nm was measured using an automated plate reader. The comparisons were performed between the activities measured within linear range, before onset of substrate (nitrocefin) depletion. The results were analyzed by one-way ANOVA followed by Dunnett’s test.

**Molecular Graphics—**The Swiss-PDBViewer was used to generate the molecular model of TM2 dimer. The sequence of GAT1 TM2 (AFLIPYFLTLIFFAGVYFLLECS) was fitted onto the α-carbon backbone of the two GCN-4 (2ZTA) leucine zipper helices. The improbable side chain conformations were excluded by selection of allowed conformers following energy minimization of the whole structure by Swiss-PDBViewer implementation of GROMOS99. The derived model was rendered by POV-Ray 3.0.

**RESULTS**

**Site-directed Mutagenesis—**The hydrophobic environment of the membrane does not provide any driving force for the assembly or stabilization of a leucine zipper (9, 10). Thus, the amino acid residues of TM2 were examined for their ability to form hydrogen bonds and selected by sequence comparison between the members of the NSS family. For lack of structural information, we considered the TM2 region as the sequence between Ala⁶¹ and Leu¹⁰⁴ (Fig. 1A); the leucine heptad repeat comprises residues Leu⁶³–Leu¹⁰⁴. We identified residues tyrosine 86 (Tyr⁶⁶), threonine 89 (Thr⁹⁰), glutamate 101 (Glu¹⁰¹), cysteine 102 (Cys¹⁰²), and serine 103 (Ser¹⁰³) as the candidates for site-directed mutagenesis (marked by the arrows in Fig. 1A).

**Table I**

| Primer         | Sequence                                      |
|----------------|-----------------------------------------------|
| Y86A-fw       | 5'-ctgctattcactgctttctg-3'                    |
| Y86A-rr       | 5'-ggatcggaggaagctggtta-3'                   |
| Y86F-fw       | 5'-ctgctattcactgctttctg-3'                    |
| Y86F-rr       | 5'-ggatcggaggaagctggtta-3'                   |
| T89A-fw       | 5'-ctattttgcggctc-3'                          |
| T89A-rr       | 5'-gttattttgcggctc-3'                          |
| T89F-fw       | 5'-ctattttgcggctc-3'                          |
| T89F-rr       | 5'-gttattttgcggctc-3'                          |
| E101A-fw      | 5'-ctttctttcttggtgctg-3'                      |
| E101A-rr      | 5'-ctttctttcttggtgctg-3'                      |
| E101D-fw      | 5'-ctttctttcttggtgctg-3'                      |
| E101D-rr      | 5'-ctttctttcttggtgctg-3'                      |
| C102A-fw      | 5'-cctttgagggctcctgg-3'                       |
| C102A-rr      | 5'-cctttgagggctcctgg-3'                       |
| S103A-fw      | 5'-ctttggctgagggct-3'                         |
| S103A-rr      | 5'-ctttggctgagggct-3'                         |
mains of polytopic proteins. Tyr^{86} and Glu^{101} are conserved in all members of the family including bacterial and archaeal transporters (three examples shown in Fig. 1A). This suggests similar or related functional roles in different transporters. Ser^{103} is present in close relatives of GAT1, namely glycine and proline transporters. The equivalent positions in TM2 of more distantly related transporters are predominantly occupied by alanine. Thr^{89} and Cys^{102} are present exclusively in GAT1. Thus, we searched for the hydrogen donor that may support oligomer formation by generating the following point-mutated versions of GAT1 (see also Fig. 1A); the Tyr^{86} residue was mutated to alanine (the smallest side chain, incapable of forming hydrogen bonds) and phenylalanine (lacking only the hydroxyl group when compared with tyrosine). Thr^{89} was substituted by alanine and phenylalanine (present in analogous positions of dopamine transporter, norepinephrin transporter, GAT2, GAT3, etc.). Glu^{101} was changed to alanine and aspartate (a charged residue one -CH_{2}- group shorter than glutamate) and glutamine (devoid of charge but same size as glutamate). Cys^{102} and Ser^{103} were substituted by alanine residues.

Transport of GABA by GAT1 Mutants—Cellular uptake assays showed that the mutated transporters were capable of transporting GABA with a high rate and that transport was blocked by the competitive inhibitor tiagabine. By definition, cellular uptake demonstrates that the transporters are expressed on the cell surface. When assayed at a low fixed concentration of[^3]H[GABA], the transport velocities of the mutants Y86F, C102A, and S103A were comparable with that of the fluorescently tagged wild type transporter (Fig. 1B). Accordingly, in saturation experiments, these mutants had $K_m$ values that were indistinguishable from those of wild type GAT1 (Table II). In contrast, substitutions of Thr^{89} by alanine and by phenylalanine progressively reduced the affinity for substrate (see Fig. 2A and Table II). The $K_m$ reflects the overall process of substrate transport (i.e. binding of substrate and the cosubstrates Na$^+$ and Cl$^-$, their translocation, and intracellular release). Because there is no suitable radioligand for GAT1 binding assays, it is not possible to determine substrate binding affinity directly. We probed the binding pocket of GAT1 indirectly by determining the IC$_{50}$ of tiagabine for GAT1 at a very low substrate concentration. Under these conditions, differences in substrate affinity are irrelevant, and the IC$_{50}$ approaches the $K_i$ value of the inhibitor. As can be seen from Fig. 2B, mutation of Thr^{89} did not impair binding of the inhibitor regardless of whether threonine was substituted by alanine or by the bulky phenylalanine (see also Table II). These findings argue against a role of Thr^{89} in initial inhibitor recognition and binding but imply a role in translocation of the substrate.

Y86A-GAT1 transported substrate with a significantly lower rate when compared with wild type GAT1 (second bar from left in Fig. 1B). In experiments performed with E101A, E101D, and E101Q-GAT1, the transport of[^3]H[GABA] could barely be de-

![Fig. 1](image-url)
GAT1 is unlikely to be due to misfolding. is only modestly different from that of the wild type transfection that binds and translocates substrate with an affinity that out the possibility that the latter two mutants are misfolded, it E101A-GAT1 and E101Q-GAT1. Thus, while we cannot rule transfected cells and measured vesicular [3H]GABA uptake. As transporters. To assess the conformational integrity of the mu- cells. This may have resulted from misfolding of these mutated GAT1, E101A-GAT1, E101D-GAT1, and E101Q-GAT1; these fluorescence recorded over the plasma membrane with Y86A-CFP-GAT1 (and the other mutants), there was little, if any, transfected HEK-293 cells (Fig. 1). Substitutions were expressed at the surface of heterologously expressed YFP-tagged GAT1, the transporter was found at the plasmic reticulum (Fig. 4). In contrast, in the cell that only expressed YFP-tagged GAT1, the transporter was found at the plasma membrane (top right corner of Fig. 4B). We have also verified this conclusion by using DRAP-FRET microscopy. FRET microscopy relies on the energy transfer that occurs if donor and acceptor fluorophore are in close vicinity; in the pair CFP (donor) and YFP (acceptor), this Foerster distance is in the range of 50 Å (Fig. 5A) (21) and thus requires the formation of an oligomer. Here we used approaches that rely either on the protection of the donor against bleaching in the presence of acceptor (donor photobleaching FRET) or on the increase in donor emission after acceptor bleaching (donor recovery after acceptor photobleaching). Donor photobleaching FRET did not reveal any difference in the lifetime (τ) of the fluorescent decay upon bleaching the mutant transporters expressed in the plasma membrane (Fig. 5, B and C). Thus, the mutations of residues Thr89, Cys102, and Ser103 as well as substitution of Tyr86 to phenylalanine did not impede the interaction with wild type GAT1. In other words, the hydrophilic side chains of these residues are unlikely to participate in forming the oligomeric interface.

We have also verified this conclusion by using DRAP-FRET microscopy, an independent approach that relies on bleaching of the acceptor rather than the donor. The data set depicted in Fig. 5D shows a representative experiment for GAT1-D37 (top row) and for GAT1–Y86A (bottom row), where the fluorescence intensity is shown in pseudocolors. We used GAT1–D37 to gauge the oligomeric status of those mutants that are retained within the cell; because GAT1–D37 is trapped in the endoplasmic reticulum, it serves as a good control and obviates a comparison over different membrane compartments. The increase in fluorescence that can be seen for GAT1–D37 (cf. CFP images

### Table II

| TM2 mutation | $K_m$ (whole cell)$^a$ | $K_m$ (vesicular uptake)$^b$ | $IC_{50}$ for tagamine-induced inhibition thereof |
|--------------|----------------------|-----------------------------|-----------------------------------------------|
| WT GAT1      | $4.2 \pm 1.0$       | $4.1 \pm 0.6^c$            | $408 \pm 26$                                  |
| Y86A         | $9.2 \pm 3.0$       | $233 \pm 63$               | $244 \pm 91$                                  |
| Y86F         | $2.0 \pm 0.4$       | $57.8 \pm 15.0$            |                                               |
| T89A         | $17.6 \pm 2.3$      |                             |                                               |
| T89F         | $1.6 \pm 0.5$       | $1.5 \pm 1.0$              |                                               |
| E101A        | NM$^c$              |                             |                                               |
| E101D        | $3.0 \pm 0.5$       |                             |                                               |
| E101Q        | $4.7 \pm 0.9$       |                             |                                               |
| C102A        | $3.0 \pm 0.5$       |                             |                                               |
| S103A        | $4.7 \pm 0.9$       |                             |                                               |

$^a$ $K_m$ values were calculated from uptake assays in vesicular membrane preparations ($n = 4$).

$^b$ $K_m$ values were calculated from whole cell uptake experiments (performed as shown in Fig. 1A); the differences between wild type GAT1, T89A-GAT1, and T89F are statistically significant judged by one-way ANOVA followed by Tukey’s multiple comparison test ($n = 4$–6).

$^c$ $K_m$ was determined for intracellularly retained GAT1–D37.

$^d$ NM, not measurable.
before and after bleaching in the top row is not readily detectable for the Y86A mutant (bottom row). Fig. 5E summarizes the results from DRAP-FRET microscopy (Fig. 5, D and E), which allows for quantitative comparisons of energy transfer efficiencies; it is evident that the FRET efficiency is comparable for wild type GAT1 and Y86F (white columns) and that they are similar in magnitude to the C-terminally truncated GAT1-D37. In contrast, the FRET efficiency is reduced by about 50% for the mutants Y86A-GAT1 and E101A/D/Q-GAT1 regardless of whether they are allowed to interact with GAT1-D37 or with themselves (Fig. 5E). Consistent with these DRAP measurements, donor photobleaching experiments showed reduced photobleaching lifetimes with the representative pairs of the intracellularly retained mutants: Y86A versus GAT1 and E101D versus GAT1 (black columns in Fig. 5C). Taken together, these results indicate that the phenyl ring of Tyr86 and the side chain of Glu101 are crucial for formation of the GAT1 oligomer. Two different FRET-based methods that monitor protein-protein interaction lead to the same conclusions. Furthermore, FRET microscopy provides evidence for a homophilic TM2-driven oligomerization. The mutated transporters fused to CFP and YFP comprise the FRET pairs with FRET efficiencies roughly equal to those of pairs with the acceptor molecule being GAT1-D37. Mutation of a single residue (Tyr86 or Glu101) in a TM2 of one of the interacting molecules was sufficient to impair this interaction. Mutation of the respective residue in both interacting subunits did not increase the difference in FRET efficiency, compared with the GAT1-D37 control. This suggests that the mutated residues form contacts in a mutually dependent fashion, constituting a homophilic (TM2-TM2) interaction domain.
A decrease in FRET efficiencies may arise from an increase in the distance between the fluorophores, a change in the relative orientation of the fluorophore dipoles, or a decline in the steady state number of oligomeric complexes. The data in Fig. 4 also suggest that the mutations reduced the level of oligomeric complexes. We obtained independent evidence for this interpretation by employing a third approach, namely a \( \beta \)-lactamase fragment complementation assay (17). This method of proving interaction between two proteins relies on reconstitution of enzyme activity (cleavage of nitrocefin) upon interaction of two proteins that are tagged by two split parts of the enzyme \( \beta \)-lactamase. Membranes were prepared from HEK-293 cells that co-expressed the indicated pairs of CFP- or YFP-labeled GAT1 mutant constructs were co-expressed in HEK-293 cells at a 1:1 ratio of plasmids encoding donor and acceptor. Donor photobleaching (B, C) and DRAP (D, E) FRET microscopy experiments were performed 24–28 h after transfection. A, scheme illustrating the principle of FRET. B, photobleaching of the donor (CFP) was achieved by illuminating the cell at the center of the visual field with a mercury arc lamp (Zeiss HBO; 100-watt intensity) for 60 s. Images were captured every 3 s, digitized, and stored. Decay of intensity (in the regions of interest) was quantified in successive images to generate the decay curves and calculate photobleaching lifetimes, \( \tau \). C, a summary of \( \tau \) values determined as shown in B for the indicated mutants. The \( \tau \) values for all mutants that were expressed at the cell surface (white bars) were not significantly different from those of the wild type GAT1; for mutants that were retained within the cell (represented by black bars), there was a significant difference between the ability of self-association of the control protein GAT1-D37 and its association with the Y86A and the E101 mutant (*, \( p < 0.05 \); **, \( p < 0.01 \); significance judged by one-way ANOVA followed by Dunnett’s test; values represent mean \( \pm \) S.E. from 15–22 individual curves). D, to measure DRAP, a donor (CFP) image was acquired before (left image) and after photobleaching (middle image) using the YFP setting for 90 s (excitation 500 nm, dichroic mirror 525 nm, and emission 535 nm); shown are pseudocolor images for the control protein (GAT1-D37) and a representative, intracellularly retained mutant, Y86A-GAT1; the YFP image prior to bleaching is also shown to document the presence of acceptor within the same cell. E, summary of FRET efficiencies obtained from DRAP experiments carried out as in C. The FRET efficiency (mean \( \pm \) S.E.; \( n = 11–29 \), calculated as described under “Experimental Procedures”) determined for GAT1-D37 versus GAT1-D37 was significantly different from that determined for all other intracellularly retained mutants (represented by black bars; \( p < 0.05 \); one-way ANOVA followed by Dunnett’s test) but comparable with the transporters expressed at the cell surface (indicated by white bars) (i.e. self-association of wild type GAT1 (abbreviated as \( \text{CrG} \) versus \( \text{YrG} \)) and Y86F versus wild type GAT1 (abbreviated as Y86F versus YrG)).
TM2 Controls GABA Transporter-1 Oligomerization

**DISCUSSION**

For many membrane proteins, a correct quaternary assembly is a prerequisite for efficient exit from the endoplasmic reticulum and subsequent delivery to the plasma membrane. Prominent examples are GABA<sub>B</sub> receptors and ATP-sensitive K<sup>+</sup> channels (23, 24). The mechanisms that drive the oligomeric assembly of proteins with multiple transmembrane segments are, in many instances, poorly understood. Neurotransmitter transporters are of particular interest, because they contain multiple motifs known to support the association of transmembrane segments: a glycoporin-like motif in TM6 (9), a leucine heptad repeat in TM2 (7), and additional interaction motifs in TM4 (22) and TM11–12 (18).

**β-Lactamase Fragment Complementation by Isolated Transmembrane Domains**—It is evident that the point mutations in TM2 (at positions Tyr<sup>86</sup> and of Glu<sup>101</sup>) neither fully eliminated resonance energy transfer (Fig. 5E) nor completely abolished β-lactamase complementation (Fig. 6B). These observations suggest that there are additional contact sites in the oligomeric interface (e.g. TM4 (22) and TM11/12 (18)). To study the homophilic interaction between TM2 segments, we generated constructs that comprised TM1, TM2, and either the β-lactamase fragment F[1] or F[2] (schematic rendering in Fig. 5E). TM1, which functions as a (noncleavable) signal sequence, was included to ascertain the correct orientation of the polypeptide. These short GAT1-derived constructs carrying mutations Y86A/F and a representative of the glutamate 101 (E101D) were co-expressed as F[1]- and as F[2]-tagged versions. The activities of these pairs of fusion constructs were compared with those of the wild type pair; Y86F was again indistinguishable from wild type, whereas Y86A and E101D had lower activities (Fig. 6D). A comparison of Fig. 6, C and D, shows that the differences in association of Y86A and E101D mutants was more pronounced when presented in the context of the short TM1-TM2 construct.

In the present work, we focused on TM2 of GAT1 and searched specifically for the polar residue that may be required to drive the association of leucine heptad repeats in the membrane (9). Our observations exclude Tyr<sup>86</sup>, Thr<sup>89</sup>, Cys<sup>102</sup>, and Ser<sup>103</sup> as hydrogen bond donors required for stabilization of the oligomer. In contrast, Glu<sup>101</sup> meets several essential criteria. (i) Glu<sup>101</sup> occupies the a-position in the leucine heptad repeat (i.e. it is sandwiched between Leu<sup>97</sup> and Leu<sup>104</sup>, which are one helical turn above and below Glu<sup>101</sup>) and thus occupy the d-position in their respective heptad repeat (where positions 1–7 are denoted a–g) (9); (ii) replacement of Glu<sup>101</sup> by mutation greatly reduced the ability of the resulting mutants to support oligomerization: cell surface delivery, resonance energy transfer, and β-lactamase complementation; and (iii) the loss of oligomerization cannot be attributed to a major misfolding. Although E101A and E101Q failed to translocate substrate, the activity of E101D-GAT1 was readily measurable and its affinity for substrate was within the range observed with the wild type transporter. This unequivocally shows that E101D-GAT1 adopts an active conformation. We therefore rule out the possibility that the E101D mutant is retained in the endoplasmic reticulum due to misfolding at the level of tertiary structure. Loss of function in Glu<sup>101</sup> to misfolding. Loss of function in E101D-GAT1 to misfolding at the level of quaternary structure. Loss of function in Glu<sup>101</sup> to misfolding. Loss of function in E101D-GAT1 to misfolding at the level of quaternary structure.
The side chain of Glu\textsuperscript{101} is a likely hydrogen bonding entity that may provide the driving force for TM2 dimerization in the hydrophobic environment of the membrane. In accordance with our hypothesis, the protonated E101 may donate its hydrogen for a hydrogen bond with a heteroatom of the Glu\textsuperscript{101} of a juxtaposed helix. The acidic residues in TM spans have been shown to cause strong hydrogen bond-based association within the lipid bilayer of ErbB2 receptor and cystic fibrosis transmembrane conductance regulator Cl\textsuperscript{−} channel (30, 31). Glu\textsuperscript{101} is flanked along its helical side by two leucine residues, Leu\textsuperscript{97} and Leu\textsuperscript{104}, which may protect Glu\textsuperscript{101} from the aqueous cytosolic milieu (Fig. 7B). Mutation of Leu\textsuperscript{97} to alanine destabilizes the dimer, whereas mutation of both Leu\textsuperscript{97} and Leu\textsuperscript{104} completely abolishes TM2-TM2 dimerization (7). This may be explained by a loss of hydrophobic contacts between leucine residues, which allows for access of water and thus results in deprotonation of the ω-carboxyl group of Glu\textsuperscript{101} (Fig. 7C). Our interpretation is that the hydrogen bonding of Glu\textsuperscript{101} occurs in the context of hydrophobic side chains of its neighbors and is strictly directional; mutations to Asp or Gln are detrimental to hydrogen bond formation. The E101Q mutant may not form the equivalent hydrogen bond. Likewise, the transporter with E101D substitution is unable to form the hydrogen bond and may be more easily accessible to water from the cytosol, leading to deprotonation and subsequent mutual helix repulsion. In serotonin transporter, the substitution of the homologous Glu\textsuperscript{136} by cysteine renders this residue sensitive to modification by a hydrophilic sulphydryl-reactive compound (32). However, this observation may be compatible with our model if replacement by cysteine creates a water-accessible cavity; as mentioned earlier, in the hydrophobic environment of the lipid bilayer, the stable association of leucine heptad repeats requires a hydrogen bond donor in the α-position, which is sandwiched between two leucines that are one helical turn below and above in the d-position of the repeat (9, 13). If the side chain of cysteine is too short to stabilize the leucine zipper, the driving force that keeps the leucine side chains aligned may be reduced, and the cysteine at the bottom of TM2 is likely to become accessible to water.

These conjectures are well supported by a statistical assessment of Adamian and Liang (33); their survey scored the probabilities for residues in TM segments to interact with each other within the membrane. In agreement with our results, Adamian and Liang reported that a Glu-Glu pair is frequently found in contact regions within the membrane, whereas Asp-Asp and Gln-Gln pairs are very unlikely to occur (33). Thus, the interaction between two Glu residues are expected to support a strong intramembrane association of two transporter molecules. Recent work suggests that the sequence context strongly modulates transmembrane hydrogen bonding interactions (34). Accordingly, the specificity of hydrogen bonding between GAT1 TM2 helices could be provided by intrinsic properties of the Glu\textsuperscript{101} side chain and by the surrounding residues, which would form a hydrophobic cage.

Our model is strongly supported by the evidence obtained from the β-lactamase complementation experiments performed with full-length transporters and fragments thereof. The association patterns are similar, and, in fact, the effects of the mutations are more pronounced when assessed in the isolated TM1-TM2 segments. This approach demonstrates that the putative transmembrane domain interface involving Tyr\textsuperscript{86} and Glu\textsuperscript{101} is present in full-length GAT1 as well as in its severed TM1-TM2 segment. Thus, the available evidence supports a model where the interface depicted in Fig. 7A may play a role in helical packing; this is apparently true even when the constraints are relieved that are projected on TM1-TM2 in the con-
text of an intact transporter and despite the concomitant increase in the conformational flexibility of isolated TM segments.

It is worth pointing out that a cysteine scanning mutagenesis of TM2 also identified the homologous residues (i.e. Tyr^{121} and Glu^{136}) in serotonin transporter as essential for cell surface expression and for transporter activity (32). Previously, the residue Glu^{101} of GAT1 was proposed to act as the counterion for binding of the cosubstrate Na\(^+\) (35). This interpretation fails to account for the reduction in cellular \([^{3}H]GABA\) uptake if we assume that aspartate can still provide a counterion for Na\(^+\) binding. However, the reduction in cellular uptake and in transporter-associated currents is readily explained by the impaired cell surface expression of the Glu^{101} mutants that we documented in the present study. Accordingly, we consider our model more plausible, where the carboxyl group of Glu^{101} is shielded from the aqueous phase and is thus unavailable for binding of Na\(^+\).

Our results rather emphasize the central role of this polar residue Glu^{101} in driving GAT1 oligomerization by intramembrane contacts via adjacent TM2 helices. In our opinion, this association is supported by a meshwork of hydrophobic residues (leucine heptad repeat and Tyr^{386}) dispersed along the helical interface. It is worth pointing out that the residues involved are conserved among the homologues of GAT1 from archaea to humans (Fig. 1A). In addition, the leucine zipper-like motifs are present in other transport proteins (e.g. GLUT4, Sglt1, etc.). Thus, the mode of transmembrane association that we propose for GABA transporter molecules may also govern the oligomerization of other members of the transporter family. Finally, our work proves that the concepts developed for artificial single transmembrane segments (9, 10, 34) are applicable for proteins with a more complex architecture (i.e. several transmembrane α-helices). Related combinations of hydrophobic and polar interactions are likely to support oligomer formation in other proteins with multiple transmembrane spans.

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REFERENCES
1. Busch, W., and Snier, M. H., Jr. (2002) Crit. Rev. Biochem. Mol. Biol. 37, 287–337
2. Torres, G. E., Gainetdinov, R. R., and Caron, M. G. (2003) Nat. Rev. Neurosci. 4, 13–25
3. Zahniser, N. R., and Doolen, S. (2001) Pharmacol. Ther. 92, 21–55
4. Horiuichi, M., Nica, A., Gomez, J., Aschrafi, A., Schmalzing, G., and Betz, H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1448–1453
5. Sitte, H. H., and Freissmuth, M. (2003) Eur. J. Pharmacol. 479, 229–236
6. Schmid, J. A., Scholze, P., Kudlacek, O., Freissmuth, M., Singer, E. A., and Sitte, H. H. (2001) J. Biol. Chem. 276, 3805–3810
7. Solzhe, P., Freissmuth, M., and Sitte, H. H. (2002) J. Biol. Chem. 277, 43682–43690
8. Torres, G. E., Carneiro, A., Seumann, K., Fiorentini, C., Sweeney, A., Yao, W. D., and Caron, M. G. (2003) J. Biol. Chem. 278, 2731–2739
9. Zhou, F. X., Meri, H. J., Brunger, A. T., and Engelman, D. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2250–2255
10. Gurekza, R., and Langosch, D. (2001) J. Biol. Chem. 276, 45580–45587
11. Zimmerman, H. K., Kobayashi, Y. M., Atruy, J. M., and Jones, L. R. (1996) J. Biol. Chem. 271, 5941–5946
12. Seubert, N., Riker, Y., Staer, K., Kubatzyk, K. F., Moucadel, V., Krishnakumar, S., Smith, S. O., and Constantinescu, S. N. (2003) Mol. Cell. 12, 1239–1250
13. Dawson, J. P., Weinger, S., and Engelman, D. M. (2002) J. Mol. Biol. 316, 799–805
14. Farhan, H., Korkov, V. M., Paulitschke, V., Dorostikar, M. M., Scholze, P., Kudlacek, O., Freissmuth, M., and Sitte, H. H. (2004) J. Biol. Chem. 279, 28552–28563
15. Okayama, H., and Berg, P. (1985) Mol. Cell. Biol. 5, 1138–1142
16. Miyawaki, A., and Tsien, R. Y. (2000) Methods Enzymol. 327, 472–500
17. Galan, J. A., Primeau, M., Trudeau, L. E., and Michnick, S. W. (2002) Nat. Biotechnol. 20, 619–622
18. Just, H., Sitte, H. H., Schmol, J. A., Freissmuth, M., and Kudlacek, O. (2004) J. Biol. Chem. 279, 45045–45048
19. Hastrup, H., Karlin, A., Javitch, A. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10055–10060
20. Hsin, M. K., Robertson, D., and Blakely, R. D. (2003) J. Neurosci. 23, 4470–4478
21. Schmid, J. A., Sitte, H. H. (2003) Curr. Opin. Oncol. 15, 55–64
22. Hastrup, H., Sen, N., Javitch, A. J. (2003) J. Biol. Chem. 278, 45045–45048
23. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2000) Neuron 27, 97–106
24. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) Neuron 22, 537–548
25. Schmid, J. A., and Sitte, H. H. (2003) Curr. Opin. Oncol. 15, 55–64
26. Chelli, R., Gervasio, F. L., Proacci, P., Schettino, V. (2002) J. Am. Chem. Soc. 124, 6133–6143
27. Agelli, A., Bannister, M. L., Bell, M., Boden, N., Findlay, J. B., Hunter, M., Knowles, P. F., and Yang, J. C. (1998) Biochemistry 37, 8121–8131
28. O'Shea, E. K., Klemm, J. D., Kim, P. S., and Alber, T. (1991) Science 254, 539–544
29. Cordes, F. S., Bright, J. N., and Sansom, M. S. (2002) J. Mol. Biol. 323, 951–960
30. Schmid, J. A., Sitte, H. H. (2003) Curr. Opin. Oncol. 15, 55–64
31. Therien, A. G., Grant, E. F., and Deber, C. M. (2001) Nat. Struct. Biol. 8, 597–601
32. Sitte, H. H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10055–10060
33. Adamian, L., and Liang, J. (2001) Mol. Biol. Lett. 311, 891–907
34. Dawson, J. P., Melnyk, R. A., Deber, C. M., and Engelman, D. M. (2003) J. Mol. Biol. 331, 255–262
35. Keshet, G. I., Bendahan, A., Su, H., Mager, S., Lester, H. A., and Kanner, B. I. (1999) FEBS Lett. 436, 39–42