Proximity of Transmembrane Domains 1 and 3 of the γ-Aminobutyric Acid Transporter GAT-1 Inferred from Paired Cysteine Mutagenesis*

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GAT-1 is a sodium- and chloride-dependent γ-aminobutyric acid transporter and is the first identified member of a family of transporters that maintain low synaptic neurotransmitter levels and thereby enable efficient synaptic transmission. Because transmembrane domains 1 and 3 contain amino acid residues important for transport activity, we hypothesized that these domains may participate in the formation of the binding pocket of the transporter. Pairwise substitutions have been introduced in several predicted transmembrane domains and in the first extracellular loop of GAT-1. In the double mutant W68C/I143C, in which the cysteines were introduced at locations at the extracellular part of transmembrane domains 1 and 3, respectively, ~70% inhibition of transport was observed by cadmium with an IC50 of ~10 μM. This inhibition was not observed in the corresponding single mutants and also not in >10 other double mutants, except for V67C/I143C, where the half-maximal effect was obtained at ~50 μM. The inhibition by cadmium was only observed when the cysteine pairs were introduced in the same polypeptide. Our results suggest that transmembrane domains 1 and 3 come in close proximity within the transporter monomer.

The overall process of synaptic transmission is terminated by neurotransmitter transporters located in the plasma membranes of cells surrounding the synapse. Most neurotransmitters are removed from the synaptic cleft by sodium- and chloride-dependent transporters, which form a family that also includes (besides the transporters for γ-aminobutyric acid (GABA))3 those for serotonin, dopamine, norepinephrine, and glycine (for reviews, see Refs. 1 and 2). The GABA transporter GAT-1 (3, 4), the first identified member of this family, catalyzes electrogenic sodium:chloride:GABA cotransport with a stoichiometry of 2:1:1 (5–8). The role of chloride in this process is still under debate, because it has been proposed that, during sodium-dependent conversion of the leak mode to line the permeation pathway and appears to form a more extended structure than expected from a membrane-embedded α-helix (18, 19), contains several amino acid residues critical for function. They have been implicated in playing an important role in the interaction with the neurotransmitter (20, 21), in the determination of the apparent affinity for sodium (21, 22), and in the sodium-dependent conversion of the leak mode of the transporter into the coupled mode (21). These observations suggest that TMDs 1 and 3 may participate in the formation of the binding pocket in this family of transporters and therefore may be close in space. In this study, we have provided the first evidence for this idea.

EXPERIMENTAL PROCEDURES

Generation and Subcloning of Mutants—Mutations were made by site-directed mutagenesis of the wild-type GAT-1 in the vector pBlueScript SK(-) (Stratagene) using single-stranded uracil-containing DNA as described previously (23, 24). Briefly, the parent DNA was used to transform Escherichia coli CJ236 (dut, ung-) (Stratagene) to transform E. coli using helper phage R408. This yields the sense strand, and consequently mutagenic primers were designed to be antisense. Double cysteine mutants were prepared by subcloning mutants in TMD 3 into a construct containing mutants in TMD 1 using unique phospholipid loops with the amino and carboxyl termini located inside the cell, and strong experimental support for this model has been obtained using the serotonin transporter SERT (10).

GAT-1 has 15 endogenous cysteine residues of which three are located on extracellular loops. Studies on the related dopamine and serotonin transporters indicate that the cysteine residues, equivalent to their GAT counterparts at positions 164 and 173 (which are located in the second extracellular loop), form a disulfide bond (11, 12). This leaves cysteine 74, located on the first extracellular loop, as the only cysteine that reacts with impermeant methanethiosulfonate reagents. GABA transport by wild-type GAT-1 is only very modestly inhibited by the membrane-impermeant (2-trimethylammonium)ethyl)methanethiosulfonate (13), indicating that this position is not easily accessible. The reagent (2-aminoethyl)methanethiosulfonate has a definite membrane permeability and can react with cysteine 399, located on the intracellular loop connecting TMDs 8 and 9 (14). Attempts to create a functional cysteineless GAT-1 have been unsuccessful (14), and the same is true for DAT, another member of the SLC6 family (15).

Mutagenesis studies, of GAT-1 and SERT, in particular (but also of other members of the family), have identified a conserved tyrosine (tyrosine 140 and 176 in GAT-1 and SERT, respectively) in TMD 3 critical for neurotransmitter binding (16, 17). Two TMD 3 residues of SERT, corresponding to leucine 136 and isoleucine 143 of GAT-1, are located one turn of a putative α-helix below or above the critical tyrosine 176, respectively (17). The highly conserved TMD 1, which appears to line the permeation pathway and appears to form a more extended structure than expected from a membrane-embedded α-helix (18, 19), contains several amino acid residues critical for function. They have been implicated in playing an important role in the interaction with the neurotransmitter (20, 21), in the determination of the apparent affinity for sodium (21, 22), and in the sodium-dependent conversion of the leak mode of the transporter into the coupled mode (21). These observations suggest that TMDs 1 and 3 may participate in the formation of the binding pocket in this family of transporters and therefore may be close in space. In this study, we have provided the first evidence for this idea.

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‡ The abbreviations used are: GABA, γ-aminobutyric acid; TMD, transmembrane domain; CuPh, CuII(1,10-phenanthroline)2Cl; SERT, serotonin transporter; WT, wild-type.
The indicated concentrations of cadmium chloride in sodium solution were washed once with choline solution and preincubated with the indicated concentration of CuPh. The CuPh stock solution was prepared for each experiment by mixing 0.4 ml of 1.25 M 1,10-phenanthroline in water:ethanol (1:1) and 0.6 ml of 250 mM CuSO₄.

Inhibition by Cd²⁺—HeLa cells transfected with the indicated construct were washed once with choline solution and preincubated with the indicated concentration of Cd²⁺ (150 mM NaCl, 5 mM K⁺, pH 7.4, 0.5 mM MgSO₄, and 0.3 mM CaCl₂) and preincubated with the indicated concentration of Cd²⁺. The solution was aspirated, and the cells were assayed for transport in the presence of the same concentration of cadmium chloride.

RESULTS

Effects of Thiol Cross-linking and Cd²⁺ on Transport by W68C Double Mutants—The pioneering studies of Kaback and co-workers (27) to detect proximity relationships in lactose permease were based on creating, under oxidative conditions, a disulfide bond between two single cysteines, each located on a different TMD or loop. Extracellular loop 1, is the only extracellular loop between TMDs 1 and 3. If we could engineer a protease-sensitive site in this loop, it might be possible to detect cross-links between TMDs 1 and 3 by detection of the full-length transporter after cross-linking subsequent to proteolytic cleavage. However, this loop is almost completely conserved between the members of the SLC6 transporter family. Moreover, cysteine 74 located on this loop reacts poorly with (2-(trimethylammonium)ethyl)methanethiosulfonate (13), and therefore the protease would unlikely be able to cut at such an engineered site.

On the other hand, we have shown in the glutamate transporter GLT-1 that functional criteria, such as inhibition of transport by CuPh, which is apparently due to the fact that these transporters undergo extensive conformational changes during the translocation process, can be used as evidence for proximity (28). In fact, the recently published high resolution structure of a glutamate transporter homologue (29) confirmed the close proximity of a pair of cysteines, each located on a different re-entrant loop (28). Our assumption is that positions that are accessible to a sulfhydryl reagent are excellent candidates to be accessible to reactive oxygen species or divalent metal ions.

Based on the studies of SERT (17), in GAT-1, the candidate positions in TMD 3 are 136 and 143. In the external part of TMD 1, the positions, where an externally accessible cysteine can be introduced so that functionality is maintained, are 67, 68, and 70 (19). Unless stated otherwise, all of the studies described below were done in the background of C74A.

We started with the W68C/I143C double mutant in the background of C74A. Potent inhibition of the transport of this double mutant was observed by CuPh (Fig. 1). No such inhibition was observed by the I143C single mutant (data not shown) or by the W68V/I143C double mutant (Fig. 1). This indicates that the inhibition by CuPh observed in the double mutant is unlikely to be caused by the cross-linking of cysteine 143 with a previously buried endogenous cysteine, which became exposed because of the replacement of tryptophan 68. However, significant inhibition of transport by the single mutant W68C was observed with CuPh, and the same was true when, simultaneously, a mutation to another residue, such as valine (Fig. 1) or to alanine (data not shown) was introduced at position 143.

The concentration dependence of the inhibition by CuPh of the W68C/L136C double mutant by CuPh was similar to that observed in W68C alone (data not shown). We tried to reduce the inhibition by CuPh of transport by W68C by removing the endogenous cysteines of GAT-1 one at a time or in combinations in the background of W68C, yet the inhibition remained the same as in W68C alone. We also considered the possibility that the cysteine introduced at position 68 might be cross-linked to the same cysteine in another transporter monomer. However, no higher molecular weight species than the monomer was found after the CuPh treatment of W68C followed by surface biotinylation (data not shown).

Although the strongest inhibition of transport by CuPh was observed in the W68C/I143C double mutant, we looked for additional evidence that these two positions could be close in space and examined the ability of the W68C/I143C double mutant to form a high affinity Cd²⁺ binding site. This divalent cation interacts with cysteinyl side chains (30, 31), and the affinity of the interaction is dramatically increased when the Cd²⁺ can be coordinated by two cysteines (32).

Exposure of the single mutant W68C or the double mutants W68C/I143V or W68C/I143A to up to 500 µM Cd²⁺ had very little effect on [³H]GABA uptake (Fig. 2), and the same is true for the W68V/I143C double mutant (Fig. 2) and the single mutant I143C (data not shown, but see Fig. 5). In contrast to these controls, an inhibition of ~70% is observed on uptake by the W68C/I143C mutant, with a half-maximal effect at ~10 µM (Fig. 2). This inhibition is reversible when, after preincubation with Cd²⁺, the cells are washed with NaCl-containing medium supplemented with 0 mM EDTA (data not shown).

The inhibition by Cd²⁺ was only observed when the cysteine pairs were introduced in the same polypeptide (Fig. 3) but not when the single mutants were coexpressed. This suggests that the cysteines introduced at positions 68 and 143 come in close proximity within the transporter monomer but not at the interface of two transporter monomers. The specificity of the ability of the two cysteines introduced at positions 68 and 143...
to generate a Cd\(^{2+}\) binding site is illustrated by the fact that very little inhibition of \(^{3}H\)GABA transport was observed by concentrations as high as 500 \(\mu M\) Cd\(^{2+}\) in the double mutants W68C/L136C, G79C/I143C, and A81C/I143C (with position 79 located in extracellular loop 1 and position 81 at the extracellular end of TMD 2) (Fig. 3). The same was true for each of the single mutants W68C, L136C, and I143C, each in the wild-type background (here a cysteine is present at position 74; in contrast, the single mutants shown in the other figures were in the C74A background). Cd\(^{2+}\) sensitivity in the latter cysteine mutants would have been indicative of their ability to form a Cd\(^{2+}\) binding site with the cysteine at position 74 in the extracellular loop 1 (Fig. 3).

Effects of Cadmium Ions on V67C/I143C and F70C/I143C Double Mutants—Adjacent to tryptophan 68 is valine 67, and the V67C mutant also has significant transport activity (19). Cd\(^{2+}\) also inhibited uptake by the double mutant V67C/I143C up to a maximum extent of \(-70\%\) (Fig. 4), but its apparent affinity, with an IC\(_{50}\) of \(-50 \mu M\), was considerably lower than that observed with W68C/I143C (Fig. 2). No significant inhibition by Cd\(^{2+}\) was observed in the single mutants V67C (Fig. 4) or I143C (Fig. 5) or in the double mutants V67C/I143A (Fig. 4), V67S/I143C, or V67C/L136C (Fig. 5). Further evidence for the specificity of the formation of the Cd\(^{2+}\) binding site comes from the fact that uptake by V67C in the wild-type background (probing the ability of the cysteines at positions 70 and 143 to form such a site) also was not significantly inhibited by the divalent cation (Fig. 5). Again, as was the case for the pair W68C and I143C (Fig. 2), Cd\(^{2+}\) did not inhibit when the single mutants V67C and I143C were coexpressed (Fig. 5).

There was only a very modest inhibition by Cd\(^{2+}\) on transport by the F70C/I143C double mutant, which was only slightly increased as compared with that on the single mutant F70C and the double mutant F70C/I143V (Fig. 6). However, Cd\(^{2+}\) inhibition of transport in the double mutant F70C/I143A was almost the same as in F70C/I143C (Fig. 6). Thus it appears that the weak inhibition observed is not necessarily because of the simultaneous presence of cysteines at positions 70 and 143.

**DISCUSSION**

We have created a Cd\(^{2+}\) binding site formed by cysteines introduced at TMD 1 position 68 and TMD 3 position 143 (Figs. 2 and 3). The simultaneous presence of both of these cysteines is required. The site is not formed 1) with the single cysteine replacements (Figs. 2 and 5); 2) with one cysteine introduced at
either position and a mutation to an amino acid other than cysteine at the other position (Fig. 2); 3) when, besides the cysteine at position 143, another is introduced at position 74 or at position 79 in the first extracellular loop or at position 81 at the top cysteine at position 143, another is introduced at position 74 or at position 143 and this results in a view that the cysteines introduced at positions 68 and 143 are crucial for GABA binding (41). However, the apparent affinity for Cd\(^{2+}\) in the latter case (\(K_d \sim 5 \mu M\)) was lower than in the former (\(K_d \sim 0.1 \mu M\)), indicating that other parameters are relevant as well. Nevertheless, it is very well possible that the higher affinity for Cd\(^{2+}\) in the W68C/I143C pair is the result of a contribution of another (yet unknown) contact site within the W68C/I143C transporter and that this additional site contributes less to the binding site in V67C/I143C. Such contributions have been observed during zinc sensitivities in DAT mutants (42).

The effects of CuPh on the W68C mutants also support the idea that the cysteines introduced at positions 68 and 143 are in close proximity (Fig. 1). Even though the W68C/I143C mutant was much more sensitive to CuPh than W68C, there was still a significant inhibition in this mutant as well as in those where the W68C mutation was paired with a non-cysteine mutation at position 143 (Fig. 1). We failed to identify an endogenous cysteine as a potential partner for the cysteine introduced at position 68, and the reason for this inhibition by CuPh is not yet clear. Another potential target to find such relationships may be TMD 2, which is highly conserved in the SLC6 family. This TMD is possibly close to the external part of TMD 1, because extracellular loop 1 is very short. The activity of TMD 2 cysteine mutants, except for A81C, is not inhibited by methanethiosulfonate reagents (data not shown), and the same is true in SERT (43). However, the cysteines introduced into TMD 2 may still be accessible to the much smaller Cd\(^{2+}\). Thus probing pairs of cysteines introduced into TMD 2 together with those in TMD 1 and/or 3 with Cd\(^{2+}\) may potentially lead to the identification of additional structural constraints in GAT-1.

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**FIG. 6. Sensitivity of F70C/I143C/C74A to cadmium ions.** Conditions are the same as described in the legend to Fig. 2. Values represent the mean ± S.E. of at least three separate experiments done in triplicates. Mutants investigated and their activity as percentage of C74A-GAT-1 activity (in parentheses) were F70C (29.5 ± 5.5%), F70C/I143V (12.1 ± 2.1%) (▲), F70C/I143A (9.6 ± 0.5%) (●), and F70C/I143C (14.9 ± 3.7%) (●). (n = 4).
