Identification of Tryptophan Residues Critical for the Function and Targeting of the γ-Aminobutyric Acid Transporter (Subtype A)*

(Received for publication, July 30, 1993)

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The γ-aminobutyric acid transporter is localized in nerve terminals. It catalyzes coupled electrogenic translocation of the neurotransmitter with two or three sodium ions and one chloride ion. The transporter contains 599 amino acids and 12 putative membrane spanning α-helices. It is the first described member of a neurotransmitter transporter superfamily. Using site-directed mutagenesis we have investigated the role of all 10 tryptophan residues predicted to reside in these helices. All 10 have been changed to serine as well as to leucine residues. Expression of mutant cDNAs in which the tryptophans, located in positions 68, 222, and 230, are replaced by either of these two amino acids reveals that they are severely impaired in γ-aminobutyric acid transport. Mutants in which a phenylalanine residue is introduced, at either position 68 or 230, are active. On the other hand, at the 222 position replacement of the tryptophan by the aromatic amino acids results in inactive transport. After prelabeling of the proteins with [35S]methionine, immunoprecipitation of mutant transporters indicates that their expression levels are similar to those of the wild type. Reconstitution experiments, aimed to reveal the activity of transporter molecules not apparent in the plasma membrane, indicate that the lack of activity of the W223S transporter in intact cells is by and large due to its inefficient targeting to the plasma membrane. Tryptophan residues 68 and 222 appear to be required for the intrinsic activity of the transporter. Based on several observations, including one that tryptophan residue 222 is conserved in all amino acid transporter members of the superfamily, but not in those transporting biogenic amines, we hypothesize that the σ electrons of this tryptophan could be involved in the binding of the amino group of these neurotransmitters.

The overall process of synaptic transmission is thought to be terminated by high affinity sodium-dependent transport of neurotransmitters (Iversen, 1971; Bennett et al., 1974). The systems involved in this process catalyze sodium-coupled transport of neurotransmitters (reviewed in Kanner and Schuldiner (1987)) into presynaptic nerve terminals and fine processes of glial cells which are in close contact with the synapse (Radian et al., 1990; Danbolt et al., 1992). GABA1 is the predominant inhibitory neurotransmitter in the mammalian brain. Its transporter has been purified to near homogeneity using a rapid reconstitution assay (Radian and Kanner, 1985; Radian et al., 1986) and is an 80-kDa glycoprotein which represents 0.1% of the membrane protein. This protein, which is predominantly located in nerve terminals (Radian et al., 1990) catalyzes the electrogenic cotransport of the neurotransmitter molecule with one chloride and two or three sodium ions (Kynan and Kanner, 1988; Kavanaugh et al., 1992; Mager et al., 1993). It was the first neurotransmitter transporter to be cloned. Its cDNA clone was termed GAT-1, and it was expressed (Guastella et al., 1990). The deduced protein has 599 amino acids with a molecular weight of 67,000 (Guastella et al., 1990) which agrees well with the molecular weight of the deglycosylated GABA transporter (Kanner et al., 1989). Its analysis suggests the presence of 12 transmembrane regions (Guastella et al., 1990). Subsequently many other neurotransmitter transporters were cloned and shown to be related to GAT-1 (reviewed in Uhl (1992) and Schloss et al. (1992)). GABA is a zwitterionic molecule, and the substrates are charged species. Therefore, charged amino acids located in the 12 putative transmembrane helices are likely to be important for the binding and translocation of the substrates. Glutamate 467 is the only negatively charged amino acid in the membrane. Therefore, it was reasoned that it might participate in the binding site for the sodium ions. However, site-directed mutagenesis showed that it is not essential for activity. Only one of the five charged amino acids located in the transmembrane domain of the GABA transporter, arginine 69, is absolutely essential for its activity (Pantanowitz et al., 1993). Other residues which could serve as sodium binding sites are those capable of electrostatic interactions with positive charges. Studies of the enzymes acetylcholinesterase (Sussman et al., 1991) and barnase (Loewenthal et al., 1991) highlight the importance of aromatic amino acids, and especially tryptophans, in the structure and function of these proteins. We have examined the 10 tryptophan residues within the membrane domain of the GABA transporter, using site-directed mutagenesis. Two of these residues (Trp68 and Trp222) have a profound influence on the transporter's activity. A third residue (Trp230) may be involved in targeting the transporter to the plasma membrane.

EXPERIMENTAL PROCEDURES

Methods

Site-directed Mutagenesis — Mutagenesis was performed as described by Kunkel et al. (1987). The shortened GAT-1 clone (Pantanowitz et al., 1993) was used to transform Escherichia coli CJ 236 to ampicillin resistance. From one of the transformants single-stranded uracil containing DNA was isolated upon growth in a uridine-containing medium according to the standard protocol from Stratagene, using helper phage R46. This yields the sense strand, and consequently the mutagenic primers were designed to be antisense. The following primers were used to make the following mutations (names in parenthesis; the added antisense codons are in bold char-

* This work was supported by United States-Israel Binational Science Foundation Grant 90-00039, Bundesministerien fur Forschung und Technologie (Germany) Grant 1000, National Institutes of Health Grant NS 16708, and the Bernard Katz Minerva Center for Cell Biophysics. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviation used is: GABA, γ-aminobutyric acid.
Site-directed Mutagenesis of Tryptophan Residues

Materials

Polynucleotide kinase, DNA-polymerase, and DNA-alkaline phosphatase (all from New England Biolabs) were used.

Restriction enzymes were from U. S. Biochemical Corp. [3H]ATP (1,000 Ci/mmol) and [35S]methionine (1,000 Ci/mmol) were from Amersham Corp. [3H]GABA (47.6 Ci/mmol) was from the Nuclear Research Center, Negev, Israel. Protein A-Sepharose CL-4B, asolectin (P-5638, type 12), and Protein A-Sepharose CL-4B were used.

The results are shown in Fig. 1. A putative model of the GAT-1 transport protein. Tryptophan residues located within the transmembrane domains of the transporter are marked: ●, residues conserved in all known members of the superfamily of neurotransmitter transporters; □, residues where tryptophan is replaced only by other aromatic amino acids; ▲, residues which are not aromatic in at least one of the transporters of the family.

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RESULTS

Effects of Tryptophan Substitutions on GABA Transport—Fig. 1 shows the location of the 10 tryptophan residues present in the 12 putative transmembrane helices of the GABA transporter and indicates the degree of their conservation throughout this family of neurotransmitter transporters. Using site-directed mutagenesis, each of these residues was changed to leucine as well as to serine. After the mutations were verified by sequencing, the mutant cDNAs were expressed in HeLa cells using the recombinant vaccinia/T7 virus (Fuerst et al., 1986) as described (Keynan et al., 1992). The results are shown in Fig. 2.

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Usually, the leucine mutations preserve a higher capacity of GABA uptake than their serine counterparts. W135 and W538 retained around 80% of the expressed GABA transport activity when the replacement was by leucine (Fig. 2). Mutation of 5 other tryptophan residues, 381, 473, 496, 496, and 500, to leucine led to a 70% or greater, reduction in expressed transport activity and the effect was usually even more severe when replacement was by serine (Fig. 2). Three out of the 10 mutated tryptophans are the most critical for expression of GABA transport activity. These are Trp68 (helix I), Trp222, and Trp309 (helix IV). In these cases replacement of the tryptophan to either serine or leucine results in mutations causing a reduction of over 90% of the GABA transport activity. Therefore, we decided to further characterize Trp68, Trp222, and Trp309.

In addition to the serine and leucine mutations, each of these tryptophan residues was conservatively replaced by phenylalanine and tyrosine. The 12 mutants (W68S, W68L, W68Y, W68F; W222S, W222L, W222Y, W222F; and W305S, W305L, W305Y, W230F) were subcloned into the wild type background. Sequencing of the subcloned mutant DNA fragments from both
directions was then performed to ensure that only the indicated mutation had occurred without any additional changes. Fig. 3 shows a time course of GABA transport by HeLa cells, where each of the subcloned mutants is expressed. Cells expressing the wild type exhibit \(^{3}H\)GABA transport which is absolutely sodium dependent. In harmony with the results of Fig. 2, W68L, W68S; and W230L, W230S to the native protein in an immunoprecipitation assay. The results are shown in Fig. 4. The wild type transporter was expressed in HeLa cells, labelled with \(^{35}S\)methionine, lysed, and immunoprecipitated with anti PCOOH, as described under "Experimental Procedures." Lane 1, wild type; 2, W68L; 3, W230S; 4, W230L; 5, W222L; 6, W222S; 7, W222Y; 8, W222F; 9, vector without insert.

The first possibility was tested by comparing W68L, W68S; W222L, W222S, W222Y, W222F; and W230L, W230S to the native protein in an immunoprecipitation assay. The results are shown in Fig. 4. The wild type transporter was expressed in HeLa cells, labelled with \(^{35}S\)methionine and immunoprecipitated by an antibody directed against a sequence located in the carboxyl terminus of the GABA transporter. On SDS-polyacrylamide gel electrophoresis it runs as a 67-kDa polypeptide (Keynan et al., 1992). This band was not observed in HeLa cells expressing the vector alone (Fig. 4, lane 9). In contrast, all the mutant clones examined (including W68S which is not shown in this experiment) produce a band of the same mobility and of similar intensity as that of the wild type (Fig. 4, lanes 1–8).

Thus, the severe reduction of transport activity in the mutants does not seem to be due to reduced levels of the transporter proteins. The specificity of the immunoprecipitation with this antibody was demonstrated by the fact that the 67-kDa band from wild type as well as from mutant transporters was abolished by the homologous peptide \(\text{PCOOH} \) (against which the antibody was raised) but not by a heterologous peptide, \(\text{P}_{189-206} \) (data not shown).

Since the mutants with impaired activity still produce normal transporter levels, it is possible that they are inefficiently targeted to the plasma membrane. This possibility is explored in the experiment depicted in Fig. 5. One would expect that cells expressing a mutant transporter, which is intrinsically active but is inefficiently targeted to the plasma membrane, would have a cryptic transport activity. Detergent extraction of the cells expressing such transporters by reconstitution of the solubilized proteins is likely to yield transport activity in the proteoliposomes even if they were originally residing in internal membranes. Under such conditions the GABA transport activity of the reconstituted transporter from wild type is 8.4 pmol/min/mg of protein (Fig. 5). This activity is completely sodium-dependent (data not shown). In agreement with the expression in whole cells (Fig. 3), the sodium-depend
ent transport of W68L comprises 5% or less of the corresponding activity of the wild type. The serine mutation is slightly more active than its leucine counterpart. All four mutants, W222L, W222S, W222Y, W222F, hardly show any sodium-dependent transport. Reconstitution of the W230L transporter yields about 14% of its wild type counterpart (Fig. 5). This is slightly higher than its relative activity in intact cells (Fig. 3). However, when the W230S transporter is reconstituted, transport activity increases to around 60% of that of the wild type. Thus, the defect in mutant W230S observed in intact cells (Fig. 3) appears to be largely due to defective targeting to the plasma membrane.

**DISCUSSION**

In this study we have investigated the role of 10 tryptophan residues of the (Na+/Cl−)-coupled GABA transporter on its function. We hypothesized that the π electrons of the tryptophan residues could participate in the binding sites for the sodium ions or of the amino group of GABA. Thus, we have concentrated on the ten tryptophan residues predicted to be located in the 12 putative membrane spanning α-helices (Guastella et al., 1990). Each of these ten residues was replaced by a serine as well as by a leucine residue using site-directed mutagenesis. Out of these, three turned out to be the most critical (Figs. 1 and 2). Tryptophan 68 is located in that part of the transporter most conserved between all members of this superfamily (Uhl, 1992; Schloss et al., 1992). This residue is adjacent to arginine 69, previously documented by us to be the only critical charged amino acid located in the membrane part of the transporter (Pantanowitz et al., 1993). This further emphasizes the importance of this highly conserved part of putative transmembrane helix 1. Our studies have identified another part of the GABA transporter important for its function, namely helix 4. It contains two tryptophan residues (222 and 230) important for its functional expression. Tryptophan 222, just like tryptophan 68, is important for activity of the transporter rather than its stability (Fig. 4) or translocation to the plasma membrane (Fig. 5). Tryptophan 230 appears to be important for the appropriate targeting of the transporter to the plasma membrane. This is revealed when this residue is replaced by serine (Fig. 5). Substitution of this tryptophan with leucine causes a more severe defect in transport activity (Fig. 3) and the low activity persists after reconstitution (Fig. 5). It appears that the consequences of replacement of the tryptophan are a function of which amino acid is introduced at that position. Perhaps the W230L transporter is both poorly targeted to the plasma membrane as well as defective in function. Recently another case of defective translocation of a mutated glucose transporter to the plasma membrane was reported (Garcia et al., 1992). It is of interest to note that also in this case the defective translocation occurred when a tryptophan, residue 388, was mutated. Another interesting parallel is that in both cases the tryptophan residues are located in the transmembrane α-helices at the cytoplasmic side of the membrane. This suggests that determinants on this side play a prominent role in the processing of these transporters.

Tryptophan 68 is conserved in all cloned transporters of the superfamily. This together with the observation that the W68L and W68S transporters are almost completely inactive, indicates an important role for this residue in a function common to all these transporters. We have hypothesized that the sodium ions can compete with the positively charged and essential arginine 69 interacting with the π electrons of the tryptophan (Pantanowitz et al., 1993). While this hypothesis is feasible, we have not been able to test it since the activity of the W68S mutant is too low to examine its detailed dependence on the sodium concentration. The fact that its substitution by phenylalanine or tyrosine yielded active transporters (Fig. 3) is consistent with the above idea. On the other hand, the observation that phenylalanine or tyrosine but not tryptophan residues participate in a high affinity tetraethylammonium ion binding site of a Shaker K+ channel (Heginbotham and MacKinnon, 1992) could argue against it. Alternatively tryptophan 68 could be important in maintaining the appropriate structure necessary for an active transporter. It could fulfill a similar function as tryptophan 94 in barnase. In this case it has been shown that the π electrons interact with the positive histidine 18, thereby stabilizing its structure (Loewenthal et al., 1992).

A possible clue for the function of tryptophan 222 could come from comparing its position in all the members of the superfamily. It is fully conserved among all members which transport amino acids or their analogues (Guastella et al., 1990; Yamashita et al., 1992; Clark et al., 1992; Borden et al., 1992; Lopez-Corcuera et al., 1992; Liu et al., 1992a, 1992b, 1993; Smith et al., 1992; Guastella et al., 1992; Freneau et al., 1992; Uchida et al., 1992; Guimbal et al., 1993). Only in the biogenic amine transporters it is not conserved; a leucine, valine or phenylalanine occupy this position in the dopamine (Shimada et al., 1991; Kitly et al., 1991; Usdin et al., 1991), norepinephrine (Pacholszky et al., 1991) and serotonin (Hoffman et al., 1991; Blakely et al., 1991) transporters, respectively. It seems plausible that this tryptophan is important for recognition of a motive present in the amino acids and their analogues, but not in the biogenic amines. In the former case the amino group (which might, as in betaine, even be a quaternary amine) located near a carboxyl group (or sulfonate group in the case of taurine). The π electrons of the tryptophan could bind the amino group of these compounds. This tryptophan could represent the binding site of the amino group itself. Alternatively, it could serve to guide the substrate toward its actual binding site, just as has been proposed for acetylcholine esterase (Sussman et al., 1991). The fact that tyrosine and phenylalanine cannot fulfill this function (Fig. 3) could be due to structural restrictions. Interestingly, in the case of the biogenic amine transporters, all of these have a common aspartate residue located in helix 1, instead of a glycine residue in all the other transporters. This aspartate residue has been implicated in amine binding by the dopamine transporter (Kitayama et al., 1992). The progress in our knowledge of these transporters by
molecular cloning and site-directed mutagenesis, further underlines the great importance of independent structural approaches to complement and extend our insight on the structure and function of these transporters.

Acknowledgments—We thank Dr. Reinhard Jahn for graciously providing us with the anti-PCoOH serum as well as the PcoO and P180-205 peptides. Thanks also to Dr. Bernard Moss for the provision of the recombinant virus vTF7-3, to Dr. Nicola Mahjeesh for critical reading of the manuscript, and to Beryl Levene for expert secretarial help.

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