Glutamate-101 is critical for the function of the sodium and chloride-coupled GABA transporter GAT-1

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Abstract **We have investigated the possible role of selected negatively-charged amino acids of the sodium and chloride-coupled GABA transporter GAT-1 on sodium binding. These residues located adjacent to putative transmembrane domains and which are conserved throughout the large superfamily of neurotransmitter transporters were changed by site-directed mutagenesis. The functional consequences were that one of the residues, glutamate-101, was critical for transport. Its replacement by aspartate left only 1% of the activity, and no activity could be detected when it was replaced by other residues. Expression levels and targeting to the plasma membrane of the mutant transporters appeared normal. Transient sodium currents were not observed in the mutants, and increased sodium concentrations did not affect the percentage of wild type transport of the E101D mutant. It is concluded that residue glutamate-101 is critical for one or more of the conformational changes of GAT-1 during its transport cycle.**

Key words: GABA transport; Conserved negatively-charged residues; Heterologous expression; Sodium binding; Conformational changes

1. Introduction

Transporters which couple the flux of neurotransmitters to that of sodium are thought to play an important role in the termination of synaptic transmission (for a review see [1]). GAT-1 is a transporter for the neurotransmitter γ-aminobutyric acid (GABA) which was reconstituted and purified to near homogeneity [2]. It was the first to be cloned [3] and serves as a prototype of a large superfamily of sodium- and chloride-dependent neurotransmitter transporters (see [4] and [5] for reviews). It catalyses the electrogenic cotransport of the neurotransmitter molecule with one chloride and two or three sodium ions [6–8]. Using a site-directed mutagenesis approach we have attempted to identify amino acid residues of the transporter involved in sodium binding. We have changed charged amino acids located in the 12 putative transmembrane domains (TD) [9]. This revealed that only arginine-69 located in the first TD is critical. Based on evidence that aromatic amino acids, and especially tryptophans, may interact with positively charged substrates via their π-electrons [10], the tryptophan residues located in the putative TD's of the transporter were changed [11]. One out of the two residues identified to be important for function – tryptophan-222 – is not conserved throughout the superfamily and therefore is unlikely to be involved in sodium binding. The other – tryptophan-68 – is conserved throughout and may play a role in sodium binding. However, as significant activity is observed for a mutant where this residue was replaced with a serine (see [11] and Mager, S., unpublished experiments), it appears likely that other determinants are involved in the process. In this paper we have investigated the role of conserved negatively charged residues which are predicted to be located close to the putative TD's. Due to uncertainties in the predictive ability of hydrophaty plots, such residues could well be located in the TD's. It appears that one of these residues – glutamate-101 – is essential for the function of the transporter.

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Abbreviations: GABA, γ-aminobutyric acid; TD: transmembrane domain.

2. Materials and methods

2.1. Materials

Polynucleotide kinase, DNA-polymerase, and DNA-ligase (all from T4) were from Boehringer Mannheim. Restriction enzymes were from New England Biolabs and Boehringer Mannheim. Sequenase kits (version 2.0) were from U.S. Biochemical Corp. D-[^{35}S]αATP (1,000 Ci/mmol) and [^{35}S]methionine (1,000 Ci/mmol) were from Amersham Corp. [^{3}H]GABA (47.6 Ci/mmol) was from the Nuclear Research Center, Negev, Israel. The tissue culture medium, serum, penicillin/streptomycin and L-glutamine were from Biological Industries, Kibbutz Bet Ha'Emek, Israel. Transfection reagent (DOTAP) was from Boehringer Mannheim. Brain lipids were prepared from bovine brain as published [12]. Protein A-Sepharose CL-4B, asolectin (P-5638, type II S), valinomycin, uridine, cholic acid, and all other materials were obtained from Sigma. The antiserum against residues 571–586 of the GAT-1 transporter, IQPSEDIVRPENGPEQ (P_{COOH}, part of the carboxyl terminal) was prepared as described [13]. The P_{COOH} peptide was a generous gift from Dr. Reinhard Jahn (Yale University Medical School, New Haven, CT).

2.2. Site-directed mutagenesis

Mutagenesis was performed as described [14]. The shortened GAT-1 clone [9] 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 R408. 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 altered antisense nucleotides are underlined): 5′-GCC TAG GGA GCA CCC CAA AAG GAA GAG A-3′ (E101G); 5′-GCC TAG GGA GCA (G/A)TC CAA AAG GAA GAG A-3′ (E101D); 5′-CTA GGG AGC ACT GCA AAA GGA AGA GAG GAA-3′ (E101Q); 5′-GCC TAG GGA GCA TGC CAA AAG GAA GAG A-3′ (E101A); 5′-ATC TGG GTG GCG GCG CCA AGC CAC ACC TCA GAA T-3′ (D287G); 5′-AGG ACA TGA GGA AGC CGA AGC GTC CCT T-3′ (D52G); 5′-AAC AAT GAT GGA GCC CCT GTA CAC ATT G-3′ (D319G).

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Mutations were confirmed by DNA sequencing and subcloned into wild type. For the mutations at position 101 and 52 the enzymes *Avr*II and *Xho*I were used. For the D287G mutation the enzymes *Ppu*MI and *Nhe*I were used and for D319G we used *Ppu*MI and *Bsm*I. The subcloned DNAs were sequenced from both directions between the sites of the indicated restriction enzymes.

2.3. Cell growth and expression

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 200 units/ml penicillin, 200 μg/ml streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 and subsequent transfection with plasmid DNA, GABA transport and immunoprecipitation were done as published previously [15]. Protein was determined as described by Bradford [16]. SDS-polyacrylamide gel electrophoresis was as described by Laemmli [17] using a 4% stacking and 10% separating gel. Size standards (Pharmacia LKB Biotechnology Inc.) were run in parallel and visualized by Coomassie Blue staining. Reconstitution of transport was done as follows: For each experiment infected/transfected cells from two large wells (35-mm diameter) were used for each mutant. They were washed twice with 1 ml of phosphate-buffered saline and taken up in a small volume of phosphate-buffered saline using a rubber policeman. To 35 μl of this suspension were added (in this order) 15 μl of 0.1 M KP_i (pH 7.5), and 10 μl of 20% cholic acid (neutralized by NaOH). After 10 min of incubation on ice, the mixture was reconstituted with asolectin/brain lipids using spin columns, and transport was measured exactly as described [18].

2.4. Electrophysiology

Wild type and mutant GAT constructs were transferred to pAMV-PA [19] and cRNA was generated by Ambion's MEGAscript kit, using T7 RNA polymerase. Standard oocyte injection procedures were used. Recording solutions were as follows: standard medium, ND96, contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CdCl_2$ and 5 mM HEPES (pH 7.4). The Li^+ substitution for Na^+ contained 96 mM LiCl, 2 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CdCl_2$ and 5 mM HEPES (pH 7.4). Two-microelectrode voltage-clamp procedures were used. Steady state and transient currents were measured as described [8]. Data acquisition and analysis utilized the pCLAMP program suit (Axon Instruments, Foster City, CA). For measurements of GABA currents, the membrane potential was held at −80 mV unless noted otherwise.

3. Results and discussion

There are four negatively-charged amino acids in GAT-1 which are conserved throughout the large superfamily of neurotransmitter transporters and are located adjacent to putative TD's. These are aspartate residues 52, 287 and 319 located on the amino terminal side of putative TD's I, VI and VII, respectively, and glutamate-101 located at the carboxyl terminal side of putative TD II. Each of these residues has been mutated to glycine. The cDNA's carrying these mutants as well as the wild type were cut with the appropriate restriction enzymes (see section 2) so that a relatively small fragment of the mutant cDNAs could be subcloned into the wild type. These inserted fragments were sequenced in both directions and found to be unchanged with regard to the wild type, except for the mutation itself. The cDNAs of these subcloned mutants were expressed in HeLa cells using the recombinant vaccinia/T7 virus [20] as described [15]. Fig. 1 shows that D52G, D287G and D319G exhibit considerable sodium-dependent [^{3}H]GABA transport. On the other hand there is no detectable activity in E101G. These results indicate that glutamate-101 may be important for the function of the GABA transporter GAT-1. In order to study the functional role of side chain structure at position 101, the wild type glutamate residue was mutated to several other amino acids. These mutations were subcloned and verified by sequencing as above. The results of expression experiments with the mutant cDNAs are summarized in Fig. 2. The results indicate that it is the glutamate residue itself which is important, as also the E101A and E101Q mutants do not exhibit significant transport activity. A small but consistent transport activity –around 1% of the wild type – is observed with the E101D mutant. Thus, while it is clear that a negative charge at this position is important, this is not sufficient. Optimal transport is only observed when it is occupied by glutamate.

The impaired transport of the mutants is not due to lower transporter levels. Wild type and mutant transporters were expressed in HeLa cells, labelled with [^{35}S]methionine and immunoprecipitated by an antibody raised against a peptide located in the carboxyl terminal of GAT-1 (Fig. 3). The transporter monomer runs as a 67 kDa polypeptide on SDS-polyacrylamide gels [3,9,11,13] and the wild type is displayed in lane 1. It is not expressed in HeLa cells transfected with the vector alone (lane 2). In contrast, all of the mutant clones in which glutamate-101 was replaced, express high transporter levels (Fig. 3, lanes 3–6). Although it appears that in this experiment the expression levels of the mutant transporters is higher than the wild type, in other experiments the level of wild type appeared similar to that of the mutants.

Because the mutants still produce normal transporter levels, it is possible that they are inefficiently targeted to the plasma

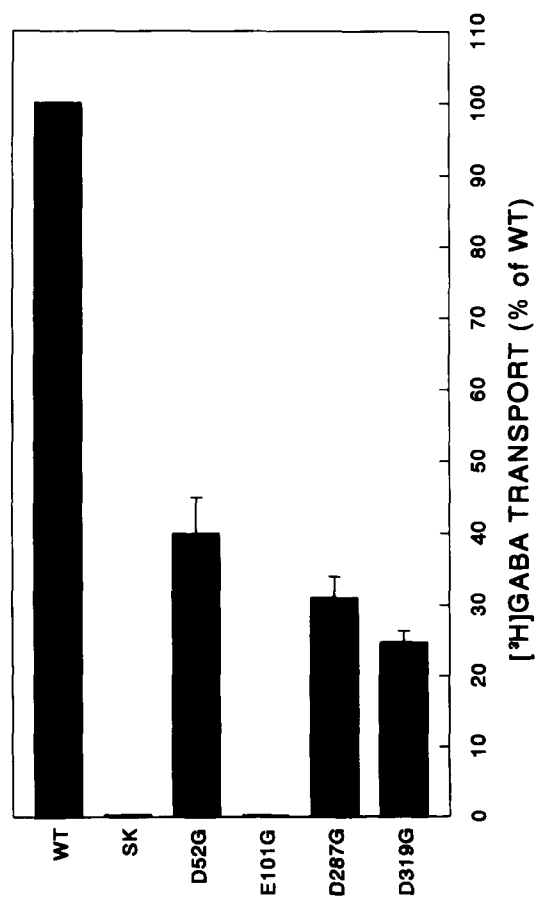

Fig. 1. [^{3}H]GABA uptake by the various mutant proteins. HeLa cells were infected with recombinant vaccinia/T7 virus and transfected with pBluescript containing wild type, the vector without the insert (SK) or the indicated mutants. Results are given as percent of sodium-dependent transport of the mutants relative to that of the wild type. Each bar is the mean ± S.E.M. of six to eight different experiments.

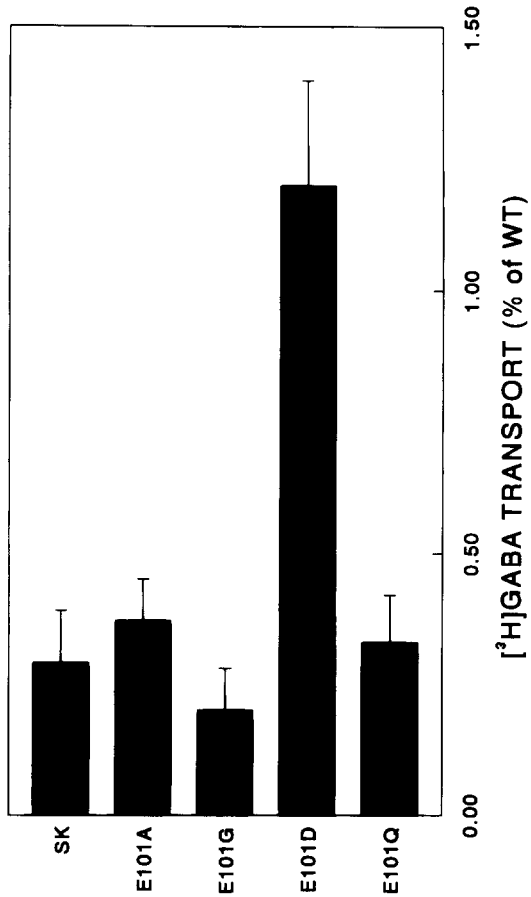

Fig. 2. [^{3}H]GABA uptake by the mutants in position 101. HeLa cells were infected with recombinant vaccinia/T7 virus and transfected with pBluescript containing wild type, the vector without the insert (SK) or the indicated mutants. Results are given as percent of sodium-dependent transport of the mutants relative to that of the wild type. Each bar is the mean ± S.E.M. of seven to eight different experiments.

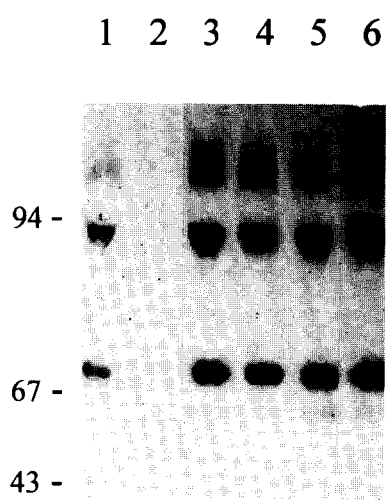

Fig. 3. Immunoprecipitation of wild type and non-active mutant GAT-1 proteins synthesized in HeLa cells. HeLa cells were infected with recombinant vaccinia/T7 virus and transfected with pBluescript containing wild type, the indicated mutants or the vector without insert. The cells were labelled with [^{35}S]methionine, lysed and immunoprecipitated with anti P_{COOH}, as described under section 2.2. Lane 1, wild type; 2, vector alone; 3, E101A; 4, 101G; 5, E101D; 6, E101Q.

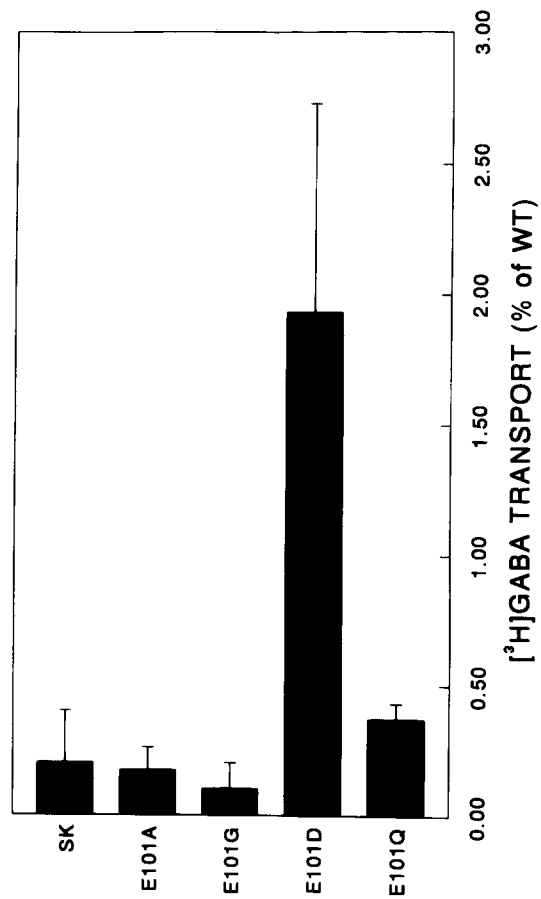

Fig. 4. [^{3}H]GABA uptake in proteoliposomes containing mutant transporters or vector alone. HeLa cells were infected with recombinant vaccinia/T7 virus and transfected with pBluescript containing wild type, the indicated mutants or the vector without the insert (SK). The cells were treated with cholate, and solubilized proteins were reconstituted with asolectin/brain lipids using spin columns as described under section 2.2. The results are averages of three experiments; 10–30 μg of protein were used per transport reaction. Bars (mean ± S.E.M.) represent net values of transport in 150 mM NaCl.

membrane. One would expect that cells expressing a mutant transporter that is intrinsically active would have a cryptic transport activity. Detergent extraction of the cells expressing such a transporter followed by reconstitution of the solubilized proteins is likely to yield transport activity even if they were originally residing in internal membranes. In fact, such cryptic transport activity has been observed using this assay with some mutants of GAT-1 [11] and of the glutamate transporter GLT-1 [21]. In this series of experiments the glutamate transport in the wild type ranges from 6–10 pmol/min/mg protein. This is similar to the values observed in whole cells. This activity is completely sodium-dependent and is not observed with cells expressing the vector alone (Fig. 4).

The E101A, E101G and E101Q mutants which do not exhibit detectable transport activity in whole cells, also remain completely void of activity after solubilization and reconstitution (Fig. 4). The small but consistent activity of the E101D mutant is only slightly enhanced under these conditions (Figs. 2 and 4). In view of these results, it appears highly unlikely that defective targeting is the cause of the impaired GABA transport of mutants at the glutamate-101 position.

The activity of the E101D transporter is too low to analyze

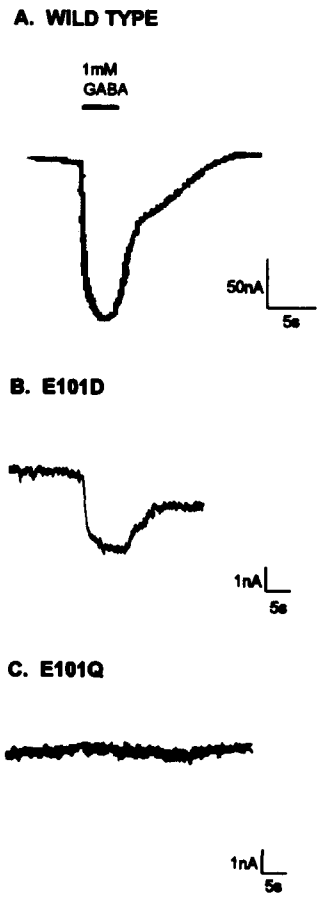

Fig. 5. GABA-induced currents for normal or mutant transporters expressed in *Xenopus* oocytes. GABA-induced currents were measured in standard perfusion medium. Oocytes were injected with cRNA for (A) wild type, (B) E101D, or (C) E101Q. Note the increased amplification for traces in (B) and (C).

for systematic dose-response studies with [^{3}H]GABA flux. Therefore, transport was measured by electrophysiological methods, utilizing the observation that transport is electrogenic [8]. It can be observed that even at 1 mM GABA, a concentration 2 orders of magnitude larger than its K_m for the transporter, the sodium-dependent transport current of the E101Q transporter is undetectable – similar to those of E101G and E101A (data not shown). The transport current of the E101D mutant is about 1% of that of the wild type (Fig. 5). As this is similar to the results obtained with tracer flux experiments (Fig. 2) in which the GABA concentration was 42 nM, it is obvious that it is not the affinity for GABA which is affected in the mutant. As transporter levels of E101D are not decreased (Fig. 3) and a targeting problem does not appear to underlie the defect, the results are consistent with a defect of sodium binding in this mutant. Furthermore, the transient sodium binding current observed with the wild type [8] is not observed in the E101D mutant (data not shown), but it should be mentioned that only transients of at least 10% of the wild type could have been detected. This transient is thought to reflect sodium binding to the transporter followed by some charge redistribution, but is independent of GABA binding [8]. Therefore, this result is consistent with the idea that a defect in the binding or debinding of sodium is caused by the E101D mutation. We have tested this idea using the reconstituted system to increase external sodium and chloride 4-fold to 0.6 M, and simultaneously by elevating the internal potassium phosphate by the same factor. The percentage of transport by the E101D mutant was not increased under these conditions (data not shown). This result indicates that a lowered affinity to either of these two ions is not likely to underlie the defective transport of the E101 mutation. Our results are best explained by either of the following two scenarios.

One is that glutamate-101 is required for the execution of the conformational change of the transporter induced upon sodium binding, e.g. the above-mentioned charge distribution. Alternatively, the residue could fulfill a critical role in the ability of the unloaded transporter to undergo a conformational change, such that the mutant transporter would be locked in the inward facing conformation. Future biochemical studies on the various conformational changes – along the lines of those already initiated [22] – may help to distinguish between these possibilities.

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