

Identification of domains of a cloned rat brain GABA transporter which are not required for its functional expression

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The sodium and chloride coupled γ -aminobutyric acid (GABA) transporter purified from rat brain, belongs to a superfamily of neurotransmitter transporters. They are involved in the termination of synaptic transmission and are predicted to have 12 membrane spanning α -helices with both amino- and carboxyl-termini oriented toward the cytoplasm. In order to define the domains not required for functional expression, we have constructed and expressed a series of deletion mutants in GAT-1, the cDNA clone encoding for the transporter. Transporters truncated at either end until just a few amino-acids distance from the beginning of helix 1 and the end of helix 12, retain their ability to catalyze sodium and chloride-dependent GABA transport.

GABA transport; Truncated cDNA clone; Expression in HeLa cells; Vaccinia/T7 recombinant virus; Rat brain

1. INTRODUCTION

The GABA_A transporter is located in nerve terminals [1] and catalyses coupled electrogenic uptake of the neurotransmitter with 2 or 3 sodium and one chloride ion [2–4]. The transporter has been purified to near homogeneity using a rapid reconstitution assay and is an 80 kDa glycoprotein which represents 0.1% of the membrane protein [5,6]. It contains 599 amino acids and is predicted to have twelve putative membrane spanning α helices with both amino- and carboxyl termini oriented toward the cytoplasm [7]. It is the first described member [7] of a large superfamily of neurotransmitter transporters [8] and is inhibited by ACHC but not by β -alanine [9]. The physiological role of these transporters is to terminate the overall process of synaptic transmission [10–12].

Often transporter molecules also contain domains which are not required for transport. For instance, in the case of the anion transporter from erythrocytes [13] and the sodium-proton exchanger [14], a substantial part of the molecule is required for binding to the cytoskeleton, whereas transport activity resides in a different domain. We have shown that upon treatment of the GABA_A transporter with proteases, fragments of 60–65 kDa can be isolated, which are functional and truncated at their amino- and carboxyl termini [15,16]. In order to define these domains – which are not essential for the transport function – we have constructed and expressed

a series of deletion mutants in GAT-1, the cDNA clone encoding for the transporter.

2. MATERIALS AND METHODS

2.1. Materials

Polynucleotide kinase, DNA polymerase and DNA ligase (all from T4) were from Boehringer. Restriction enzymes were from New England Biolabs and Boehringer. Sequenase kits (version 2.0) were from United States Biochemicals. [α -³⁵S]dATP (1,000 Ci/mmol) and [³⁵S]methionine (1,000 Ci/mmol) were from Amersham. [³H]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. The vaccinia/T7 recombinant virus was a gift from Dr. Bernard Moss (NIH). Brain lipids were prepared from bovine brain as published [17]. 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 carboxy terminal), the P_{COOH} peptide as well as P_{189–206}: VVEFWERNMHQMTDGLDK (part of the loop connecting helices 3 and 4) were generous gifts from Dr. Reinhard Jahn (Yale University Medical School, New Haven, CT).

2.2. Methods

2.2.1. Site directed mutagenesis

The original GAT clone contains about 2 kb of 3' untranslated region [7]. We have eliminated this sequence as follows: the clone was digested with *Pst*I and with *Stu*I and the 5 kb fragment was isolated after agarose electrophoresis. A synthetic linker was prepared by annealing primers [5'-CCTACATCTAGGGGTACAGCTGCA-3'] and [5'-GCTGTACCCCTAGATGTAGG-3']. The product was ligated with the 5 kb fragment and was used to transform *E. coli* strain DH-5. The closed plasmid DNA was isolated from one of the transformants. This cDNA, pT7-GAT-1, contains the open reading frame of GAT-1 behind the T7 promoter with the natural stop-codon TAG followed by 8 bases before reaching the *Pst*I site of the polylinker of pBluescript SK, and is fully active when expressed using the vaccinia/T7 recombi-

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nant virus. Site-directed mutagenesis was performed basically as described [18]. The above DNA was used to transform *E. 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:

[5' -GGGCTGCAGCTGTACCCCTAGCCCTTTAGGGTGAAGAACA-3']
to make Δ 561-599;
[5' -GGGCTGCAGCTGTACCCCTAGGGGATGAGCACCATTGGAGG-3']
for Δ 549-599;
[5' -GGGCTGCAGCTGTACCCCTAAACGTAGCTTCCCATGGTGA-3']
for Δ 528-599;
[5' -TTCCATGTGTCCCGGTGAGCGCCATGTCTCGGAGCACGA-3']
for Δ 3-41, and
[5' -GACATGAGGAAGTGAAGCGCGCCATGTCTCGGAGCACGA-3']
for Δ 3-49.

Mutations were verified by DNA sequencing and subcloned into wild type. For deletion mutations Δ 3-41 and Δ 3-49, *Eco*RI was used and orientation was verified using *Pvu*II. Age I was used together with *Sca*I for Δ 561-599, Δ 549-599 and Δ 528-599. The subcloned DNAs were sequenced between the sites of the indicated restriction enzymes.

The deletion construct Δ 576-599 was made after digestion of the GAT-1 clone by *Eco*RV and separation of the insert from the vector. The insert was ligated into the *Cl*aI site of Bluescript SK which was filled in with the Klenow fragment of *E. coli* DNA polymerase. The orientation of the insert was verified using *Bgl*II. The insert encodes the first 576 amino acids followed by arginine and the stopcodon TAA.

2.2.2. Cell growth and expression

HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum (heat-inactivated), 100 Units/ml penicillin, 100 μ /ml streptomycin and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 and subsequent transfection with plasmid DNA was done exactly as described [9]. GABA transport and immunoprecipitation were done as published previously. Protein was determined after lysis of the cells by addition of 2% Na₂CO₃ in 0.1 N NaOH (200 μ /well). Aliquots were taken for protein determination [19]. SDS-PAGE was as described [20] using a 4% stacking and 10% separating gel. Size standards (Pharmacia) were run in parallel and visualised by Coomassie blue staining. For each reconstitution experiment infected/transfected cells from 2 large wells (3 cm diameter) were used. They were twice washed with 1 ml of PBS and taken up in a small volume of PBS using a rubber policeman. To 20 μ l of this suspension were added (in this order) 2.5 μ l of saturated ammonium sulfate (pH of 20-fold dilution equals 7.5) and 1.9 μ l of 20% cholic acid (neutralised by NaOH). After a 10 min incubation on ice, the mixture was centrifuged in an Eppendorf centrifuge for 3 min. The supernatant (20 μ l) was reconstituted with asolectin/brain lipids using spin columns and transport was measured exactly as described [5].

3. RESULTS AND DISCUSSION

The transporter encoded by GAT-1 has a predicted open reading frame of 599 amino acids, and the inward oriented carboxyl tail begins at threonine-558. In order to assess its possible importance in the functional expression of the transporter, a number of progressive deletions from the terminus were constructed cutting with restriction enzymes and subcloning (Δ 576-599) or by loop-out site-directed mutagenesis [18]. These included Δ 561-599, Δ 549-599 and Δ 528-599 (the first res-

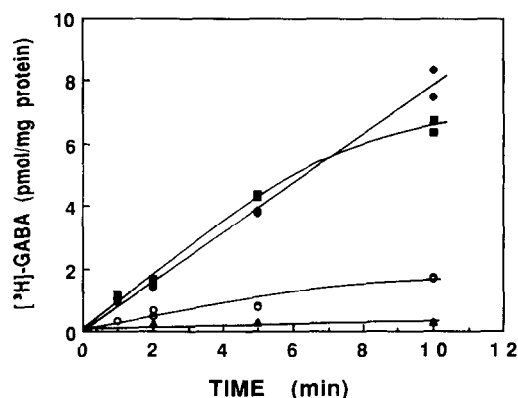


Fig. 1. Time course of [³H]GABA transport in vaccinia/T7-infected HeLa cells transfected with pBluescript containing cDNA encoding for transporters truncated from the carboxyl terminal. HeLa cells were infected with vTF7-3 and transfected with pT7-GAT-1 (wild type, ◆), Δ 576-599 (■), Δ 561-599 (○) or Δ 549-599 (▲). 50 μ g of protein was present in each well. Duplicate time points were taken, but in some of the cases the error was so small that they fell within the width of the symbol.

idue number indicates the last amino acid before the natural stop codon TAG). After the deletions were verified by sequencing, the mutant cDNA was expressed in HeLa cells using the recombinant vaccinia/T7 virus [21] as described [9,22]. Deletion of more than half of the tail (Δ 576-599) does not affect GABA transport, and even its almost complete removal (Δ 561-599) still leaves about 25% of the activity (Fig. 1). On the other hand deletion of part of helix 12 (Δ 549-599) causes abolishment of transport activity (Fig. 1). The same result is obtained when all of helix 12 is deleted (Δ 528-599, data not shown). In all cases where deletions were functional, transport was dependent on both sodium and chloride and retained its pharmacological specificity – inhibition by ACHC but not by β -alanine (data not shown).

Truncation at the amino terminal was performed starting from threonine-3, thereby leaving the start methionine and alanine-2 as well as the 5'-upstream sequences unaffected. Deletion until proline -42 (Δ 3-41) has no effect whatsoever on GABA transport (Fig. 2). However, deletion until arginine-50 (Δ 3-49), three amino acids from the start of helix 1, results in defective transport.

Defective expression could be due to importance of the deleted regions in the activity of the transporter, but also to defects in synthesis and/or turnover, or impaired targeting. Fig. 3 illustrates that the amounts of the transporters truncated at the amino terminus are similar to that of the full-length transporter. After transfection with the appropriate plasmid DNAs the cells were labelled with [³⁵S]methionine. Subsequently the labelled transporters were immunoprecipitated by an antibody directed against a sequence located in the carboxyl terminus of the transporter, but not by preimmune serum (Fig. 3). The 'wild type' transporter synthesized in this

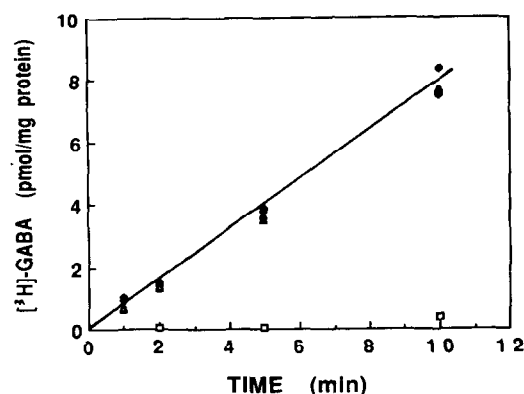


Fig. 2. Time course of [3 H]GABA transport upon expression of GAT-1 truncated from the amino terminus. HeLa cells were infected with vTF7-3 and transfected with wild type pT7-GAT-1 (\blacklozenge), $\Delta 3-41$ (\triangle) or $\Delta 3-49$ (\square).

system runs as a 67 kDa polypeptide [9]. The truncated transporters have a larger mobility, as expected from their decreased molecular mass, but they are present at similar levels to that of the 'wild-type' transporter. As an additional control for the specificity of the immunoprecipitation reactions we demonstrated that it was abolished by the homologous P_{COOH} peptide (against which the antibody was raised) but not by a heterologous peptide P189-206 (data not shown). In similar experiments – using an antibody raised against the whole transporter [6] – we have shown that all the transporters truncated from the carboxyl terminal were present at similar levels to those of the wild-type transporter (data not shown).

The lack of activity observed in some of the deletion mutants also does not seem to be due to defective targeting to the plasma membrane. In the absence of high titer antibodies against external epitopes – which would enable the performance of immunofluorescence studies on intact cells – we adopted an alternative approach to this issue. As the inactive deletion mutants $\Delta 3-49$, $\Delta 528-599$ and $\Delta 549-599$ make the truncated transporters, it could be located in internal membranes. If this were to be the reason for lack of activity, detergent extraction of the cells and reconstitution of the solubilized transporters should reveal activity in the proteo-liposomes. While such a procedure yielded sodium and chloride dependent GABA transport in the wild type, it did not in the case of these mutants as illustrated for $\Delta 3-49$ and $\Delta 549-599$ (Fig. 4). Therefore, it appears that the large majority of the hydrophilic parts of amino acid carboxyl termini are not essential for functional expression. In proteolysis experiments we performed on the isolated transporter it appeared that active fragments cleaved at carboxy and amino termini could be isolated [15,16]. The reduction in apparent molecular mass of these fragments based on the mobility in SDS-PAGE was 15–20 kDa. This would predict that several α -helices are not

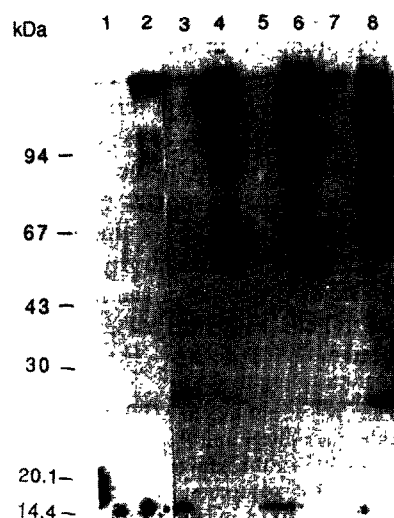


Fig. 3. Immunoprecipitation of wild type and amino terminal truncated GAT-1 proteins synthesized *in vivo* in HeLa cells. The cells were labelled with [35 S]methionine, lysed and immunoprecipitated with anti- P_{COOH} (even lanes) or preimmune serum from the same rabbit (odd lanes). GAT-1 expression was directed in the vaccinia/T7 expression system by pT7-GAT-1 (wild type, lanes 7 and 8), $\Delta 3-41$ (lanes 3 and 4) or $\Delta 3-49$ (lanes 5 and 6). A control-recombinant virus alone was also run (lanes 1 and 2).

required for the transport function, which was puzzling in view of the high conservation of helices 1 and 2 within the superfamily, as well as the presence of several critical amino acids in helix 1 as shown by site-directed mutagenesis [23,24]. In this paper we have defined the boundaries of the termini which can be cleaved off without impairing function. As the last few amino acids adjacent to helices 1 and 12 seem to be important for transport function *per se*, it appears that the proteolysis data can best be interpreted in view of the well-known

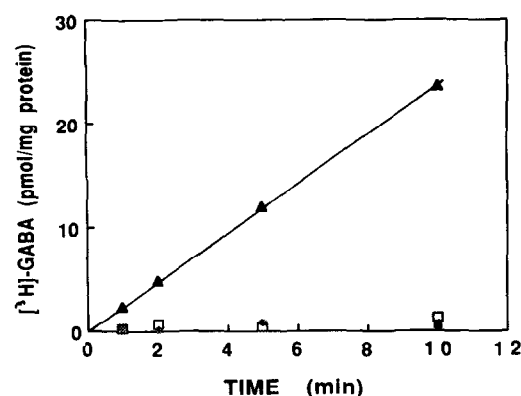


Fig. 4. Time course of [3 H]GABA transport in liposomes inlaid with solubilized HeLa cell membrane proteins. Upon infection of the cells with vTF7-3, they were transfected with pT7-GAT-1 (\blacktriangle), $\Delta 549-599$ (\square) or $\Delta 3-49$ (\blacklozenge). The cells were treated with cholate and solubilized proteins were reconstituted as described in Section 2. 10 μ g of protein was used for each time point.

atypical mobility of hydrophobic proteins as compared with that of the standard proteins [25].

It is of interest to note that truncation of most of the hydrophilic part of the carboxyl terminus of the facilitated glucose transporter does cause a complete loss of the transport function [26]. On the other hand in the proton-coupled lactose transporter of *E. coli*, the carboxyl terminal can be cleaved off almost entirely without loss of function [27], similar to the situation described here. The critical role of the hydrophilic amino acids adjacent to helix 1 in transporter function remains to be elucidated.

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