

The number of amino acid residues in hydrophilic loops connecting transmembrane domains of the GABA transporter GAT-1 is critical for its function

Baruch I. Kanner*, Annie Bendahan, Shifrah Pantanowitz, Hailing Su

Department of Biochemistry, Hadassah Medical School, The Hebrew University, PO Box 12272, Jerusalem 91120, Israel

Received 20 October 1994; revised version received 4 November 1994

Abstract Transporter proteins consist of multiple transmembrane domains connected by hydrophilic loops. As the importance of these loops in transport processes is poorly understood, we have studied this question using the cDNA coding for GAT-1, a Na⁺/Cl[−]-coupled γ -aminobutyric acid transporter from rat brain. Deletions of randomly picked non-conserved single amino acids in the loops connecting helices 7 and 8 or 8 and 9 result in inactive transport upon expression in HeLa cells. However, transporters where these amino acids are replaced with glycine retain significant activity. The expression levels of the inactive mutant transporters was similar to that of the wild-type, but one of these, Δ Val-348, appears to be defectively targeted to the plasma membrane. Our data are compatible with the idea that a minimal length of the loops is required, presumably to enable the transmembrane domains to interact optimally with each other.

Key words: GABA transport; Site-directed mutagenesis; Immunoprecipitation; Reconstitution; Rat brain

1. Introduction

High affinity sodium-dependent transporters are thought to play an important role in the overall process of synaptic transmission [1,2]. They catalyze sodium-coupled transport of neurotransmitters into presynaptic nerve terminals and fine processes of glial cells which are in close contact with the synapse [3,4]. The γ -aminobutyric acid (GABA) transporter GAT-1 was purified to near homogeneity using a rapid reconstitution assay [5,6] and is an 80-kDa glycoprotein which represents 0.1% of the membrane protein. It is predominantly located in nerve terminals [3] and catalyzes the electrogenic co-transport of GABA with one chloride and two sodium ions [7–9]. GAT-1 was the first neurotransmitter transporter to be cloned and expressed [10]. The deduced protein has 599 amino acids with a calculated molecular weight of 67,084 Da [10]. This is in good agreement with the molecular weight of the deglycosylated GABA transporter [11]. Hydrophathy plot analysis predicts twelve putative membrane-spanning α -helices [10]. After the subsequent expression cloning of NET-1, a norepinephrine transporter [12], it became clear that they are members of a novel family. Indeed, many other neurotransmitter transporters, including at least three other GABA isotoners, were then cloned and shown to be related to GAT-1 and NET-1 (for reviews see [13–16]).

We have shown that the amino- and carboxyl-terminal tails of GAT-1 are not required for its function [17,18] but the membrane domains are [18]. Indeed, we have identified several amino acid residues within the putative transmembrane domains which are critical for the function of GAT-1 [19,20]. The role of the hydrophilic loops connecting the putative transmembrane domains is thus far unexplored, not only in GAT-1 but

also in other transport proteins. In this paper we provide evidence compatible with the idea that at least one of their roles is to permit the appropriate interaction of those transmembrane helices which are adjacent to each other in the structure of the transporter.

2. Materials and methods

2.1. Site-directed mutagenesis

Mutagenesis was performed as described in [21]. The shortened GAT-1 clone [19] 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 primers used to make the mutations are presented in Table 1 (names in parenthesis).

Mutations/deletions were confirmed by DNA sequencing and subcloned into the wild-type strain using the restriction enzymes *Pvu*MI and *Age*I for Δ 348–369; *Bsm*I and *Age*I for Δ 404–408, Δ L408 and L408G and *Pvu*MI and *Bsm*I for all the others.

The subcloned DNAs were sequenced from both directions between the sites of the indicated restriction enzymes.

2.2. 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 μ g/ml streptomycin, and 2 mM glutamine. Infection with recombinant vaccinia/T7 virus vTF7-3 and subsequent transfection with plasmid DNA was done as described [22]. GABA transport and immunoprecipitation were done as published previously [19]. Protein was determined as described [23]. SDS-PAGE was as described [24] 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 transporters in proteoliposomes was used to differentiate between those exposed at the membrane surface and those located within the cell. 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 μ l of this suspension were added (in this order) 15 μ l of 0.1 M KP_i (pH 7.5) and 10 μ l of 20% cholic acid (neutralized by NaOH). After

*Corresponding author. Fax: (972) (2) 757 379.

Abbreviations: GABA, γ -aminobutyric acid; loop n –(n + 1), the hydrophilic loop connecting putative transmembrane α -helices n and n + 1; Δ X–Y, deletion of amino acids from X to Y.

Table 1

5'-TAG CTG TGT CAC AGC CTC ATG AGC CAT GAA GCC CAC-3'	(Δ348-369)
5'-GTA TGC CAA GAA TGC CAG GGA CCT CTT GGT GAC ATG-3'	(Δ353-362)
5'-CAC ATC GGC TAT GGA ATG AGC CAT GAA GCC-3'	(Δ348-351)
5'-ATC GGC TAT GGA CCT ATG AGC CAT GAA GCC-3'	(Δ348-350)
5'-GGC TAT GGA CCT CTT ATG AGC CAT GAA GCC-3'	(Δ348-349)
5'-TAT GGA CCT CTT GGT ATG AGC CAT GAA GCC-3'	(ΔV348)
5'-CAC ATC GGC TAT GGA CTT GGT GAC ATG AGC-3'	(ΔR351)
5'-CAG TCC AGG GCC TGA CGC CAC ATC GGC TAT-3'	(ΔA358)
5'-ACC TCT TGG TGC CAT GAG CCA TGA A-3'	(V348G)
5'-ACC TCT TGG TGA TAT GAG CCA TGA A-3'	(V348I)
5'-ATC GGC TAT GGA CCC CTT GGT GAC ATG A-3'	(R351G)
5'-AGT CCA GGG CCT GAG CCC GCC ACA TCG GCT AT-3'	(A358G)
5'-TGG ACC TCT TGG TGT TAA CAT GAG CCA TGA-3'	(Ins-N349)
5'-GGT ACT CAT CCA CGC CCT CCA CGG TAC AGA A-3'	(Δ404-408)
5'-GGG GTA CTC ATC CAC GGC AGT GAT GAA GCC-3'	(ΔL408)
5'-GTA CTC ATC CAC CCC GGC AGT GAT GAA-3'	(L408G)

10 min incubation on ice, the mixture was reconstituted with asolectin/brain lipids using spin columns, and transport was measured exactly as described [5].

2.3. 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 US Biochemicals. D-[³⁵S]ATP (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 [25]. 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) was a generous gift from Dr. Reinhard Jahn (Yale University Medical School, New Haven, CT) and has been documented before [18–20].

3. Results and discussion

A series of ever smaller deletions in the hydrophilic loop connecting putative transmembrane α -helices 7 and 8 of GAT-1 (loop 7–8) has been produced. None of these deleted transporters, which include Δ348–369 (a deletion from amino acid 348–369), Δ353–362, Δ348–351, Δ348–350 and Δ348–349, exhibit significant transport activity when expressed in HeLa cells using the vaccinia/T7 recombinant virus [26]. In all cases it is less than 2% of the transport activity observed with the wild-type. This phenomenon is probably not confined to loop 7–8, as a small deletion in loop 8–9, Δ404–408, also causes defective transport. In order to maximize the chance to observe transport activity, we have examined the effect of deleting individual amino acids which are not conserved within the transporter superfamily. Four such transporters have been constructed with deletions in Val-348 (ΔV348), Arg-351 (ΔR351) and Ala-358 (ΔA358) – all located in loop 7–8 – as well as one in Leu-408 (ΔL408) in loop 8–9. None of these deleted transporters exhibit significant transport activity (Fig. 1). On the other hand trans-

porter mutants where these amino acids are replaced by others, exhibit considerable activity. When Val-348 is mutated to the conserved isoleucine (V348I), activity is at least as high as in the wild-type (Fig. 2). When it is replaced by glycine, considerable activity is detected in the V348G transporter (Fig. 2). This indicates that even though at this position a hydrophobic amino acid is optimal, the Val residue is clearly not essential for activity. This is in contrast to the essential amino acids Arg-69 ([19] or Trp-222 [20] where replacement even by closely related amino acids rendered the transporter completely non-functional. Also in the case of Arg-351 and Leu-408 a similar situation occurs to that of Val-348. In all three positions replacement by a glycine results in transporters exhibiting 30–45% of wild-type activity. On the other hand, replacement of Ala-358 by glycine results in a fully active transporter. This is presuma-

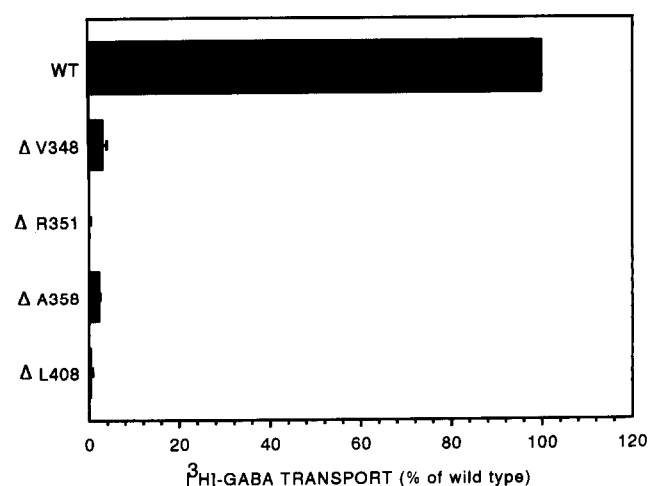


Fig. 1. [³H]GABA transport by GAT-1 transporters containing single amino acid deletions in loops 7–8 and 8–9. Sodium-dependent transport was measured in HeLa cells transiently expressing wild-type (pT7-GAT-1) or mutated transporters, as described in section 2. The results are expressed as a percent of the corresponding values of the wild-type. Each bar represents mean \pm S.E.M. of five different experiments, each done in triplicate.

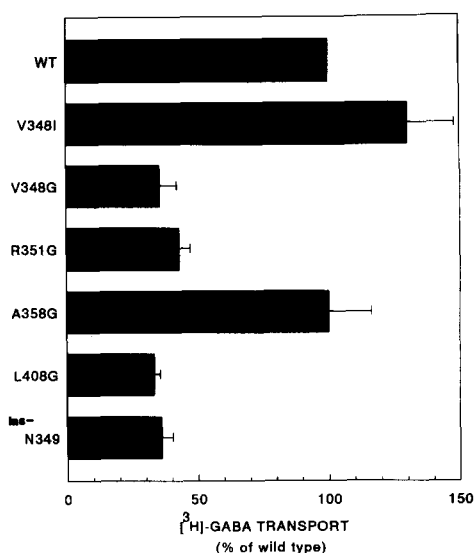


Fig. 2. [³H]GABA transport by GAT-1 transporters containing replacements or insertions in loops 7–8 and 8–9. This was performed exactly as described in the legend to Fig. 1. Each bar represents mean \pm S.E.M. of four different experiments, each done in triplicate.

bly because of the similarity of these two amino acids. The above results suggest that, at least at 4 randomly selected non-conserved positions, it is not the deleted amino acid which is critical for transport activity. Rather, it appears that the number of amino acids in the loop is the important parameter. A possible explanation may be that a minimal length of the loops is required to allow the transmembrane-spanning helices they are connecting, to occupy their correct position within the structure of the transporter. If this is correct, small insertions may be expected not to impair the activity of the transporter. As shown in Fig. 2, when an asparagine is inserted between Val-348 and Thr-349 (Ins-N349) considerable transport activity is observed.

The lack of activity of transporters in which a single amino acid was deleted at various positions in the loops is not due to decreased steady-state levels. Cells, expressing mutant or wild-type transporters, have been labelled with [³⁵S]methionine, lysed and subsequently the transporters have been immunoprecipitated using an antibody directed against the carboxyl-terminal of GAT-1 [18–20]. As published previously, the transporter runs as a band of around 67 kDa [18–20]. Even though the levels of some of the mutant transporters are somewhat lower than those of the wild-type, significant amounts are present in all of them (Fig. 3). This indicates that it is not the synthesis and turnover of the mutant transporters which are the cause for their defective activity.

The possibility that impaired activity is due to defective targeting of the mutant transporters to the plasma membrane was investigated using a solubilization/reconstitution assay [20]. One would expect that cells expressing a mutant transporter that 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 followed by reconstitution of the solubilized proteins, is likely to yield transport activity in the proteoliposomes even if they were originally residing in internal membranes. Although the activity of three of the reconstituted mutant transporters is still

low compared to the wild-type, in some cases their relative activity is increased (Figs. 1 and 4). A marked increase of transport activity is observed with mutant Δ V348. After solubilization and reconstitution, the transport of this mutant is around 30% of that of the wild-type (Fig. 4). This value is similar to that of transport of the replacement mutant V348G in intact cells (Fig. 2). Thus, it appears that the defective transport of this deletion mutant by and large is due to defective targeting. It is plausible that small deletions in the loop may have subtle effects on the structure of the transporter. This may influence targeting to the plasma membrane or the functionality of the transporter. No information as yet is available as to which helices are in direct contact with each other. It is very likely that helix 8, for instance, is not interacting with helix 7, but with others. A certain minimal length of loop 7–8 is apparently required to enable helix 8 to do so. The simplest explanation of our data is that, when this loop is shortened by as little as one amino acid residue, this interaction may be strained, causing impaired transporter function or targeting. An alternative explanation is that perhaps parts of the loops could assume an α -helical conformation. Deletion of amino acids may align a different set of residues on one side of the helix, from the point of the deletion onwards. The fact that insertion of an asparagine in this part of the loop has relatively little effect on activity as compared to deletion of single amino acids (Figs. 1 and 2) renders this alternative less likely.

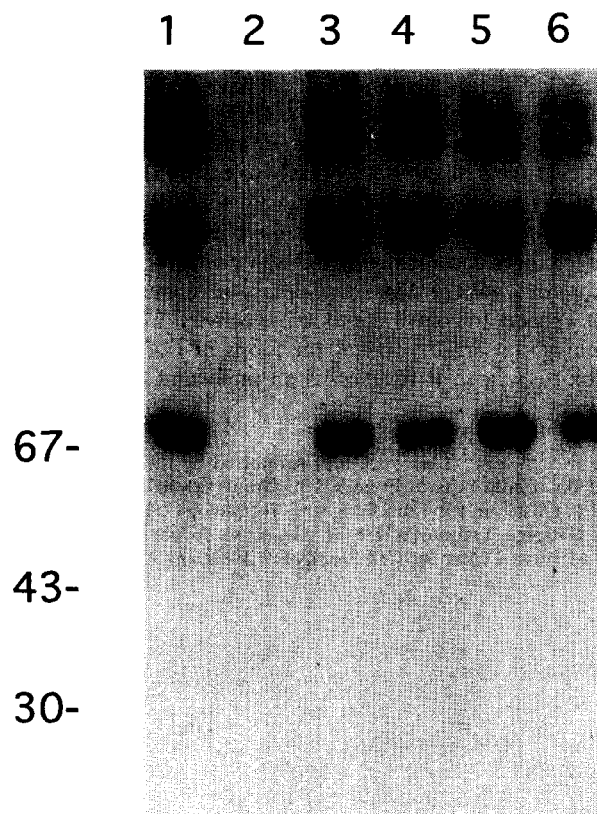


Fig. 3. Immunoprecipitation of wild-type and mutant transporters. HeLa cells were infected with the vaccinia/T7 recombinant virus and transfected as indicated below. After labelling with [³⁵S]methionine, cells were lysed and the transporters were immunoprecipitated as described in section 2. The DNA used to transfect was pT7-GAT-1 (wild type, lane 1); vector alone (pBluescript SK, lane 2); Δ V348 (lane 3); Δ R351 (lane 4); Δ A358 (lane 5); and Δ L408 (lane 6).

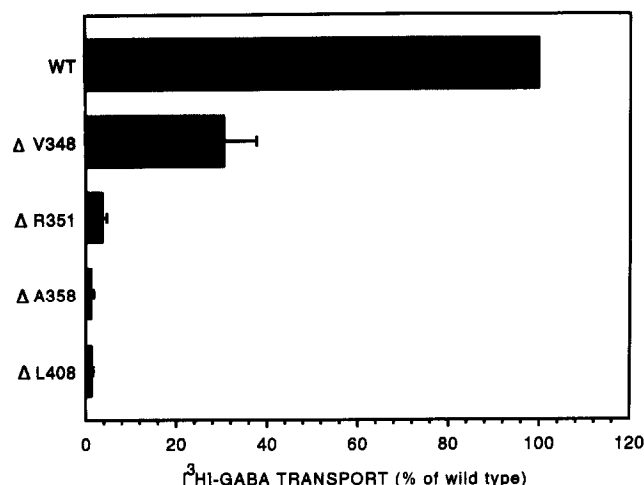


Fig. 4. [³H]GABA transport in proteoliposomes inlaid with wild-type or mutant transporters. HeLa cells transiently expressing the indicated transporters were solubilized, reconstituted and sodium-dependent transport was measured as described in section 2. The results are expressed as a percent of the corresponding values of the wild-type. Each bar represents mean \pm S.E.M. of four different experiments.

It is likely that this phenomenon is not restricted to GAT-1. It may be that the concept that a minimal length of the loops is required to allow the appropriate transmembrane domains to interact appropriately is a general one. It may be applicable to other transporters, channel proteins as well as to receptors.

Finally it should be noted that we have been studying deletions of amino acids which are poorly conserved. However, many of the loops contain highly conserved regions. It is very likely that their function is not only to ensure the appropriate length of the loops. Those amino acid residues might participate in crucial tasks such as substrate binding or in conformational changes accompanying the transport process. In fact, in the case of a voltage-dependent potassium channel, one of the loops appears to contribute to ion selectivity [27]. The participation of conserved parts of the loops of GAT-1 and other transporters in their function is an important area of future research.

Acknowledgements: This work was supported by the United States-Israel Binational Science Foundation, Grants 90-00039 and 93-0052; Bundesministerien für Forschung und Technology (Germany), Grant 1000; National Institutes of Health, Grant NS 16708; and the Bernard Katz Minerva Center for Cell Biophysics. We thank Mr. Awni Mousa

for his help in the sequencing of some of the mutant transporters. Thanks are also due to Dr. Bernard Moss for the provision of the recombinant vaccinia virus vTF7-3, and to Mrs. Beryl Levene for expert secretarial help.

References

- [1] Iversen, L.L. (1971) *Br. J. Pharmacol.* 41, 571–591.
- [2] Bennett Jr., J.P., Mulder, A.H. and Snyder, S.M. (1974) *Life Sci.* 15, 1046–1056.
- [3] Radian, R., Ottersen, O.P., Storm-Mathisen, J., Castel, M. and Kanner, B.I. (1990) *J. Neurosci.* 10, 1319–1330.
- [4] Danbolt, N.C., Storm-Mathisen, J. and Kanner, B.I. (1992) 51, 295–310.
- [5] Radian, R. and Kanner, B.I. (1985) *J. Biol. Chem.* 260, 11859–11865.
- [6] Radian, R., Bendahan, A. and Kanner, B.I. (1986) *J. Biol. Chem.* 261, 15437–15441.
- [7] Keynan, S. and Kanner, B.I. (1988) *Biochemistry* 27, 12–17.
- [8] Kavanaugh, M.P., Arriza, J.L., North, R.A. and Amara, S.G. (1992) *J. Biol. Chem.* 267, 22007–22009.
- [9] Mager, S., Naeve, J., Quick, M., Labarca, C., Davidson, N. and Lester, H.A. (1993) *Neuron* 10, 177–188.
- [10] Guastella, J., Nelson, N., Nelson, H., Czyzyk, L., Keynan, S., Miedel, M.C., Davidson, N., Lester, H.A. and Kanner, B.I. (1990) *Science* 249, 1303–1306.
- [11] Kanner, B.I., Keynan, S. and Radian, R. (1989) *Biochemistry* 28, 3722–3727.
- [12] Pacholczyk, T., Blakely, R.D. and Amara, S.G. (1991) *Nature* 350, 350–354.
- [13] Uhl, G.R. (1992) *Trends Neurosci.* 15, 265–268.
- [14] Schloss, P., Mayser, W. and Betz, H. (1992) *FEBS Lett.* 307, 76–80.
- [15] Amara, S.G. and Kuhar, M.J. (1993) *Annu. Rev. Neurosci.* 16, 73–93.
- [16] Kanner, B.I. and Kleinberger-Doron, N. (1994) *Cell. Physiol. Biochem.* 4, 174–184.
- [17] Majeesh, N.J. and Kanner, B.I. (1992) *J. Biol. Chem.* 267, 2563–2568.
- [18] Bendahan, A. and Kanner, B.I. (1993) *FEBS Lett.* 318, 41–44.
- [19] Pantanowitz, S., Bendahan, A. and Kanner, B.I. (1993) *J. Biol. Chem.* 268, 3222–3225.
- [20] Kleinberger-Doron, N. and Kanner, B.I. (1994) *J. Biol. Chem.* 269, 3063–3067.
- [21] Kunkel, T.A., Roberts, J.D. and Zarkour, R.A. (1987) *Methods Enzymol.* 154, 367–383.
- [22] Keynan, S., Suh, J.-S., Kanner, B.I. and Rudnick, G. (1992) *Biochemistry* 31, 1974–1979.
- [23] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [24] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [25] Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) *J. Biol. Chem.* 226, 497–509.
- [26] Fuerst, T.R., Niles, E.G., Studier, F.W. and Moss, B. (1986) *Proc. Natl. Acad. Sci. USA* 83, 8122–8126.
- [27] Slesinger, P.A., Jan, Y.N. and Jan, L.Y. (1993) *Neuron* 739–749.