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Tyrosine 140 of the γ-Aminobutyric Acid Transporter GAT-1 Plays a Critical Role in Neurotransmitter Recognition*

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The γ-aminobutyric acid (GABA) transporter GAT-1 is located in nerve terminals and catalyzes the electrogenic reuptake of the neurotransmitter with two sodium ions and one chloride. We now identify a single tyrosine residue that is critical for GABA recognition and transport. It is completely conserved throughout the superfamily, and even substitution to the other aromatic amino acids, phenylalanine (Y140F) and tryptophan (Y140W), results in completely inactive transporters. Electrophysiological characterization reveals that both mutant transporters exhibit the sodium-dependent transient currents associated with sodium binding as well as the chloride-dependent lithium leak currents characteristic of GAT-1. On the other hand, in both mutants GABA is neither able to induce a steady-state transport current nor to block their transient currents. The nontransportable analog SKF 100330A potently inhibits the sodium-dependent transient in the wild type GAT-1 but not in the Y140W transporter. It partly blocks the transient of Y140F. Thus, although sodium and chloride binding are unimpaired in the tyrosine mutants, they have a specific defect in the binding of GABA. The total conservation of the residue throughout the family suggests that tyrosine 140 may be involved in the liganding of the amino group, the moiety common to all of the neurotransmitters.

High affinity sodium-coupled neurotransmitter transport plays a major role in the maintenance of low synaptic levels of the transmitter (for a review, see Ref. 1). Direct proof of this has been obtained recently for the dopamine transporter, using homozygous mice in which this transporter was disrupted (2). GAT-1 is a GABA[1] transporter that was reconstituted, purified to homogeneity (3), and cloned (4). It is the prototype of a large superfamily of sodium- and chloride-dependent transporters of neurotransmitters. This includes transporters norepinephrine, dopamine, serotonin, and glycine as well as several additional GABA transporters (for a review, see Ref. 5). GAT-1 catalyzes the electrogenic transport of GABA with one chloride and two sodium ions (6–8).

Using site-directed mutagenesis we have attempted to identify amino acid residues of GAT-1 involved in substrate binding. Since GABA is a zwitterionic molecule and the cosubstrates are charged species, we have changed the charged and conserved residues likely to be located in the membrane. This revealed that arginine 69 is critical for transport (9). Although we also identified a critical negatively charged residue, glutamate 101 (10), it is not clear that it is located in the membrane. Other residues that may interact with positively charged substrates, such as sodium ions or the amino group of the neurotransmitter, are aromatic amino acids, which could do so by virtue of their π electrons (11–13). In a previous study we have modified the tryptophan residues of GAT-1 thought to be located in the membrane (14). Even though two important tryptophans were identified, it is now clear that they are not directly involved in substrate binding (15).[2]

In this study we have modified the tyrosine residues of GAT-1, which are thought to be located in the membrane and are conserved throughout the superfamily. We have identified one residue, tyrosine 140, which is critical for transport and does not tolerate replacement to other aromatic residues. Transporters in which the residue is replaced by phenylalanine (Y140F) or tryptophan (Y140W) still exhibit sodium and chloride binding but appear to be impaired selectively in the binding of GABA. The stringent conservation of the tyrosine suggests that it may interact with a moiety common to all substrates of the family, namely the amino group.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]GABA (47.6 Ci/mmol) was obtained from the Nuclear Research Center (Negev, Israel) and D,L-[3,4-^{3}H]nipecotic acid (40 Ci/mmol) was from Amersham. SFK 100330A and the recombinant vaccinia/T7 virus vTF7/3 were generous gifts of Dr. W. Blondinell (Smith Kline Beecham) and Dr. B. Moss (National Institutes of Health), respectively. Molecular mass markers were from Pharmacia Biotech Inc. Solutions of acrylamide/bisacrylamide were obtained from Bio-Rad. Restriction enzymes were from New England Biolabs Inc. and Boehringer Mannheim. T_4 polynucleotide kinase, T_4 DNA polymerase, T_4 DNA ligase, and the transfection reagent DOTAP were also from Boehringer Mannheim. Sequenase version 2.0 kits were obtained from U. S. Biochemical Corp., and kits for plasmid DNA preparation were from QIAGEN Inc. [^{35}S]dATPαS and EXPRE^{35}S^{35}S protein-labeling mixture (1,000 Ci/mmol) were from Du Pont NEN. Tissue culture medium, serum, and antibiotics were from Biological Industries (Kibbutz Beit HaEmek, Israel). P_{COOH}, a peptide located in the carboxyl terminus of GAT-1 (residues 571–586, **IQPSEDIVRPENGPEQ**), was synthesized by Dr. Lea Goldberg (Weizmann Institute, Rehovot, Israel) on an Applied Biosystems model 430A peptide synthesizer. The antibody against P_{COOH} was raised in a rabbit as described (16).

Methods

Site-directed Mutagenesis—This was performed using the method of Kunkel *et al.* (17) as described (14). Mutations were confirmed by DNA sequencing and were subcloned into wild type using pairs of unique

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[1] The abbreviations used are: GABA, γ-aminobutyric acid; SKF 100330A, *N*-(4,4-diphenyl-3-butenyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid; dATPαS, deoxyadenosine 5′-*O*-(thiotriphosphate).

[2] S. Mager personal communication.

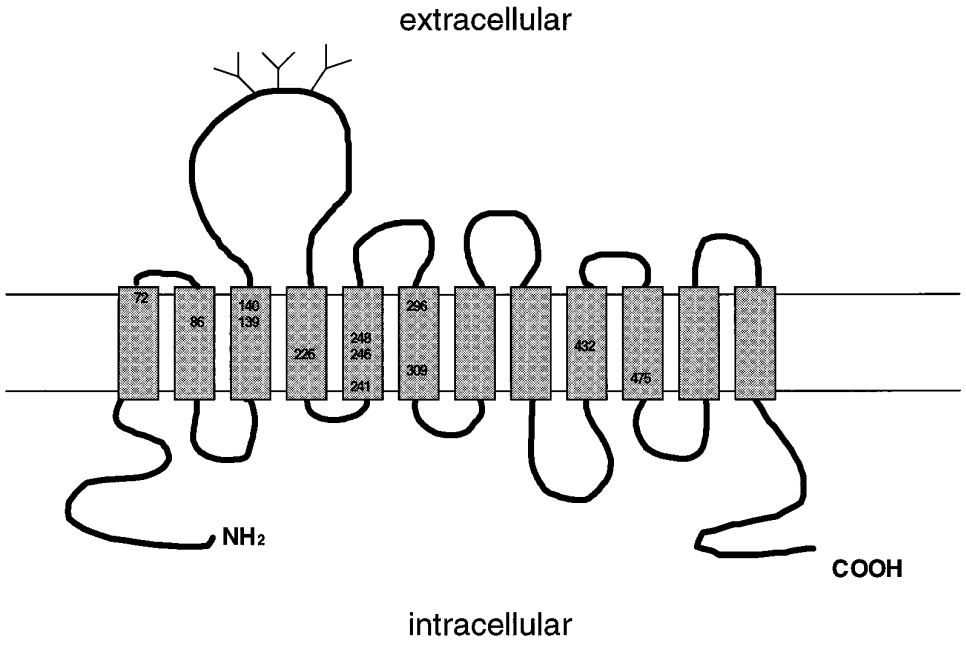

FIG. 1. **Hypothetical secondary structure of the GAT-1 transport protein.** Putative transmembrane segments are depicted as *rectangles*. The indicated tyrosines have been modified by site-directed mutagenesis.

restriction enzymes cutting the cDNA on either side of the mutation. The subcloned mutant cDNAs were then sequenced from each direction between the two restriction sites.

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 (18) and subsequent transfection with plasmid DNA, GABA transport (19), and immunoprecipitation (20) were done as published previously. Protein was determined as described by Bradford (21). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was as described by Laemmli (22) using a 4% stacking and 10% separating gel. Size standards (Pharmacia Biotech 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 (23).

Electrophysiology—The cDNAs encoding GAT-1 and the mutants Y140F and Y140W were subcloned in the oocyte expression vector pOG, which contains a 5′-untranslated *Xenopus* β-globin sequence and a 3′-poly(A) signal). Capped cRNAs transcribed from these constructs were injected into stage V–VI *Xenopus* oocytes (50 ng/oocyte), and membrane currents were recorded 2–3 days later (15, 24). Recording solution (Ringer) contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES (pH 7.4). In substitution experiments sodium ions were replaced with equimolar choline or lithium, and chloride was replaced by acetate. Two microelectrode voltage clamp recordings were performed at 22 °C with a Geneclamp 500 interfaced to an IBM-compatible PC using a Digidata 1200 A/D controlled using the pCLAMP 6.0 program suite (Axon Instruments). The data were acquired at 2 kHz and low pass filtered at 1 kHz. Microelectrodes were filled with 3 M KCl solution and had resistances of <1 megohm. In Cl^- substitution experiments, a 3 M KCl-agar bridge connected the recording chamber to a 3 M KCl reservoir containing an Ag/AgCl electrode. Records shown are typical and representative of experiments done with four or five independent oocytes. Blockade by SKF 100330A was reversible but required extensive washout periods (>5 min).

RESULTS

Identification of a Tyrosine Residue Critical for GABA Transport—Fig. 1 shows the position of the 12 conserved tyrosine residues, predicted to be located in the membrane according to the theoretical topological model (4). Tyrosine residues 72, 86, 140, 309, and 475 located in respective transmembrane domains I, II, III, VI, and X, are totally conserved in all known members of the superfamily. The others are mostly aromatic in the majority of the transporters. Using site-directed mutagenesis each of these residues was changed to serine. After the mutations were verified by sequencing, the mutant cDNAs were expressed in HeLa cells using the recombinant vaccinia/T7 virus (18) as described (19). The results of sodium-dependent [^{3}H]GABA transport of the mutants are shown in Fig. 2. Replacement of each of the five residues 241, 246, 248, 296, and 432 by serine results in transporters that exhibit significant activity. Because of the very different nature of the side chains of tyrosine and serine, we conclude that these five residues are not essential for sodium- and chloride-dependent GABA transport. It is of interest to note that all of the fully conserved tyrosines are included in the remaining seven in which replacement by serine severely compromises transport. Each of these seven tyrosines was then also changed to the other aromatic amino acids, phenylalanine and tryptophan. The results are shown in Fig. 3. It can be seen that in six out of the seven cases at least one of the aromatic replacement mutants exhibits significant transport of [^{3}H]GABA. Only in the case of tyrosine 140 are both replacement mutants, Y140F and Y140W, totally devoid of any detectable transport activity. These mutations were subcloned back into the wild type background. Sequencing of the subcloned mutant cDNA fragments from both sides was then performed to ensure that only the indicated mutation had occurred without any additional changes. Reexamination of the subcloned Y140F and Y140W transporters showed that their phenotype, lack of detectable sodium-dependent [^{3}H]GABA transport, was maintained (not shown). D,L-[3,4-^{3}H]Nipecotic acid, a ring constrained analog of GABA, is also transported by wild type GAT-1 in a sodium-dependent fashion but not by Y140F- and Y140W-GAT-1 transporters (data not shown).

The impaired transport of the mutants is not due to lower transporter levels. Wild type GAT-1 and Y140F- and Y140W-GAT-1 mutant transporters were expressed in HeLa cells, labeled with [^{35}S]methionine, and immunoprecipitated by an

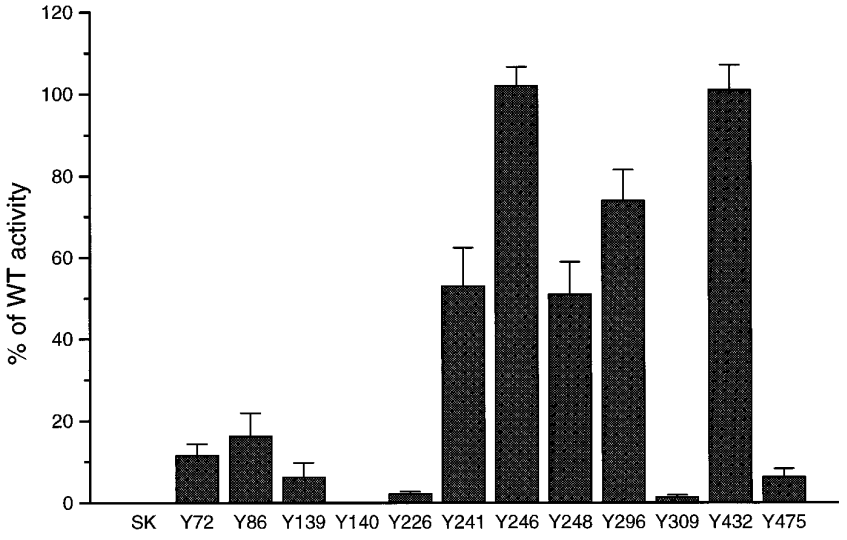

FIG. 2. **Transport activity of GAT-1 and mutant transporters in which tyrosine has been changed to serine.** HeLa cells were infected with recombinant virus and transfected with pBluescript containing wild type (*WT*) or the indicated mutants. Sodium-dependent transport of [^{3}H]GABA was performed as described under "Experimental Procedures." Results are given as a percent of wild type transport activity. Each *bar* is the mean ± S.E. of three to five different experiments.

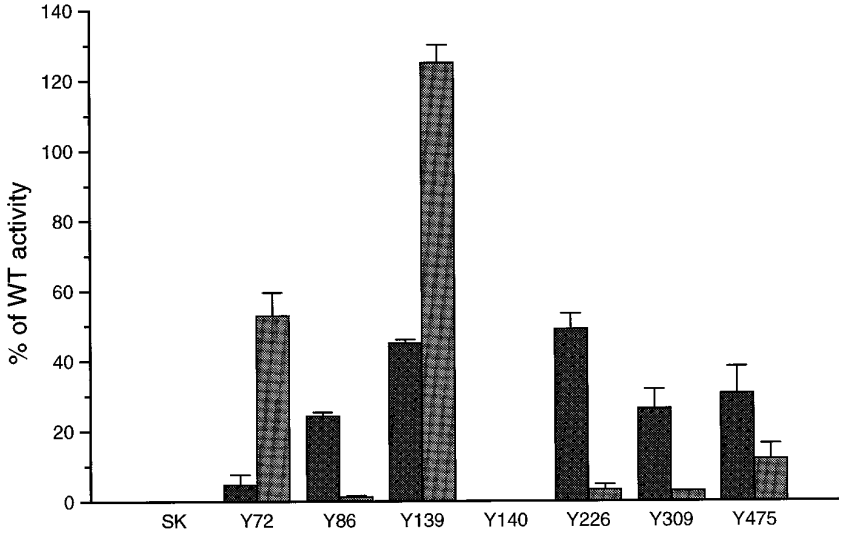

FIG. 3. **Transport activity of GAT-1 and mutant transporters in which tyrosine has been changed to phenylalanine and tryptophan.** Results of the sodium-dependent [^{3}H]GABA transport of the indicted mutants are given as a percentage of the value of the wild type (*WT*). *Dark bars*, mutations to phenylalanine; *light bars*, mutations to tryptophan.

antibody raised against a peptide located in the carboxyl terminus of GAT-1. The transporter is detected in three major forms: a monomer running as a band of ~65 kDa, a more abundant dimer, and a high molecular mass aggregate (Fig. 4, *lane 1*). In addition, a faster running minor band can be visualized (Fig. 4) which corresponds to the transporter that has not been glycosylated (20). No transporter is detected in HeLa cells transfected with the vector alone (Fig. 4, *lane 2*). In contrast each of the two mutants, Y140F- and Y140W-GAT-1, express high transporter levels (Fig. 4, *lanes 3* and *4*).

Because the mutants still produce normal transporter levels it is possible that they are targeted inefficiently to the plasma membrane. One would expect that cells expressing a mistargeted mutant transporter that is intrinsically active would have 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 (14, 25) and of the glutamate transporter GLT-1 (26). Both mutant transporters remain completely void of activity after solubilization and reconstitution, in contrast to the wild type (data not shown).

Sodium and Chloride Binding to Y140F- and Y140W-GAT-1—The above results indicate that mutant transporters Y140F and Y140W have an intrinsic defect in the transport process

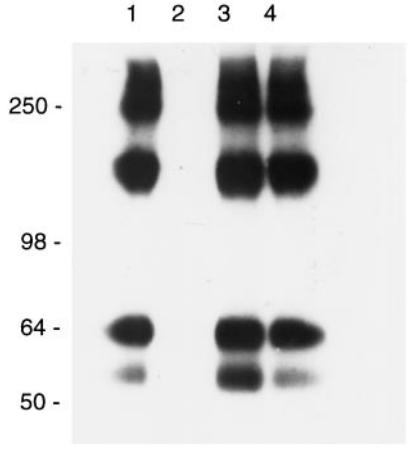

FIG. 4. **Immunoprecipitation of wild type and mutant transporters.** HeLa cells were infected with the vaccinia/T7 recombinant virus and transfected with the indicated cDNAs. After labeling with [^{35}S]methionine, cells were immunoprecipitated as described under "Experimental Procedures." The cDNA used to transfect was pT7-GAT-1 (wild type, *lane 1*), vector alone (pBluescript SK$^-$), *lane 2*), Y140F (*lane 3*), or Y140W (*lane 4*).

itself. We used an electrophysiological approach monitoring partial reactions of the translocation cycle to pinpoint the defect in the mutant transporters. One such reaction is the bind-

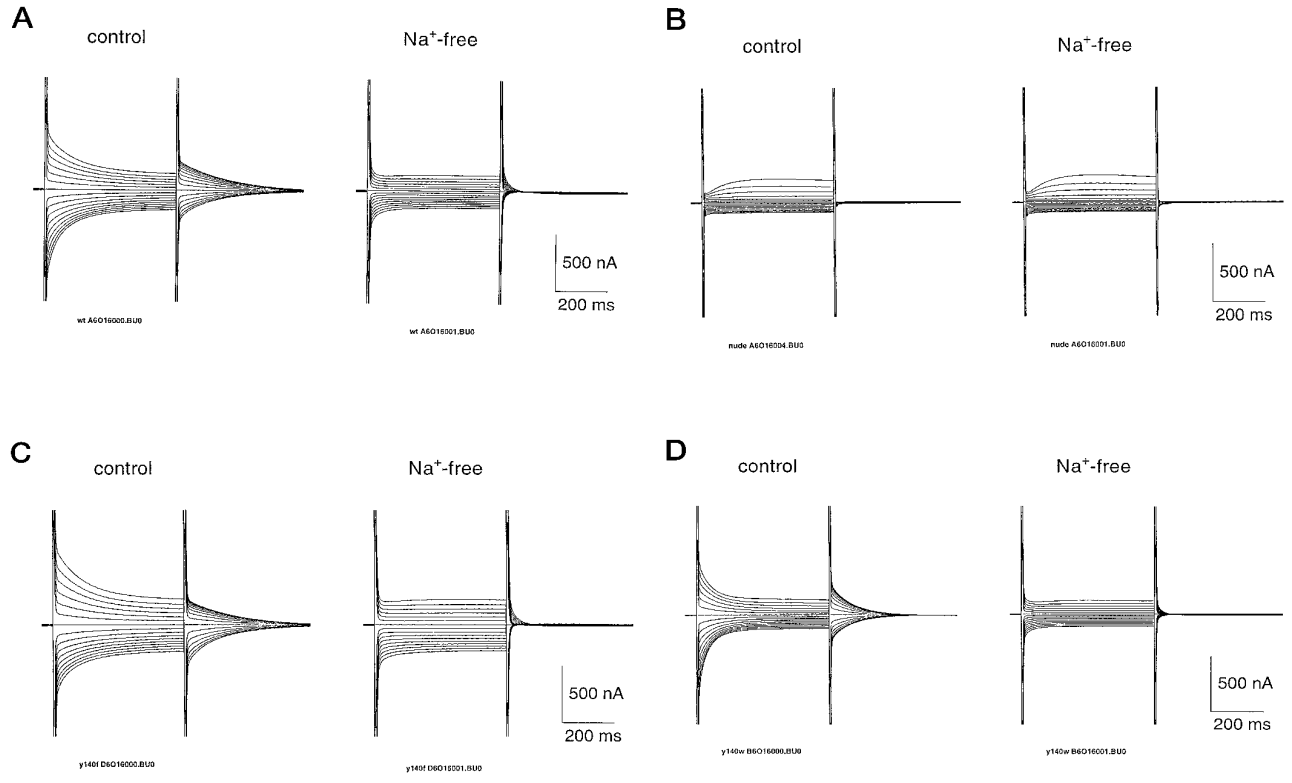

FIG. 5. **Sodium-dependent transient currents mediated by wild type (*wt*) and mutant transporters.** Transient currents were recorded in the presence and the absence of sodium (choline substitution) in oocytes expressing wild type (*panel A*) and Y140F (*panel C*) and Y140W (*panel D*) transporters as well as in noninjected oocytes (*panel B*). The voltage was jumped from a holding potential of −30 mV to potentials ranging from −120 to +30 mV. The duration of each pulse was 500 ms. Following reperfusion with sodium solutions after choline, complete recovery of the transient currents was observed (data not shown).

ing of sodium to the transporter, which can be monitored as a sodium-dependent transient current observed after changing the membrane potential (8, 15). This is exemplified in Fig. 5. Oocytes expressing GAT-1 are held at a membrane potential of −30 mV in a sodium-containing medium. Subsequently the membrane potential is jumped to a range of potentials varying from +30 mV (Fig. 5*A*, *upper trace*) to −120 mV (Fig. 5*A*, *lower trace*). In oocytes expressing GAT-1, slow current relaxations (time constants 70–130 ms) were observed following voltage jumps, and these transient currents were greatly attenuated by replacement of sodium with choline (Fig. 5*A*). These currents are distinct from the rapidly decaying membrane capacitance and are presumed to reflect transporter-mediated charge movements associated with sodium binding (8, 15). Uninjected control oocytes did not display this slow sodium-dependent relaxation (Fig. 5*B*). Analogous sodium-dependent transient currents were also observed in oocytes expressing the GAT-1 mutant Y140F and Y140W transporters (Fig. 5, *C* and *D*). In oocytes expressing the mutant transporter Y140W, the sodium-dependent transient current displayed slightly faster relaxation, with time constants ranging from 40–80 ms, whereas the relaxation times of the Y140F mutant were unchanged from wild type GAT-1.

In addition to sodium, chloride ion also interacted with both the mutant wild type transporters. As reported previously (15), wild type GAT-1 exhibits a GABA-independent and lithium-dependent leak current that is increased by the presence of chloride ions (Fig. 6). An analogous lithium-dependent current was observed in oocytes expressing the Y140F and Y140W mutant transporters, and this current was increased in the presence of chloride (Fig. 6). These results demonstrate that the mutant transporters Y140F and Y140W retain both the lithium-dependent leak current and the ability to bind chloride seen in the wild type transporter.

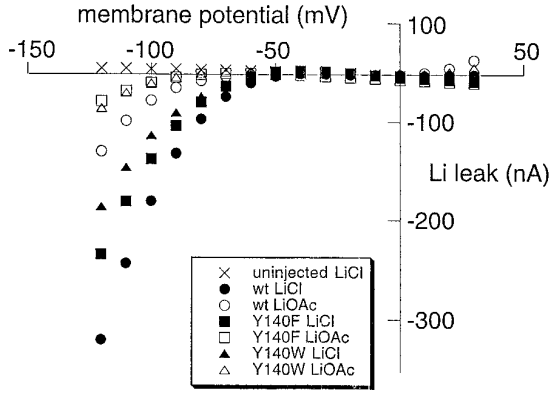

FIG. 6. **Chloride-dependent lithium leak currents mediated by wild type (*wt*) and mutant transporters.** Steady-state lithium-induced currents (obtained by subtraction of the response in sodium) in representative oocytes expressing wild type and mutant transporters were recorded at the indicated potentials, in the presence or absence of chloride (acetate substitution). Similar results were obtained in three or four oocytes.

Interaction of GABA and Uptake Blockers with Wild Type and Mutant GAT-1—The sodium-dependent charge movements induced by voltage jumps are inhibited by nontransported GABA analogs (8). This is exemplified for wild type GAT-1 (Fig. 7*A*), which shows the transient current blocked by SKF 100330A at 30 μM, a concentration 2 orders of magnitude above its apparent K_i for blocking GABA transport (27). Consistent with this, 30 μM SKF 100330A blocked 98.0 ± 0.4% (n = 3) of the off charge movement induced by stepping from −120 to −30 mV (Fig. 8*A*). In contrast, SKF 100330A was significantly less efficacious at blocking the charge movements in the

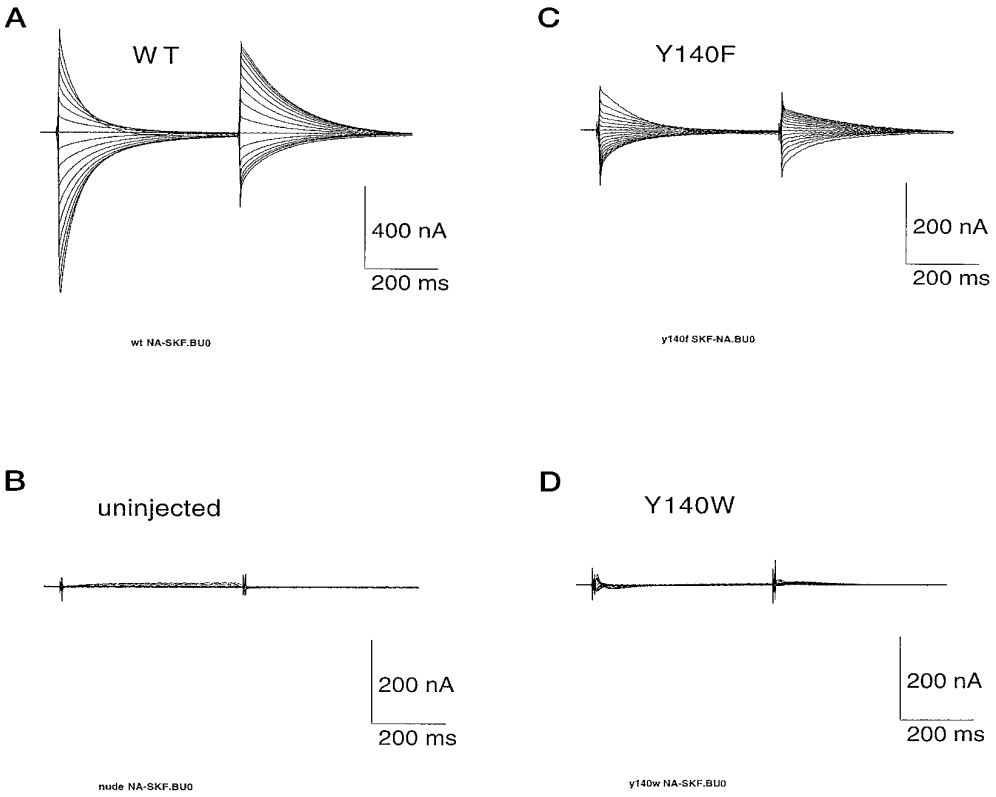

FIG. 7. **Currents blocked by SKF 100330A mediated by wild type (*WT*) and mutant transporters.** Difference records are shown representing SKF 100330A (30 μM); sensitive currents were obtained by subtraction of currents recorded in the presence of blocker at potentials between +30 and −120 mV from control currents. Superfusion of oocytes expressing wild type (*panel A*), Y140F (*panel C*), or Y140W (*panel D*) transporters was done in a sodium-containing medium. Records for noninjected oocytes are also shown (*panel B*). The conditions were similar to those in Fig. 5.

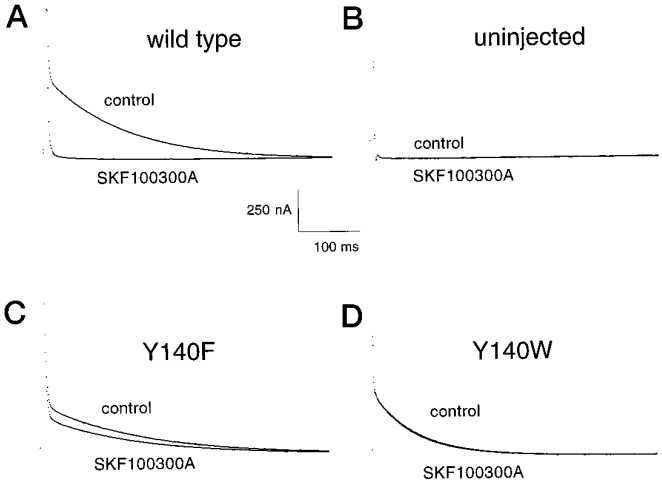

FIG. 8. **SKF 100330A effects on the off charge movements elicited by stepping back from −120 to −30 mV.** The same cells were used in Fig. 7. The transient currents obtained by stepping back from −120 to −30 mV in the presence or absence of 30 μM SKF 100330A are shown.

Y140F transporter, 33.9 ± 7.2% (n = 3), as illustrated in Figs. 7 and 8, *panels C*. Increasing the concentration of the inhibitor to 100 μM blocked 63% of the charge movement (n = 2). This difference in potency was even greater in the Y140W mutant, in which 100 μM SKF 100330A blocked only 1.9 ± 1.0% (n = 4) of the charge movement (Figs. 7 and 8, *panels D*).

GABA itself, when added in a sodium chloride-containing medium, induces a steady-state transport current in the wild type (Fig. 9*A*). In agreement with the tracer flux data (Fig. 3), GABA is not able to induce a transport current in the mutants (Fig. 9, *B* and *C*). Moreover, it is not even capable of inhibiting a transient current (Fig. 9, *B* and *C*). Thus, not only is GABA not transported by the mutant transporters, it is not even able to bind to them.

DISCUSSION

In this paper we have identified a tyrosine residue, which is critical for sodium- and chloride-dependent GABA recognition and transport. Out of the 12 conserved residues, tyrosine 140 is the only one that does not tolerate replacement to either phenylalanine or tryptophan (Fig. 3). This is neither due to reduced synthesis (Fig. 4) nor to reduced targeting (Figs. 5–7). The electrophysiological (Figs. 5–8) data clearly show the presence of sodium-dependent transient currents and chloride-dependent leak currents of lithium, strongly indicating that the mutant transporters are localized in the plasma membrane and that the structural elements critical for forming the transporter pore are intact. They strongly point to a defective GABA binding in the mutants. Not only are GABA and nipecotic acid not transported, GABA is not able to suppress the capacitative transient sodium current in Y140F- and Y140W-GAT-1 as it does in the wild type (Fig. 9). This is also supported by the total inability of SKF 100330A to suppress the clearly existent sodium transient in Y140W-GAT-1 (Figs. 5*D*, 7*D*, and 8*D*). This compound is a derivative of the GABA analog guvacine to which via the nitrogen atom a diphenylbutenyl group has been attached (27). The observation that the blocker still has some effect, albeit diminished, on the transient current of Y140F-GAT-1 indicates that the mutation does not cause a sterical alteration of the binding pocket for GABA. Although far from clear, its limited ability to interact with Y140F, but not with Y140W-GAT-1, could perhaps be due to an interaction of one of the phenyl groups of SKF 100330A with the introduced phenyl-

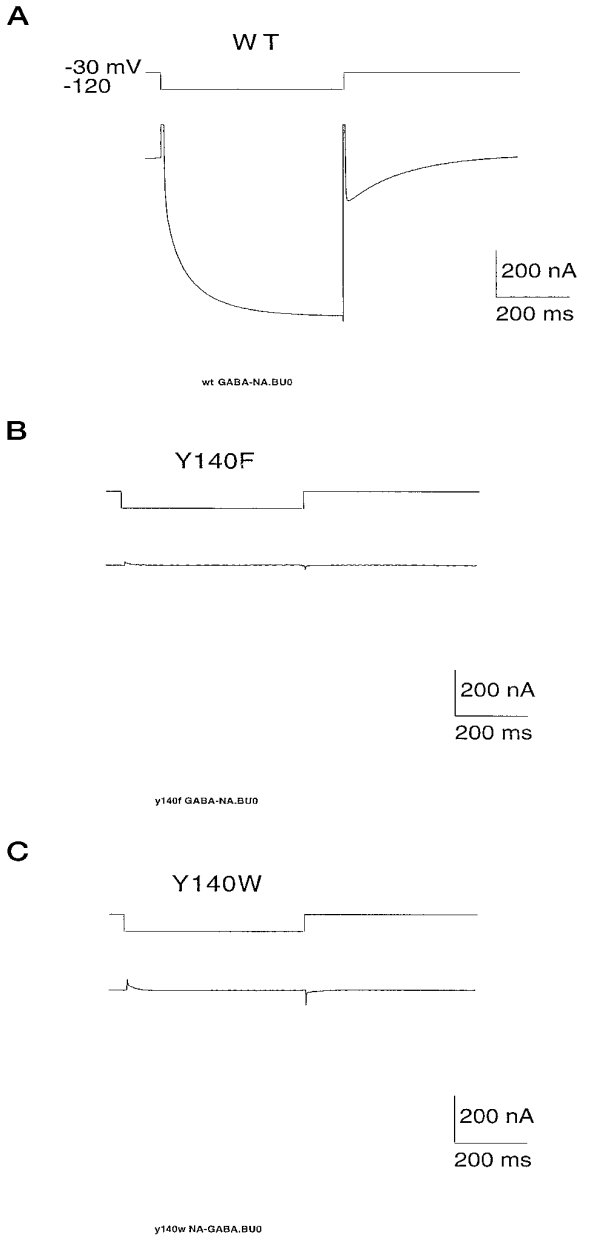

FIG. 9. **Steady-state transport currents by wild type (*WT*) and Y140F/W mutant transporters.** Subtracted records showing currents induced by GABA in representative oocytes expressing GAT-1 wild type (*panel A*) and mutants Y140F (*panel B*) and Y140W (*panel C*). The membrane potential was stepped for −30 to −120 mV and back as indicated in the *upper trace*.

alanine at the 140 position.

In view of the total conservation of the tyrosine residue in the superfamily of transporters, it is tempting to speculate that it interacts with a structural element common to all of the transported solutes. This would be the amino group that is common to both kinds of substrates: amino acids and biogenic amines. In fact, a mutant form of the serotonin transporter in which the equivalent tyrosine (tyrosine 176) was replaced with cysteine has decreased affinity for both serotonin and cocaine. Moreover, residual binding of a cocaine analog to this mutant is blocked by chemical modification of the cysteine at position 176.[3] The fact that Y140F-GAT-1 is totally void of transport (Figs. 3 and 9*B*) suggests that it is the oxygen atom, which is critical perhaps via the formation of a hydrogen bond with an amine hydrogen.

Several amino acid residues/regions of the transporters have been implicated in neurotransmitter binding. Tryptophan 222 of GAT-1 is one example (14), but recent data indicate a highly reduced V_{max} of GABA transport but an increased affinity for it in tryptophan 222 mutants.[4] The extracellular loops of GAT-1 also have been implicated (28), but it is very possible that they function as a selectivity filter. In the dopamine transporter it has been suggested that aspartic acid 79 located in the postulated first transmembrane domain and serines 356 and 359 located in seventh are involved in binding of the amine (29). These residues are conserved among the three biogenic amine transporters, and it is possible, but not proven, that in these cases they play, together with tyrosine 140, a role in neurotransmitter binding.

At this time it not possible to speculate on which other residues might be involved in the liganding of GABA or other transmitters. The theoretical model of the topology (4) may not be in harmony with novel experimental data (20, 30). Until the topology is firmly resolved, it is impossible to know which other group might be located close by tyrosine 140. It is also difficult to speculate on the identity of residues interacting with the carboxyl group of GABA. One possibility is that the determinants on some of the external loops are involved (28). These are located in the carboxyl-terminal half of the transporter. Interestingly, studies with chimeric biogenic amine transporters indicate that regions involved in the recognition of the biogenic amines are located in transmembrane domains 5–8 and even more toward the carboxyl terminus (31–34). It is anticipated that further mutagenesis studies focusing on these regions may shed further light on the determinants for neurotransmitter binding.

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