Substrate-induced Regulation of γ-Aminobutyric Acid Transporter Trafficking Requires Tyrosine Phosphorylation*

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Neurotransmitter transporters regulate synaptic transmitter levels and are themselves functionally regulated by a number of different signal transduction cascades. A common theme in transporter regulation is redistribution of transporter protein between intracellular stores and the plasma membrane. The triggers and mechanisms underlying this regulation are important in the control of extracellular transmitter concentrations and hence synaptic signaling. Previously, we demonstrated that the γ-aminobutyric acid transporter GAT1 is regulated by direct tyrosine phosphorylation, resulting in an up-regulation of transporter expression on the plasma membrane. In the present report, we show that two tyrosine residues on GAT1 contribute to the phosphorylation and transporter redistribution. Tyrosine phosphorylation is concomitant with a decrease in the rate of transporter internalization from the plasma membrane. A decrease in GAT internalization rates also occurs in the presence of GAT1 substrates, suggesting the hypothesis that tyrosine phosphorylation is required for the substrate-induced up-regulation of GAT1 surface expression. In support of this hypothesis, incubation of GAT1-expressing cells with transporter ligands alters the amount of GAT1 tyrosine phosphorylation, and substrate-induced surface expression is unchanged in a GAT1 mutant lacking tyrosine phosphorylation sites. These data suggest a model in which substrates permit the phosphorylation of GAT1 on tyrosine residues and that the phosphorylated state of the transporter is refractory for internalization.

γ-Aminobutyric acid (GABA)¹ is the major inhibitory neurotransmitter in the central nervous system. The inhibitory action of GABA, mediated through both GABAₐ and GABAₐ receptors, is regulated by GABA transporters (1–3), integral membrane proteins located perisynaptically on neurons and glia (4). GABA transporters also play a pathophysiological role in temporal lobe epilepsy (5) and are the targets of pharmacological interventions in epilepsy treatment (6). Recent evidence suggests that GABA transporters, and neurotransmitter transporters in general, are not passive players in regulating neuronal signaling; rather, transporter function can be altered by a variety of initiating factors and signal transduction cascades (for review see Refs. 7 and 8). In general, this functional regulation occurs in two ways, by changing the rate of transmitter flux through the transporter (9) or by changing the number of functional transporters on the plasma membrane. A recurring theme in transporter regulation is the rapid redistribution of the transporter protein between intracellular locations and the cell surface. This functional modulation occurs in part through activation of second messengers such as kinases, phosphatases, arachidonic acid, and pH. These factors may act directly on the transporter protein (e.g. by phosphorylation; see Refs. 10–13). However, the mechanisms underlying transporter phosphorylation and transporter redistribution have yet to be fully elucidated. Also unknown is whether such second messenger-correlated effects alter rates of transporter internalization, externalization, or both.

Previously, we showed that inhibitors of tyrosine kinases decreased GABA uptake in hippocampal neurons that endogenously express GAT1. The decrease in uptake seen with tyrosine kinase inhibitors was correlated with a decrease in direct tyrosine phosphorylation of GAT1 and resulted in a redistribution of the transporter from the cell surface to intracellular locations (14). In the present report, we show that two tyrosine residues on GAT1 are responsible for both the phosphorylation of GAT1 and for its subcellular distribution. The increase in surface GAT1 expression that correlates with tyrosine phosphorylation is because of a decrease in the internalization rate of the transporter. Furthermore, substrate-induced up-regulation of GAT1 expression (15) requires direct tyrosine phosphorylation. These data suggest a model in which GAT1 substrates permit the phosphorylation of the transporter on tyrosine residues and that the phosphorylated state of the transporter limits transporter internalization.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary (CHO) cells were maintained in α-minimal essential medium supplemented with 5% fetal bovine serum, l-glutamine, and penicillin/streptomycin. Transfections were carried out using LipofectAMINE (Life Technologies, Inc.) in OptiMEM I (Life Technologies, Inc.). The lipid/DNA mix was incubated with the cells for 5 h; cells were then rinsed and fed with complete medium.

Site-directed Mutagenesis—GAT1 tyrosine residue mutants were made using Altered Sites I (Promega). Five putative intracellular tyrosine residues (Tyr-107, Tyr-317, Tyr-412, Tyr-481, and Tyr-598; see Ref. 14) were mutated to alanine individually or together. The mutants were subcloned into pcDNA3 for subsequent transfections. All mutations were confirmed by sequencing.

³H/GABA Uptake Assays—Pre-assay drug incubations (5–15 min) were performed in HBSS. Following incubation, cells were rinsed three times in 1× HBSS and allowed to equilibrate for 10 min in the final wash. Buffer was then exchanged with control HBSS or drug-containing HBSS. GABA was added to initiate the assay. The final [³H]GABA concentration of the assay solution was 40 nM; the total GABA concentration of the assay solution was 30 μM. The assay was terminated by rapidly rinsing the cells 3 times with 1× HBSS, followed by solubilization in 300 μl of 0.001–0.005% SDS at 37 °C for 2 h. Aliquots were used

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¹ The abbreviations used are: GABA, γ-aminobutyric acid; CHO, Chinese hamster ovary; HBSS, HEPES-buffered saline solution; Mes, 4-morpholineethanesulfonic acid; DAT, dopamine transporter.

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for scintillation counting and to determine protein concentrations. Statistical analyses of the uptake data were performed using SPSS. Two-sample comparisons were made using t tests; multiple comparisons were made using one-way analysis of variances followed by Tukey’s honestly significant difference post-hoc test.

Surface Biotinylation—Biotinylation experiments were performed essentially as described (17, 18). Cells were grown in 60-mm tissue culture dishes to 80% confluence. The cells were rinsed twice with 37 °C phosphate-buffered saline/CaCl2 (Pierce) in phosphate-buffered saline/Ca2+/Mg2+/CaCl2 (PH9262) with the appropriate drug, cells were lysed in buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris) and then incubated at 37 °C for 60 min with gentle shaking. The biotinylation solution was removed by two washes in phosphate-buffered saline/Ca/Mg plus 100 mM glycine and quenched in this solution by incubating the cells at 4 °C for 45 min with gentle shaking. The cells were lysed with 1 ml of radioimmune precipitation buffer (in mM: 100 Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mM aprotinin, 250 μg phenylmethylsulfonyl fluoride) at 4 °C for 60 min. The cell lysates were centrifuged at 20,000 × g at 4 °C for 60 min. The supernatant fractions (300 μl) were incubated with an equal volume of ImmunoPure immobilized monomeric avidin beads (Pierce) for 60 min. The beads were washed three times with radioimmune precipitation buffer, and adsorbed proteins were eluted with SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM mercaptoethanol) at room temperature for 30 min. For the internalization experiments, cells were labeled with biotinylation reagent at 4 °C, brought up to 22 °C for different time periods, and then placed immediately on ice. Removal of already surface-bound NHS-SS-biotin was performed using Mes-Na+ as described (19). Briefly, dishes were washed with buffer (150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris) and then incubated for 10 min with buffer, for 30 min with buffer containing freshly dissolved 10 mM Mes-Na+, and twice for 60 min with buffer with Mes-Na+. Control dishes were treated identically, except the Mes-Na+ was omitted. The dishes were then thoroughly rinsed three times with buffer to remove Mes-Na+.

Immunoprecipitations and Immunoblotting—Following treatment with the appropriate drug, cells were lysed in buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 250 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM activated sodium orthovanadate, 5 mM sodium pyrophosphate) for 1 h at 4 °C. Lysates were precleared with 10 μl of protein G-agarose conjugate (Sigma), followed by immunoprecipitation of GAT1 using anti-GAT1 antibody 346J (20) and protein G-agarose. The product was washed in radioimmune precipitation buffer and run on a 10% acrylamide gel. Protein was transferred to a nitrocellulose membrane by electroblotting. Western blotting was performed using the anti-phosphotyrosine antibody PY99 (Santa Cruz Biotechnology) and visualized using ECL reagents (Amer sham Pharmacia Biotech).

RESULTS

Previously we showed that GAT1, endogenously expressed in hippocampal neurons and heterologously expressed in CHO cells, was a substrate for tyrosine-mediated phosphorylation, and that a GAT1 mutant lacking all five putative intracellular tyrosine residues was refractory for tyrosine phosphorylation (14). To determine the site or sites of tyrosine phosphorylation of GAT1, we made additional GAT1 mutants that contained only one of five intracellular tyrosine residues; the other four putative intracellular tyrosine residues in each construct were changed to alanine residues. These mutant transporters were expressed in CHO cells and subjected to immunoprecipitation with an anti-phosphotyrosine antibody and subsequent immunoblotting with an anti-GAT1 antibody. These results are shown in the upper immunoblot of Fig. 1. Wild-type GAT1 and constructs containing tyrosine residues only at amino acid positions 107 or 317 (16) showed immunoreactive bands of the appropriate size; constructs containing individual tyrosine residues at amino acid positions 412, 481, and 598 and the GAT1 mutant lacking all five putative intracellular tyrosine residues (5YA) did not show immunoreactive bands. These results suggest that two tyrosine residues in GAT1, Tyr-107 in the putative first intracellular loop and Tyr-317 in the putative third intracellular loop, can be phosphorylated in vivo. To determine whether the amount of phosphorylation of these residues could be modulated, similar experiments were repeated in the presence of the tyrosine kinase inhibitor genistein. These results are shown in the lower immunoblot of Fig. 1. Both the Tyr-107 construct and the Tyr-317 construct showed reduced levels of phosphorylation in the presence of genistein.

In GAT1 expressed endogenously in hippocampal neurons, decreases in tyrosine phosphorylation correlate with the following two events: (i) an approximate 50% decrease in GABA uptake and (ii) a decrease in the amount of GAT1 protein on the plasma membrane, accompanied by a concomitant increase of GAT1 protein in intracellular locations (14). To determine which of the two tyrosine phosphorylation sites may be associated with these functional and trafficking effects, we examined GABA uptake and GAT1 subcellular distribution of the Tyr-107 and Tyr-317 mutants. These results are shown in Fig. 2. Genistein treatment of CHO cells expressing either the Tyr-107 phosphorylation site or the Tyr-317 phosphorylation site alone resulted in an approximate 25–35% reduction in radiolabeled GABA uptake, compared with ~55% in wild-type GAT1-expressing cells (Fig. 2A). None of the other GAT1 constructs containing individual tyrosine residues, nor the GAT1 mutant lacking all putative intracellular tyrosine residues, showed reduced uptake. These data suggest that either tyrosine phosphorylation site is sufficient to mediate the genistein inhibition of GABA uptake.

To determine whether the decrease in function mediated by tyrosine phosphorylation correlated with a decrease in surface expression of GAT1, we performed surface biotinylation experiments on wild-type GAT1 and various GAT1 tyrosine mutants. Representative immunoblots are shown in Fig. 2B, and their quantitation is shown in Fig. 2C. In wild-type GAT1 expressed in CHO cells, approximately half of the GAT1 protein, in the untreated state, was found on the plasma membrane. Treatment with genistein reduced the amount of GAT1 found on the plasma membrane by half, which correlated well with the 50% reduction of GABA uptake seen in these cells following genistein treatment. The loss in plasma membrane expression of GAT1 also correlated with an accumulation of GAT1 intracellularly. CHO cells expressing GAT1 mutants containing only the Tyr-107 phosphorylation site or the Tyr-317 phosphorylation site showed basal expression and genistein-mediated redistribution that was comparable with wild-type GAT1; that is, in either phosphorylation site mutant ~50% of the transporter was found on the plasma membrane in the basal state, and this amount was reduced in the presence of genistein. In a GAT1 mutant in which both tyrosine phosphorylation sites
Two tyrosine residues in GAT1 are required for both the up-regulation of GAT1 function and the subcellular redistribution of GAT1 induced by tyrosine kinases. A, GABA uptake is reduced by genistein in transporters containing one of two tyrosine phosphorylation sites. CHO cells were transfected with wild-type GAT1 (WT), GAT1 mutants containing only one of five putative intracellular tyrosine residues (Y107, Y317, Y412, Y481, and Y598), or a GAT1 mutant with all five putative intracellular tyrosine residues removed (5YA). Data are from four separate experiments, six wells/condition/experiment. GABA uptake under control conditions ranged from 115 to 556 fmol/min/mg protein. The genistein concentration was 10 μM. Experimental conditions that resulted in a significant change (p < 0.05) from control values are denoted by the asterisk.

B, the subcellular distribution of a mutant GAT1 protein lacking tyrosine phosphorylation sites is unchanged following genistein treatment. CHO cells were transfected with wild-type GAT1, GAT1 mutants containing only one putative intracellular tyrosine residue (Y107 or Y317), or a GAT1 mutant lacking both tyrosine phosphorylation sites (Y107A and Y317A). Cells were incubated for 20 min prior to biotinylation in control medium or medium containing 10 μM genistein. Representative immunoblot shows GAT1 immunoreactivity in intracellular (I; non-biotinylated) and surface (S; biotinylated) fractions. C, surface expression of GAT1 is reduced by genistein and by GAT1 mutants lacking tyrosine phosphorylation sites. CHO cells were transfected with wild-type GAT1 (WT), GAT1 mutants containing only one of two intracellular tyrosine phosphorylation sites (Y107 and Y317), or GAT1 mutants lacking two (Y107A and Y317A) or five (5YA) intracellular tyrosine residues. Data are from densitometry measurements made from three separate experiments and plotted with respect to total cellular amounts of GAT1 protein. Experimental conditions that resulted in a significant change (p < 0.05) from control values are denoted by the asterisk.

Tyrosine phosphorylation of GAT1 controls the rate of transporter internalization from the plasma membrane. A, representative immunoblots of the internalization rate of untreated wild-type GAT1 (upper blot), wild-type GAT1 treated with 10 μM genistein (middle blot), or untreated GAT1 5YA mutant (lower blot). Values under each blot refer to the time permitted for internalization at 22 °C. The total amount of surface GAT1 at the start of the assay is denoted by $S$. B, quantitation of the internalization rate of GAT1 as shown in A. The same amount of internalized transporter was greater at all time points measured. In a set of control experiments, wild-type GAT1-expressing cells were labeled with biotin and then immediately stripped; no bands were seen in subsequent immunoblots (data not shown), suggesting that all of the immunoreactivity seen in these experiments came from internalized transporters.
transporter. The rates of internalization (Fig. 3B) were quantified by comparing the immunoreactivity at each time point with the initial surface levels of GAT1 (denoted as S in Fig. 3A). These calculations showed that untreated wild-type GAT1 internalized at ~10% per min at 22 °C; both genistein-treated wild-type GAT1 and tyrosine phosphorylation-deficient GAT1 mutants internalized approximately twice as fast. Although we do not yet know whether there are also changes in the rates of GAT1 externalization, the alterations in transporter internalization are sufficient to account for the 50% decrease in both GABA uptake and surface transporter protein in tyrosine phosphorylation-deficient transporters.

Thus, tyrosine phosphorylation correlates with increases in surface GAT1 protein levels and GABA uptake, and this is consistent with decreases in transporter internalization rates. Previously, we showed that substrates of GAT1 such as GABA and the transporter-specific substrate nipecotic acid also increase surface GAT1 protein levels and GABA uptake (15). These data suggested that this effect of substrates may occur by similarly decreasing transporter internalization rates. To test this hypothesis, wild-type GAT1-expressing cells were treated with nipecotic acid and examined for changes in function and trafficking. These data are shown in Fig. 4. Treatment for 10 min with nipecotic acid resulted in a significant increase in radiolabeled GABA uptake; this increase was blocked by co-application of the GABA transporter-specific antagonist SKF89976A (Fig. 4A). Changes in transporter internalization rates in the presence of nipecotic acid were performed as described in Fig. 3. Nipecotic acid treatment decreased the internalization of GAT1 compared with untreated cells (Fig. 4B). Quantification of the internalization rate showed that the decrease in internalization rates was ~50%, consistent with the approximate 2-fold increase in GABA uptake following nipecotic acid treatment.

The data from Figs. 3 and 4 revealed that tyrosine phosphorylation and substrate incubation resulted in a similar phenotype, namely increased function, increased surface transporter expression, and decreased internalization rates. This raised the possibility that these two modulatory events share a common mechanism. Thus, we tested the hypothesis that GAT1 ligands regulate tyrosine phosphorylation of GAT1. These results are shown in Fig. 5. Cells expressing wild-type GAT1 were untreated or treated with nipecotic acid or SKF89976A, subjected to immunoprecipitation with an anti-phosphotyrosine antibody, and immunoblotting with an anti-GAT1 antibody. Nipecotic acid treatment increased the amount of tyrosine phosphorylation of GAT1 (Fig. 5A, upper blot) whereas SKF89976A caused a decrease in the amount of tyrosine phosphorylation of GAT1 (Fig. 5A, lower blot). As a control, the GAT1 mutant lacking all putative intracellular tyrosine residues did not show immunoreactive bands, either in the untreated state or in the nipecotic acid-pretreated state (Fig. 5A, upper blot). Quantitation of the immunoblots of wild-type GAT1 phosphorylation in the presence of GAT1 ligands is shown in Fig. 5B.

The increase in tyrosine phosphorylation of GAT1 in the presence of GABA transporter substrates and the decrease in tyrosine phosphorylation of GAT1 in the presence of GABA transporter antagonists suggested that ligand-induced regulation of GAT1 function and surface expression may be mediated by tyrosine phosphorylation. To test this hypothesis, we examined ligand-induced regulation of wild-type GAT1 and GAT1 mutants that cannot be tyrosine-phosphorylated. These results are shown in Fig. 6. Cells expressing wild-type GAT1 or GAT1 5YA were treated with nipecotic acid and subjected to surface biotinylation (Fig. 6A). Whereas the amount of surface expression of wild-type GAT1 increased in the presence of nipecotic acid, no increase in surface expression was detected in the GAT1 5YA mutant. In addition, the nipecotic acid-mediated increase in surface expression of wild-type GAT1 was blocked by co-application of genistein (Fig. 6B, upper blot), and the decrease in surface expression of GAT1 in the presence of SKF89976A was not further affected by co-application of genistein. Quantification of these results (Fig. 6C) showed that prevention of tyrosine phosphorylation (either by genistein or by removal of tyrosine phosphorylation sites) eliminated the substrate-induced up-regulation of GAT1 surface expression. Furthermore, the presence of transporter antagonists (SKF89976A) reduced surface GAT1 expression, and this reduction was concomitant with a decrease in antagonist-mediated tyrosine phosphorylation (see Fig. 5). These data suggest that GABA transporter ligands mediate their trafficking effects on GAT1 via tyrosine phosphorylation.

**DISCUSSION**

Neurotransmitter transporters exhibit a number of functional properties that enable them to influence extracellular
neurotransmitter levels. For example, transport rates are dependent on substrate concentrations, with half-maximal effective concentrations for transport occurring in the submicromolar to low micromolar range (21, 22). Transporter function is bidirectional, and non-vesicular efflux of transmitter may contribute to ambient extracellular transmitter levels. Transporter efflux may be related to pathophysiological conditions (5, 23, 24), but it can also be a primary mechanism of transmitter release (25, 26). Neurotransmitter transporter expression can also be regulated, and changes in the number of functional transporters will contribute to the control of synaptic neurotransmitter levels either through transport directly (27) or by changing the number of synaptic binding sites for released transmitter (28).

One trigger for alterations in surface transporter expression is feedback from extracellular transmitter. The serotonin transporter is differentially regulated by psychostimulants that act as either serotonin transporter antagonists or substrates (29). Norepinephrine transporters are down-regulated following chronic treatment with the norepinephrine transporters antagonist desipramine, perhaps through changes in protein expression and/or transporter turnover (30). In contrast to serotonin transporter, the norepinephrine transporters agonists norepinephrine and amphetamine also cause decreases in expression (31). In dopamine transporters (DATs), DAT agonists dopamine and amphetamine cause a net accumulation of intracellular DAT; this internalization is blocked by the DAT antagonist cocaine (32). GABA and other GABA transporter substrates and antagonists interact with the GABA transporter GAT1 to directly regulate transmitter uptake by up-regulating or down-regulating surface transporter expression, respectively (15). In the present report, we show that GABA transporter ligands alter tyrosine phosphorylation of GAT1 and that this event is required for ligand-induced changes in surface GAT1 expression. Increases in tyrosine phosphorylation of either of two tyrosine residues in GAT1 expression, respectively (15). In the present report, we show that GABA transporter ligands alter tyrosine phosphorylation of GAT1 and that this event is required for ligand-induced changes in surface GAT1 expression. Increases in tyrosine phosphorylation of either of two tyrosine residues in GAT1 results in a decrease in the rate of GAT1 internalization from the plasma membrane, effectively increasing the functional expression of GAT1.

It is hypothesized that direct phosphorylation of the serotonin transporter by protein kinase C serves as a tag that identifies transporters to be internalized (12). This might occur, because the tag is indicative of a transporter in an appropriate conformational state for internalization. The evidence that serotonin transporter substrates prevent protein kinase C-dependent phosphorylation and increase surface transporter expression supports a role for conformational changes in the transporter as being important for redistribution (29). We suggest a similar model for GAT1; substrates produce a conformational change in the transporter protein that increases the likelihood that the transporter will become tyrosine-phospho-
rylated. Tyrosine phosphorylation is then the molecular switch for preventing internalization.

Tyrosine kinase activity has been shown to regulate redistribution of receptors (33) and channels (34), although the mechanism for these effects is not completely understood. Tyrosine phosphorylation is important in G protein-coupled receptor internalization (35), and tyrosine phosphorylation enhances the interaction of the receptor with proteins involved in trafficking (36). Transporters have been shown to associate with a number of trafficking proteins (9, 37, 38), and it is possible that tyrosine phosphorylation of GAT1 regulates the ability of the transporter to interact with such components. In addition to decreasing the rate of transporter internalization, tyrosine phosphorylation of the transporter may also affect other aspects of the transporter trafficking process, such as externalization. For the type 4 glucose transporter, insulin causes both a decrease in internalization rates and an increase in externalization rates, leading to net increases in surface transporter expression (39). For GAT1, the magnitude of the decrease in transporter internalization rates would appear to account for the net change in GAT1 surface expression, but we cannot rule out a change in transporter externalization rates in the presence of tyrosine phosphorylation. We are presently pursuing this question, although to date our direct externalization measurements are confounded by ongoing transporter internalization during the assay. Regardless, the ability of transporter ligands to regulate at least one arm of the transporter recycling process via tyrosine phosphorylation reveals a link between the triggers for transporter redistribution and their underlying molecular mechanisms.

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