Regulation of a γ-Aminobutyric Acid Transporter by Reciprocal Tyrosine and Serine Phosphorylation*

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A feature of the rat brain γ-aminobutyric acid transporter GAT1, and other members of the neurotransmitter transporter family, is its regulated redistribution between intracellular locations and the plasma membrane. Recent studies have focused upon defining the signaling molecules that facilitate this redistribution. Agents that promote direct tyrosine phosphorylation of GAT1 promote a relative increase in surface GAT1 levels, and this results from a slowing of the transporter internalization rate. Agents that act to increase protein kinase C (PKC) activity promote a relative decrease in surface GAT1 levels; whether this effect is caused by direct transporter phosphorylation is unknown. The opposing actions of tyrosine kinase activity and PKC activity raise the possibility that the subcellular distribution of GAT1 can be associated with mutually exclusive transporter phosphorylation events. The present experiments show that GAT1 is phosphorylated on serine residues in a PKC-dependent manner, but this state is only revealed when GAT1 tyrosine phosphorylation is eliminated or greatly reduced. The relative levels of serine phosphorylation and tyrosine phosphorylation are negatively correlated. The amount of serine phosphorylation is regulated by agents that affect tyrosine phosphorylation, and vice versa. In addition, the ability of agents that affect tyrosine kinase activity to regulate GAT1 serine phosphorylation requires a change in its tyrosine phosphorylation state. These data support the ideas that affect tyrosine kinase activity to regulate GAT1 serine phosphorylation states and that the relative abundance of these states determines in part the relative subcellular distribution of the transporter.

Neurotransmitter transporters are integral membrane proteins, expressed at or near the synapse on neurons and glia, that function by coupling the uptake of neurotransmitter to the movement of co-transported ions down their electrochemical gradients. Homologous recombination experiments in which various neurotransmitter transporter genes have been manipulated demonstrate a critical role for these transporters in maintaining appropriate levels of transmitter in the synaptic cleft and the importance of this maintenance on behavior (1–3). Studies such as these raise the likelihood that neurons and glia have developed means by which to rapidly regulate transporter function to control these extracellular transporter levels. Recent evidence suggests this to be true. Transporter function can be altered by a variety of triggering factors and signal transduction cascades (for review, see Refs. 4–8). In general, this functional regulation occurs either by changing the rate of substrate translocation through the transporter, or by changing the number of functional cell surface transporters. A recurring theme in transporter regulation is the rapid redistribution of the transporter between intracellular locations and the plasma membrane. This 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. 9–12).

The γ-aminobutyric acid (GABA)1 transporter GAT1 (13), one member of the Na⁺- and Cl⁻-dependent transporter family SLC6 (14), regulates the activity of GABA_a and GABA_b receptors at some synapses (15–17), plays a pathophysiological role in temporal lobe epilepsy (18), and is the target of drugs used in seizure treatment (19). Much is known about the characteristics of GAT1's regulated trafficking. Intracellularly localized GAT1 resides on a small (50 nm), clear vesicle that recycles on and off the plasma membrane of hippocampal neurons on a time scale of seconds to minutes in a calcium- and activity-dependent manner (20). GAT1 associates with, and is regulated by (21, 22) soluble N-ethyl maleimide-sensitive factor attachment protein receptor (SNARE), which is part of the apparatus involved in the docking and fusion of neurotransmitter-containing synaptic vesicles to the plasma membrane (23). For example, interaction with syntaxin 1A increases GAT1 expression on the cell surface (24). GAT1 internalization is associated with clathrin- and dynamin-dependent processes (20), and ~5% of surface-localized GAT1 is internalized each minute in the basal state (25). Thus, a balance of recurrent exocytosis and endocytosis determines GAT1 expression.

In cultures of hippocampal neurons that endogenously express GAT1, the relative subcellular distribution of the transporter in the basal state is ~60% surface to 40% intracellular (26, 27). However, a number of signals can alter this balance. Transporter substrates increase relative surface expression, whereas transporter antagonists decrease it (26). Phorbol esters that activate PKC (28–31) and G protein-coupled receptors that are linked to PKC activation (32) cause a relative increase in intracellularly localized protein. In contrast, agents that promote tyrosine phosphorylation cause a relative increase in surface localized protein (27). This effect is caused by direct phosphorylation of GAT1 residues Y107 and Y317, and the relative increase in surface expression results from a slowing of the rate of transporter internalization (25). The serotonin transporter is directly phosphorylated by PKC (33), and it is

1 The abbreviations used are: GABA, γ-aminobutyric acid; PKC, protein kinase C; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; 4a-PDD, 4α-phorbol 12,13-didecanoate.

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hypothesized that this event tags the transporter for internalization (34). Taken together, these observations suggest a hypothesis in which GAT1 is phosphorylated either on tyrosine residues or on serine/threonine residues, and that the relative abundance of these states determines the relative subcellular distribution of the transporter.

EXPERIMENTAL PROCEDURES

Cell Culture and GAT1 Mutagenesis—Hippocampal cultures were prepared from rats aged from postnatal day 0 to 3 (32). Experiments were performed after 10–14 days in vitro. CHO cells were maintained in α-minimal essential medium supplemented with 5% fetal bovine serum, 5 mM l-glutamine, and penicillin/streptomycin. Serum-deprived neuronal cultures were maintained in neurobasal medium supplemented with B-27 (Invitrogen). Two GAT1 tyrosine residue mutants that are refractory for tyrosine phosphorylation were made using Altered Sites I (Promega). In GAT1–5YA, all five putative intracellular tyrosine residues (Tyr-107, Tyr-317, Tyr-412, Tyr-481, and Tyr-598) are mutated to alanine (27), Tyr-107,Tyr-317 is an alanine double mutant that eliminates two sites known to be tyrosine-phosphorylated (24). Mutations were confirmed by sequencing. All constructs were subcloned into pcDNA3 (Invitrogen) for subsequent transfections. Transfections were carried out using FuGene 6 (Roche) in Opti-MEM I (Invitrogen).

[3H]GABA Uptake Assays—Pre-assay drug incubations were performed in HEPES-buffered saline. After incubation, cells were rinsed three times in 1× HEPES-buffered saline solution and allowed to equilibrate for 10 min in the final wash. Buffer was then exchanged with control HEPES-buffered saline or drug-containing HEPES-buffered saline. 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 three times with HBSS, followed by solubilization in 300 μl of 0.001% SDS at 37 °C for 2 h. Aliquots were used for scintillation counting and to determine protein concentrations. Statistical analyses were performed using SPSS (SPSS Inc.). Two-sample comparisons were made using t tests; multiple comparisons were made using one-way analyses of variance followed by Tukey’s honestly significant difference post hoc test.

Surface Biotinylation—Biotinylation experiments were performed essentially as described previously (34, 35). Cells were grown to 80% confluence in 60-mm tissue culture dishes. The cells were rinsed twice with 37 °C phosphate-buffered saline/calcium/magnesium buffer (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 9.6 mM Na2HPO4, 1 mM MgCl2, and 0.1 mM CaCl2, pH 7.4). The cells were next incubated with 2 ml of a solution containing 1 mg/ml sulfo-N-hydroxysuccinimide biotin (Pierce) in phosphate-buffered saline/calcium/magnesium buffer for 20 min at 4 °C with gentle shaking. The biotinylation solution was removed by two washes in phosphate-buffered saline/calcium/magnesium buffer at 37 °C. Cells were thenbuffered 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 radioimmunoprecipitation assay buffer (100 mM Tris-Ci, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM leupeptin, 1 μg/ml aprotinin, and 250 μM phenylmethylsulfonyl fluoride) at 4 °C for 60 min. The supernatant fractions (300 μl) were incubated with an equal volume of Immunopure Immobilized Membronic Avidin beads (Pierce) for 60 min. The beads were washed three times with radioimmunoprecipitation assay buffer, and adsorbed proteins were eluted with SDS sample buffer (62.5 mM Tris-Ci, pH 6.8, 2% SDS, and 100 mM mercaptoethanol) at room temperature for 30 min. Analysis was performed on aliquots taken before incubation with beads (total cell lysate), 2) of the supernatant fraction after adsorption and centrifugation (intracellular fraction), and 3) of the bead eluate(biotinylated fraction). Western blotting was then performed using anti-GAT1 antibody 346J, anti-phosphotyrosine (Santa Cruz Biotechnology), anti-phosphoserine (Zymed Laboratories Inc.), or anti-phosphothreonine (Zymed Laboratories Inc.) antibodies.

Phosphorylation—Experiments were performed essentially as described previously (11). Briefly, cells were rinsed in Dulbecco’s modified Eagle’s medium and then incubated for 1 h at 37 °C with Dulbecco’s modified Eagle’s medium containing 1 mM/mL [32P]orthophosphate for 1 h to equilibrate the intracellular ATP pools. Various drugs or control solutions were then added. At the end of the incubation period, the cells were washed twice in phosphate-buffered saline and lysed in 500 μl well ice-cold radioimmunoprecipitation assay buffer containing phosphatase inhibitors (10 mM sodium fluoride, 50 mM sodium pyrophosphate, and 1 μM okadaic acid) for 1 h at 4 °C. Extracts were then centrifuged at 20,000 × g for 30 min at 4 °C. Immunoprecipitations were performed as described above using anti-GAT1 antibody 346J and protein A-agarose. Radiolabeled proteins were detected and quantified by direct imaging (Alpha Innotech).

RESULTS

Previous data showed that agents that promote tyrosine kinase activity (and tyrosine phosphorylation of GAT1) are associated with increased surface GAT1 expression (25, 27), and agents that promote PKC activity are associated with decreased surface GAT1 expression (21, 29). How do these two activities interact? To begin to address this question, subcellular GAT1 expression and GAT1 function were assessed in CHO cells stably expressing GAT1 (Fig. 1). As shown previously, in untreated cultures, ~50–60% of GAT1 was found on the cell surface based upon biotinylation with an impermeant biotiny-
lating reagent (Fig. 1A). Two control experiments supported these findings: first, the intracellular cytoskeletal protein actin was not labeled by the biotinylating reagent, suggesting that only surface proteins were being labeled. Second, immunoreactive bands were not seen in parental CHO cells immunoblotted with GAT1 antibody (data not shown).

In cultures treated with the tyrosine phosphatase inhibitor pervanadate and the PKC inhibitor bisindolylmaleimide, more than 80% of GAT1 protein was found in surface fractions, as expected given that tyrosine kinase activity should dominate in this treatment condition. In addition, as expected given that PKC activity should dominate, when cells were treated with the PKC activator PMA and the tyrosine kinase inhibitor K252a, more than 80% of GAT1 was found in intracellular fractions. When cultures were treated such that both tyrosine kinase and PKC activities were probably inhibited (using k252a and bisindolylmaleimide), or when tyrosine phosphatases were inhibited and phorbol esters were activated (using pervanadate and PMA), subcellular distribution of GAT1 was indistinguishable from untreated cultures. These results are consistent with the idea that the relative balance of these two activities is associated with the subcellular distribution of the transporter rather than the absolute activities associated with either or both pathways. In addition, the level of GAT1 surface expression correlated highly with GABA uptake in cultures treated in parallel, suggesting that these signaling pathways regulate function at least in part via subcellular transporter redistribution (Fig. 1B).

In general, this balance of activities that regulate transporter expression and function could be managed in one of two ways. Each activity could be independent of the other, or the activity of one pathway could regulate the activity of the other. To examine these possibilities, CHO cells expressing wild-type GAT1 were examined for levels of tyrosine phosphorylation in the presence of increasing concentrations of PMA (Fig. 2). As the PMA concentration increased, the relative amount of GAT1 that was recognized by a phosphotyrosine antibody decreased in a concentration-dependent manner. This was probably a result of PKC activation by PMA, because a non-active phorbol ester analog of PMA (4αPDD) did not alter the amount of GAT1 recognized by the phosphotyrosine antibody and because co-application of the PKC inhibitor bisindolylmaleimide eliminated the PKC-mediated effect. The concentration range over which PMA exerted its effects was comparable with that associated with redistribution of GAT1 to intracellular locations (21), and correlated with a decrease in GAT1 function (Fig. 2B). These data suggest that PKC activity regulates GAT1 tyrosine phosphorylation.

An attractive model resulting from the above data is that GAT1 can exist in either a PKC-phosphorylated state or a tyrosine kinase-phosphorylated state, and the relative abundance of these states determines the subcellular distribution of the transporter. One problem with this hypothesis is that although direct tyrosine phosphorylation of GAT1 has been shown (25), direct PKC-mediated phosphorylation has not. PKC-mediated effects on GAT1 subcellular distribution and GAT1 function are present even in GAT1 mutants in which consensus serine and threonine phosphorylation sites have been removed (29). However, several considerations prompted revisiting this possibility. First, direct PKC-mediated phosphorylation occurs for another member of this transporter family (11). Second, phosphorylation often occurs at non-consensus sites. Third, the data from Fig. 2 showing that PMA reduced tyrosine phosphorylation raised the possibility for the opposite effect (i.e. that PKC-mediated phosphorylation might be obscured by tyrosine kinase activity).

Therefore, to increase the probability of detecting PKC-mediated GAT1 phosphorylation, experiments were carried out using two different GAT1 mutants that are refractory for tyrosine phosphorylation: GAT1–5YA (27) and Tyr-107,Tyr-317 (24). CHO cells stably expressing these mutants were incubated with PMA, the phosphatase inhibitor okadaic acid, and [32P]orthophosphate and subjected to subsequent immunoprecipitation (Fig. 3A). Precipitation with a GAT1-specific antibody revealed a radioactive band of the size expected for GAT1 protein. This band was not present when the antibody was pre-adsorbed by a GST fusion protein containing the antibody recognition site, when using pre-immune sera for the precipitation, or in parental CHO cells. A nonspecific signal was present in all experimental conditions, although the source of this signal is unknown. To confirm this phosphorylation event, experiments were performed using antibodies that specifically recognize phosphoserine or phosphothreonine residues. CHO cells stably expressing GAT1–5YA were subjected to immunoprecipitation using each phospho-specific antibody and subsequently immunoblotted using a GAT1-specific antibody (Fig. 3B). GAT1 immunoreactivity was present in the phosphoserine-precipitated lysates but not in phosphothreonine-
precipitated lysates or in parental CHO cell lysates. Taken together, these data suggest that GAT1 is a serine-mediated phosphoprotein.

To further characterize this phosphorylation event, CHO cells stably expressing GAT1–5YA were treated with agents that enhance or diminish PKC activity, subjected to immunoprecipitation using a GAT1 antibody, and then evaluated for the relative amount of GAT1–5YA that was recognized by the phosphoserine antibody (Fig. 4A). Compared with the untreated condition, the amount of GAT1–5YA recognized by the phosphoserine antibody was significantly reduced by bisindolylmaleimide, whereas okadaic acid and PMA both significantly increased the amount of GAT1–5YA recognized by the phosphoserine antibody. PMA concentration-response experiments (Fig. 4B) revealed that the concentration range over which PMA exerted its effects was compatible with that required to reduce tyrosine phosphorylation (see Fig. 2) and to redistribute GAT1 to intracellular locations (21). The PMA effect was probably caused by PKC activation, because 4αPDD did not alter the amount of GAT1 recognized by the phosphoserine antibody and because co-application of the PKC inhibitor bisindolylmaleimide eliminated the PMA-mediated effect. These data suggest that, at least in the absence of tyrosine phosphorylation sites, GAT1 is a PKC-regulated phosphoprotein.

The data to this point indicated that GAT1 can be both tyrosine-phosphorylated (Fig. 2) and serine-phosphorylated (Fig. 3) and that the PKC activator PMA can reduce the amount of GAT1 that is tyrosine-phosphorylated. These results suggested a model in which tyrosine kinase and PKC compete to phosphorylate GAT1. If true, there should be a negative correlation between tyrosine kinase phosphorylation and PKC phosphorylation. To test this prediction, CHO cells expressing wild-type GAT1 were treated with various inhibitors and enhancers of tyrosine kinase, tyrosine phosphatase, and PKC activities, subjected to immunoprecipitation with a GAT1 antibody, and then probed for total GAT1 protein and the relative activities, subjected to immunoprecipitation with a GAT1 antibody. The representative immunoblot shows phosphoserine immunoreactivity in the precipitates (right blot) relative to total GAT1 immunoreactivity (left blot). Data from three such experiments are quantified in the graph from densitometry measurements and plotted relative to total GAT1 immunoreactivity. *, experimental conditions that resulted in a significant change (p < 0.05) from control values. B, cells measured as in A were left untreated or treated for 15 min before immunoprecipitation with increasing concentrations of PMA (○), 4αPDD (●), or PMA + 0.1 μM bisindolylmaleimide (□). Data are from three experiments.
GAT1 Reciprocal Phosphorylation

Fig. 5. GAT1 tyrosine phosphorylation and serine phosphorylation are negatively correlated. CHO cells stably expressing wild-type GAT1 were left untreated or treated for 15 min before immunoprecipitation with (applied individually or in combination) 0.1 μM bisindolylmaleimide (Bis), 0.01 μM K252a, 1.0 μM PMA, and 100 μM pervanadate (Van). Lysates were precipitated using anti-GAT1 antibody. A, representative immunoblot shows total GAT1 immunoreactivity in the precipitates (lower blot) relative to phosphotyrosine (upper blot) and phosphoserine immunoreactivity (middle blot). B and C, data from three experiments performed as in A for phosphotyrosine (filled bars) and phosphoserine (open bars) immunoreactivity, quantified from densitometry measurements and plotted relative to total GAT1 immunoreactivity. Notable experimental conditions that resulted in a significant change (p < 0.05) from control values are denoted by the asterisk; n.s., not significant.

Fig. 6. GAT1 serine phosphorylation is unchanged in a GAT1 tyrosine phosphorylation mutant. CHO cells stably expressing the GAT1–5YA mutant were left untreated or treated for 15 min before immunoprecipitation with 100 μM pervanadate (Van) or 0.01 μM K252a. Lysates were precipitated using anti-GAT1 antibody. The representative immunoblot shows phosphoserine immunoreactivity in the precipitates (right blot) relative to total GAT1 immunoreactivity (left blot). Data from three such experiments are quantified in the graph from densitometry measurements and plotted relative to total GAT1 immunoreactivity.

GAT1 recognized by the phosphotyrosine antibody. The addition of pervanadate reversed this effect.

The data in Fig. 5 are consistent with the idea that there is competition between the states of GAT1 tyrosine and GAT1 serine phosphorylation: first, GAT1 exhibited reciprocal phosphorylation; as the relative amount of tyrosine phosphorylation increased, the relative amount of serine phosphorylation decreased and vice versa. Second, tyrosine kinase inhibitors not only reduced tyrosine phosphorylation but also increased serine phosphorylation; tyrosine phosphatase inhibitors had the opposite effect. Third, PKC activators not only increased serine phosphorylation but also decreased tyrosine phosphorylation.

The ability of K252a, for example, to inhibit tyrosine phosphorylation and increase serine phosphorylation is consistent with a model in which the lack of tyrosine phosphorylation of GAT1 is necessary to permit serine-mediated phosphorylation. However, another possibility is that the lack of tyrosine phosphorylation is not required to permit serine phosphorylation; rather, the actions of K252a on each pathway are separate. To examine this idea in more detail, CHO cells expressing GAT1–5YA were treated with K252a or pervanadate and then examined for the relative amount of GAT1 that was recognized by the phosphoserine antibody (Fig. 6). Unlike in wild-type GAT1, neither pervanadate nor K252a affected the relative amount of GAT1–5YA recognized by the phosphoserine antibody. Thus, changes in serine phosphorylation induced by agents that affect tyrosine kinase activity occur only when the tyrosine phosphorylation state of GAT1 changes. This is consistent with the hypothesis that the two phosphorylation states are mutually exclusive.

In hippocampal neurons that endogenously express GAT1, direct tyrosine phosphorylation is observed (25). In an attempt to reveal endogenous serine-mediated phosphorylation of GAT1 previously not seen, conditions were chosen that increased PKC activity and decreased tyrosine kinase activity. Hippocampal cell cultures were treated with PMA, okadaic acid, and K252a and then immunoprecipitated with a phosphoserine antibody. Subsequent immunoblotting with a GAT1 antibody revealed faint immunoreactivity (Fig. 7A). This band was absent in cultures treated to enhance PKC activity alone or to inhibit tyrosine kinase activity alone. These data are comparable with the results seen in CHO cells stably expressing GAT1 and suggest that similar phosphorylation-dependent events may be occurring in neurons that endogenously express the transporter. To examine this possibility in more detail, neuronal cultures were treated in ways that modulated GAT1 function via endogenous PKC and tyrosine signaling pathways (27, 32). These included placing the cultures in serum-free medium to reduce brain-derived neurotrophic factor receptor-mediated tyrosine kinase activity (27), and applying the metabotropic glutamate receptor agonist (S)-3,5-dihydroxyphenylglycine, which leads to activation of PKC (32). Once again, under conditions in which both relative tyrosine kinase activity is reduced and PKC activity is increased, faint immunoreactivity could be observed (Fig. 7B).

**DISCUSSION**

Based upon electrophysiological analysis using GABA transport antagonists, GAT1 can regulate both the magnitude and time course of GABA<sub>A</sub> and GABA<sub>B</sub> receptor signaling at particular synapses (15–17). However, estimates of the rate at which individual GABA transporters can translocate GABA are approximately tens of molecules per second (36, 37), which is likely to be several orders of magnitude too slow to regulate synaptic transmission, at least at synapses containing GABA<sub>A</sub> receptors. This observation, as well as experiments examining
glutamate transporter function (38), has led to the idea that a significant portion of the action of transporters on cell signaling arises from the transporters acting as transmitter sinks, essentially binding transmitter away from receptor sites. Estimates of the number of GAT1 molecules present at some nerve terminals are as high as 1,000–2,000 per square micron (39), thus providing ample sites for transporter sequestration. If the number of GABA transporter binding sites is a crucial determinant of GABAergic transmission, then modulating the number of these sites via transporter trafficking will be important physiologically, not only by controlling synaptic GABA signaling but also potentially by regulating spillover of GABA onto neighboring synapses (40). The present data show that the relative distribution of GAT1 protein between surface pools and intracellular pools is regulated by reciprocal phosphorylation of tyrosine and serine residues on GAT1. These results support the idea that GAT1 exists in either of two mutually exclusive phosphorylation states and that the relative abundance of these states acts to determine the relative subcellular distribution of the transporter.

Initial experiments on PKC regulation of GAT1 (29–31) showed that drugs that alter PKC levels in cells had a dose-dependent effect on radiolabeled GABA uptake that occurred on a time scale of minutes. However, elimination of the consensus serine and threonine phosphorylation sites on GAT1 failed to eliminate the PKC effect (29). This suggested that PKC-mediated phosphorylation was occurring on non-consensus sites, that the PKC effect was indirect, or both. Indeed, PKC regulates the interaction of GAT1 and syntaxin 1A via phosphorylation of the syntaxin 1A binding partner Munc-18 (21). Phosphorylation of Munc-18, which inhibits its binding to syntaxin 1A, promotes inhibition by syntaxin 1A of GAT1 function. The present data show that PKC also exerts effects on GAT1 via direct phosphorylation. Identification of the serine residue(s) in GAT1 responsible for this effect is presently under investigation. Based upon secondary structure predictions, 12 intracellular serine residues could be responsible. Of particular interest are two serine residues located near Y107 and Y317 known to be phosphorylated in GAT1 (25).

An increase in the relative amount of GAT1 that is serine-phosphorylated correlates with an increase in GAT1 surface expression; an increase in the relative amount of GAT1 that is serine-phosphorylated correlates with an accumulation of intracellular GAT1. Thus, one possibility is that tyrosine phosphorylation is required for externalizing GAT1 or maintaining GAT1 surface expression, whereas PKC-mediated phosphorylation is required to internalize the transporter or maintain it in an intracellular pool. However, this is probably not the case. In the presence of both tyrosine kinase inhibitors and PKC inhibitors, GAT1 expresses functionally on the plasma membrane similar to that seen in the absence of these inhibitors. In addition, a GAT1 mutant that cannot be tyrosine-phosphorylated still expresses functionally on the surface and recycles, although the rate at which it internalizes is slowed (25). Therefore, it is more likely that phosphorylation states of the transporter modulate basal trafficking rates that are phosphorylation-independent.

Thus, the following model is proposed: Vesicles containing GAT1 are inserted into the membrane in a calcium-dependent manner and internalized via a clathrin-dependent manner (20). Serine phosphorylation places the transporter in a conformation that disfavors tyrosine phosphorylation and favors interactions with internalization machinery, thus speeding endocytosis and increasing the size of the intracellular GAT1 pool. Tyrosine phosphorylation would have the opposite effects. This model suggests one role for multiple transporter phosphorylation states (i.e. in regulating the availability of the transporter to be internalized); thus phosphorylation is presumed to be occurring on surface-bound GAT1. This may be true, given that the data show a correlation between the amount of GAT1 on the cell surface and the amount of GAT1 that is in either of the two phosphorylated states. Experiments are presently under way to determine whether these two phosphorylation states exist only for surface-bound GAT1.

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