Multiple Molecular Determinants in the Carboxyl Terminus Regulate Dopamine Transporter Export from Endoplasmic Reticulum*

Received for publication, November 21, 2003, and in revised form, May 5, 2004
Published, JBC Papers in Press, May 5, 2004, DOI 10.1074/jbc.M312774200

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The plasma membrane dopamine transporter (DAT) has an essential role in terminating dopaminergic neurotransmission by reuptake of dopamine into the presynaptic neurons. Therefore, the amount of DAT at the cell surface is a critical determinant of DAT function. In this study, we examined the role of the carboxyl terminus of DAT in trafficking of the transporter through the biosynthetic pathway to the plasma membrane. Live cell fluorescence microscopy and cell surface biotinylation were used to study the effects of systematic deletions and alanine substitutions in the carboxyl terminus on DAT localization. It was found that alanine substitutions of Lys-590 and Asp-600 significantly delayed the delivery of DAT to the plasma membrane because of retention of DAT in the endoplasmic reticulum (ER). Most surprising, mutation of Gly-585 to alanine completely blocked the exit of DAT from the ER and surface expression of the transporter. The effect of these three mutations on ER export of DAT was demonstrated in porcine aortic endothelial cells and in immortalized neuronal cell line 1RB_A557. In primary cultures of rat embryonic midbrain neurons, DAT G585A, K590A, and D600A mutants were restricted to the cell soma and did not traffic to the dendrites or axonal processes. These data are consistent with the model whereby the local conformation and/or intramolecular interactions of the sequences of the DAT carboxyl terminus proximal to the last transmembrane domain are essential for the ER export of the transporter.

Dopaminergic neurotransmission plays a critical role in modulation of a variety of central nervous system functions, including locomotor activity, cognition, and reward (1). Dopamine signaling is initiated by release of dopamine from the presynaptic neuron cell, and its duration and intensity are regulated primarily by the reuptake of dopamine back into the dopaminergic neurons by the plasma membrane dopamine transporter (DAT). DAT belongs to the family of Na+/Cl−-dependent plasma membrane neurotransmitter transporters, which includes the norepinephrine, serotonin (SERT), glycine, and γ-aminobutyric acid transporters (2). DAT is a 620-amino acid polypeptide embedded in the lipid bilayer by 12 hydrophobic segments. Both amino and carboxyl termini are predicted to be intracellular. The second extracellular loop contains at least three consensus N-glycosylation sites (3–5).

In the mammalian brain, DAT is found in all parts of dopamine neurons, including in cell bodies and dendrites in substantia nigra and ventral tegmental area and in axonal termini in striatum and cerebral cortex. The maximal surface expression of DAT is detected in synapses located along the axonal processes in striatum and in distal regions of dendrites in the ventral tegmental area (6, 7). This specific targeting and the normal activity of DAT in neurons require efficient export of newly synthesized transporters from the ER, sorting to transport carriers in the Golgi complex, and delivery and retention of the transporters at the presynaptic functional sites. The amount of DAT at the cell surface is also regulated by endocytosis. Although all these trafficking processes are currently being investigated, in this study we focused on the transport of DAT from the ER. The molecular mechanisms of the efficient sorting of membrane multispanning proteins into cotransport COPII vesicles in the ER are not understood. Two sequence motifs in the cytoplasmic domains of transmembrane proteins, the di-acidic ((D/E)X(E/D), where X is any amino acid) and di-phenylalanine motifs, have been shown to mediate ER export (8–11). These motifs, however, have been identified only in a limited number of transmembrane proteins, and it is likely that other sequence motifs exist that are capable of serving as ER export signals.

Recent studies of DAT in model cell expression systems have revealed a number of requirements for the normal transporter trafficking to the plasma membrane. The carboxyl terminus of human DAT (hDAT) has been demonstrated to be important for cell surface expression of DAT (12, 13). The interaction of the last three residues of human DAT (LKV620) with the PDZ domain of PICK1 (protein that interacts with C kinase) increases the efficiency of ER-to-plasma membrane trafficking of the transporter (12, 13). In contrast, DAT lacking the amino
terminus is efficiently delivered to the cell surface (13, 14). The oligomerization of DAT in the ER has also been proposed to be essential for the efficient ER export of the transporter (13, 14). A number of mutations within the transmembrane domains of DAT also resulted in inefficient ER exit (15, 16). The ER retention of these mutants is probably a consequence of either incorrect folding and/or disruption of the normal membrane topology of the mutant DAT in the ER.

In the present study we performed an extensive mutagenesis analysis of the carboxyl-terminal tail of hDAT. This analysis revealed that in addition to the most distal three residues, several residues in the carboxyl terminus region proximal to the membrane are essential for transport of DAT from the ER to the plasma membrane in three types of cells, including immortalized neuronal cells and primary cultures of embryonic midbrain neurons.

EXPERIMENTAL PROCEDURES

**Plasmids and Mutations**—YFP-DAT, CFP-DAT, YFP-NΔ-DAT (deletion of the first 65 amino acid residues), and YFP-DAT-C615 (truncation of the last 5 amino acid residues) were described previously (14). The full-length hDAT in pcDNA3.1 was kindly provided by Dr. G. W. Miller (Emory University). Single amino acid substitutions were made using the pEGFP-C1, ΔN-DAT-G585A, or ΔN-pEDGFP-C1, or ΔN-pEDCNA3.1 as templates and a QuickChange site-directed mutagenesis kit according to the manufacturer’s protocol (Stratagene Cloning Systems, La Jolla, CA). To generate ΔN-DAT, C616, C615, C610, and C576 truncation mutants, a stop codon was created at the positions corresponding to amino acid residues 619, 616, 611, and 577, respectively, in the DAT-C615 using a QuickChange site-directed mutagenesis. DAT mutants C597-LKV and C586-LKV were generated by replacing three residues following residue 597 (598–600) or 586 (587–589) by LKV, and by creating a stop codon at the positions corresponding to residues 601 and 590, respectively, using site-directed mutagenesis. The mutant ΔN-DAT-610 was generated by replacing residues 581–590 by residues 611–620 followed by a stop codon using Quick-change mutagenesis.

To generate the EGFR receptor (EGFR)-DAT chimera, the XhoI restriction site was introduced into wtEGFR-pEGFP-N1 (17) at the position corresponding to amino acid residues 645–645 of the EGFR by using site-directed mutagenesis. A DNA fragment encoding residues 581–620 followed by a stop codon was described previously (14). Briefly, the cells were washed with cold phosphate-buffered saline containing 0.1 m CaCl2 and 1 m MgCl2 (PBS) and incubated for 20 min on ice with 1 mg/ml sulfo-N-hydroxysuccinimido-biotin (EZ-Link™ sulfo-NHS-biotin, Pierce) in PBS, followed by a second incubation with fresh sulfo-NHS-biotin. After biotinylation, the cells were washed twice with cold PBS, incubated on ice with 0.1 m glycine in PBS, and washed with PBS again. The cells were then solubilized in lysis buffer (50 m NaCl, 50 m HEPES, pH 7.2, 10% glycerol, 1 m EGTA, 1 m EDTA, 10 m sodium fluoride, 1 m sodium pyrophosphate, 0.5 m sodium vanadate, 10 m sodium orthovanadate, and 1% Triton X-100, 1 m sodium deoxycholate) supplemented with 10 m Tris-HCl (pH 6.8) at 4 °C. The lysates were cleared by centrifugation for 10 min at 16,000 × g, and the biotinylated proteins were precipitated with NeutrAvidin™ beads (Pierce), washed five times with lysis buffer, and denatured by heating the beads in sample buffer at 95 °C for 5 min.

In some experiments supernatants from the NeutrAvidin precipitation were further subjected to immunoprecipitation with polyclonal anti-GFP for 2 h at 4 °C. The precipitates were washed twice with lysis buffer supplemented with 100 m NaCl, washed once without NaCl, and then denatured by heating in sample buffer. The NeutrAvidin beads were then washed five times in 5% SDS-PAGE, and the proteins were transferred to nitrocellulose membrane. Western blotting was performed with monoclonal rat antibodies to DAT followed by secondary antibodies conjugated with horseradish peroxidase, and bands were detected by using the enhanced chemiluminescence kit from Pierce. Several x-ray films were analyzed to determine the linear range of the chemiluminescence signals, and the quantifications were performed using densitometry and NIH Image software.

In co-immunoprecipitation experiments, the cells co-expressing wild-type DAT and YFP-NΔ-DAT-G585A mutant were lysed, and DAT was immunoprecipitated by using rat anti-DAT. The immunoprecipitates were then analyzed with antibodies to DAT and GFP.

**Immunofluorescence Staining**—The cells grown on glass coverslips were washed with Ca2+, Mg2+-free PBS (CMF-PBS), fixed with freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) for 15 min at room temperature, and mildly permeabilized using a 3-min incubation in CMF-PBS containing 0.1% Triton X-100 and 0.5% bovine serum albumin at room temperature. The permeabilization step was omitted in experiments with EGFR-DAT chimeras. Cells were then incubated in CMF-PBS containing 0.5% bovine serum albumin at room temperature for 1 h with primary antibodies, and subsequently incubated for 30 min with secondary antibodies labeled with CY3 or CY5 (The Jackson Laboratory, West Grove, PA). Both primary and secondary antibody solutions were washed with PBS and incubation at 100,000 × g for 20 min. After staining, the coverslips were mounted in Mowiol (Calbiochem). To obtain high resolution three-dimensional images of cells, the fluorescence imaging Mariannas work station (Intelligent Imaging Innovation, Denver, CO) was used, which consisted of a Zeiss inverted microscope equipped with a cooled CCD camera (QImaging, Cooma, CA), and was equipped with a 175-watt light source, all controlled by SlideBook 4.0 software. Typically, 5–15 serial two-dimensional images were recorded at 200-nm intervals. A z-stack of images obtained was deconvoluted using a modified version of the constrained iteration method. Final arrangement of all images was performed using Adobe Photoshop.
are shown in rats (hDAT, C'576 (ER)) and rat (hDAT, C'576 (ER-PM)). Stable expression of hDAT in HeLa cells was stably expressed in HeLa cells. The cells were incubated with sulfo-NHS-biotin at 4°C to label surface biotinylation of wild type (WT), C'586-LKV, and C'577-LKV mutant DATs at the cell surface. The cell surface was measured by incubating cells with 50 ng/ml 125I-EGF at 4°C, and the biotinylated proteins were recovered from lysates with NeutrAvidin-conjugated beads (NeuAv) as described previously. NeutrAvidin precipitates and supernatants (SN) of NeutrAvidin-conjugated beads were resolved by SDS-PAGE, and DAT was detected by Western blotting (WB). The results of these experiments are presented in small circles. The ER export of DAT are shown in small circles. These mutants were clearly detected in the plasma membrane in >80% of transfected cells. Amino acid residues that are important for the ER export of DAT, based on deletion and alanine analysis, are presented in large black circles. The ER+FM phenotype indicates that the plasma membrane localization of YFP is clearly distinguishable in 20–40% of transfected cells, although a significant amount of ER-localized YFP-DAT is also seen in these cells. ER designates a phenotype with no clearly distinguishable plasma membrane localization of YFP in transfected cells. B, cell surface biotinylation of wild type (WT), C'586-LKV, and C'577-LKV mutant DATs stably expressed in HeLa cells. The cells were incubated with sulfo-NHS-biotin at 4°C, and the biotinylated proteins were recovered from lysates with NeutrAvidin-conjugated beads (NeuAv) as described previously. NeutrAvidin precipitates and supernatants (SN) of NeutrAvidin-conjugated beads were resolved by SDS-PAGE, and DAT was detected by Western blot (WB) analysis using a monoclonal rat DAT antibody. DAT, monomeric mature form of DAT, ngDAT, nonglycosylated intracellular forms of DAT. C, amino acid sequence alignment of the carboxyl terminus of human (h) and rat (r), DAT, NET, SERT, and GAT. Residues in the positions corresponding to Gly-585, Lys-590, and Asp-600 are shown in boxes.

Fig. 1. Summary of the mutations made in the carboxyl terminus of DAT and their effects on the plasma membrane expression of the transporter. A, schematic representation of hDAT (residues 1–620) fused to YFP. The positions of the truncations and deletions are indicated by arrows. C'576, C'576, C'576, and C'576 correspond to the deletion of residues 619–620, 616–620, 611–620, and 577–620, respectively. C'577-LKV and C'586-LKV correspond to the deletion of residues 598–617 and 587–617, respectively. Δ578–619 corresponds to the deletion of residues 578–610. B, cell surface biotinylation of wild type (WT), C'586-LKV, and C'577-LKV mutant DATs stably expressed in HeLa cells. The cells were incubated with sulfo-NHS-biotin at 4°C, and the biotinylated proteins were recovered from lysates with NeutrAvidin-conjugated beads (NeuAv) as described previously. NeutrAvidin precipitates and supernatants (SN) of NeutrAvidin-conjugated beads were resolved by SDS-PAGE, and DAT was detected by Western blot (WB) analysis using a monoclonal rat DAT antibody. DAT, monomeric mature form of DAT, ngDAT, nonglycosylated intracellular forms of DAT. C, amino acid sequence alignment of the carboxyl terminus of human (h) and rat (r), DAT, NET, SERT, and GAT. Residues in the positions corresponding to Gly-585, Lys-590, and Asp-600 are shown in boxes.

RESULTS

LK Motif Is Not Sufficient for the Efficient Transport of DAT to the Cell Surface—Previous studies revealed the importance of the last three carboxyl-terminal amino acids of hDAT (618-LKV) for its transport to the cell surface (12). However, in initial fluorescence microscopy analysis of DAT deletion mutants expressed in either HeLa or HEK293 cells, we noticed that truncation of the last 2, 5, or 10 amino acids (Fig. 1A) did not completely abrogate transport of DAT to the cell surface. Rather, these truncations substantially delayed expression at the cell surface (data not shown). On the other hand, removal of the entire carboxyl terminus (C'576 truncation mutant) blocked the delivery of DAT to the cell surface (data not shown). These results suggested that there are carboxy-terminal residues other than LKV that are essential for DAT trafficking.

To map these residues, DAT mutants, in which the last 23 and 34 residues were truncated and the LKV620 motif was attached, C'597-LKV and C'586-LKV, respectively (see Fig. 1A), were stably expressed in HeLa cells. In order to label surface DAT molecules, the cells were incubated with a membrane-impermeable NHS-biotin derivative, and the biotinylated proteins were precipitated using NeutrAvidin-agarose followed by the detection of DAT by Western blotting. Cell surface biotinylation experiments demonstrated that whereas the C'597-LKV mutant was partially expressed at the plasma membrane, the C'586-LKV mutant was undetectable (Fig. 1B). Both mutants were mainly expressed as the nonglycosylated form (~50 kDa) inaccessible to biotinylation (13, 14). Moreover, attachment of the last 10 residues (611–620) to the DAT-C'577 truncation mutant (ΔLKV610) did not restore DAT trafficking to the plasma membrane (data not shown). These results suggested that the PDZ-binding motif is not sufficient for ER export of the transporter and that sequences within the juxtamembrane portion of the carboxyl terminus are critical for efficient delivery of DAT to the plasma membrane.
mutant YFP-DATs in PAE cells. PAE cells were transiently transfected with YFP-DAT, YFP-G585A, K590A, or D600A DAT mutants. After 2 or 3 days YFP images were acquired from living cells. Whereas all three mutants displayed typical ER pattern of localization after 2 days of expression (left column), K590A and D600A mutants were seen expressed at the cell surface after 3 days of expression (right column). Insets in the right column show contrasting examples of cells with ER localization of mutant YFP-DATs after 3 days of expression. Bar, 10 μm.

Analysis of DAT Alanine Substitution Mutants by Fluorescence Microscopy—In order to identify the specific amino acid residues important for DAT transport to the cell surface, the carboxyl-terminal tail (residues 575–620) was subjected to systematic alanine-scanning mutagenesis. The phenotypic screening of transiently expressed DAT mutants was performed by using single cell fluorescence microscopy analysis. To facilitate the visualization of DAT mutants in the cell, mutagenesis was performed using YFP-DAT as template, although several mutations were made in untagged full-length YFP-DAT. YFP-DAT has been shown previously to be fully functional (14, 20, 21). PAE cells were chosen as our preferred expression system for DAT structure-function studies because transiently expressed DAT mutants were located almost exclusively in the intracellular tubular network throughout the cell and often seen associated with the nuclear envelope, a pattern of distribution consistent with ER localization (Fig. 2). When YFP-DAT localization was inspected 3 days after transfection, a clearly distinguishable plasma membrane localization of K590A and D600A was observed in 20–30% of transfected cells, suggesting that these mutations did not completely block, but rather slowed down, the delivery of DAT to the plasma membrane (Fig. 2). In contrast, the G585A mutant displayed the characteristic pattern of ER localization and was not detected in the plasma membrane by microscopic analysis under any conditions.

Plasma Membrane Expression of DAT Mutants Analyzed by Cell Surface Biotinylation—Our fluorescence microscopy analysis had revealed the role of Gly-585, Lys-590, and Asp-600 in the transport of DAT to the plasma membrane. To analyze quantitatively the plasma membrane expression of YFP-DAT mutants, we performed cell surface biotinylation experiments after 2 days of expression. Nonbiotinylated YFP-DAT was immunoprecipitated from supernatants of NeutrAvidin beads with anti-GFP antibodies. As illustrated in Fig. 3, biotinylated wild-type YFP-DAT was readily detected, whereas the amounts of biotinylated K590A and D600A mutants were much smaller, indicative of the inefficient delivery of these mutants to the plasma membrane. A very small amount of biotinylated G585A was detected in some experiments (<0.5% of total cellular mutant proteins; Fig. 3), indicating that this mutant is located almost exclusively intracellularly, in agreement with the fluorescence microscopy shown in Fig. 2. In general, biotinylation assay was more sensitive in detecting surface DAT than the light microscopy because the latter technique does not allow clear detection of the very weak fluorescence of YFP-DAT diffusely distributed in the plane of the plasma membrane.

Two main forms of wild-type YFP-DAT were detected in supernatants of the NeutrAvidin beads as described previously (14). A lower molecular weight form (~75 kDa) corresponds to an immature, nonglycosylated form that is located in the ER, and a higher molecular weight form (~105 kDa) corresponds to the mature YFP-DAT. The mature form represents plasma membrane YFP-DAT that was not biotinylated, as well as endosomal and trans-Golgi YFP-DAT. Although the amounts of the nonglycosylated form of wild-type and mutant YFP-DAT were comparable, the relative amounts of matured mutant proteins were significantly lower than that of the wild-type YFP-DAT. In agreement with the microscopy experiments, the G585A mutant was predominantly present in a nonglycosylated form (>97% of total cellular immunoreactivity), suggesting that it was located in the ER. In contrast, the nonglycosylated form corresponded to only 10–20% of total immunoreactivity of the wild-type YFP-DAT. K590A and D600A mutants displayed an intermediate distribution of mature and immature forms, indicating that these mutants traveled slowly along the secretory pathway. The total amounts of mutant DAT proteins in the cells were lower than this amount of wild-type
DAT, probably because of increased degradation of the mutants retained in the ER.

Localization of G585A Mutant in the ER—Microscopic and biochemical experiments revealed that mutation of Gly-585 to alanine has the most severe effect on the transport of DAT to the plasma membrane. We therefore focused further investigation of the DAT ER export on the analysis of the G585A mutant. To confirm that the G585A mutant was localized in the ER, the localization of wild-type and mutant YFP-DAT was compared with the distribution of an ER marker, the ER quality control protein Thiols-disulfide oxidoreductase protein-disulfide isomerase (PDI), using deconvolution of the z-stack of two-dimensional images. Fig. 4 shows that very little, if any, co-localization was observed between wild-type YFP-DAT and PDI. There was, however, marked co-localization of the YFP-DAT-G585A mutant with PDI. Similar results were obtained with transiently expressed D600A and K590A mutants (data not shown). These data strengthened the conclusions drawn from Fig. 2 that, based on localization patterns in living cells, the G585A mutant is retained in the ER.

Mutagenesis Analysis of Residues Surrounding Gly-585—To analyze further how G585A mutation affects DAT trafficking, residues surrounding Gly-585 were mutated. Alanine substitutions of Leu-583, Pro-584, Ser-586, and Phe-587 did not affect the surface expression of DAT, suggesting that Gly-585 does not appear to be a part of an uninterrupted linear sequence motif (Fig. 5A). We next asked whether the location of a glycine residue in the position 585 is essential. To this end, we generated double mutants, in which residues surrounding position 585 were replaced by glycine in the G585A mutant. The resulting mutants, L583G/G585A, P584G/G585A, G585A/S586G, and G585A/F587G, were transiently expressed in PAE cells for 2–3 days. The pattern of localization of L583G/G585A, P584G/G585A, and G585A/F587G mutants was indistinguishable from that of G585A mutant (Fig. 5B). Surprisingly, a pool of G585A/S586G mutant was detected in the plasma membrane after 3 days of expression, although this mutant was ER-retracted in a significant number of cells (ER + PM phenotype) (Fig. 5C). These data showed that glycine at position 585 is important for normal exit of DAT from the ER, although glycine at the position 586 can partially compensate for the loss of Gly-585 function.

G585A Mutation Does Not Interfere with DAT Oligomerization—Previous studies (13, 14) suggested that oligomerization is necessary for ER export of DAT. Therefore, we used FRET microscopy analysis to test whether the G585A mutation inhibits DAT oligomerization. To this end, wild-type CFP-DAT and the YFP-DAT-G585A mutant were co-expressed in PAE cells for 24 h. At that time, newly synthesized wild-type CFP-DAT was located in the ER of a number of cells in the population, thus allowing examination of its oligomerization with the ER-localized YFP-DAT-G585A mutant. Fig. 6A demonstrates significant corrected FRET (FRET(C)) signals in cells containing the wild-type and mutant DATs in the ER, consistent with the interaction of the two proteins. The intensity of FRET signals between wild-type and mutant DATs was comparable with that of FRET between wild-type CFP- and YFP-DATs (data not shown). When both CFP-DAT and YFP-DAT-G585A were co-expressed at moderate levels for 2 days, wild-type CFP-DAT was localized predominantly in the plasma membrane, whereas the YFP-DAT-G585A mutant remained in the ER of the same cells (Fig. 6B).

To confirm the ability of the G585A mutant to oligomerize by using a different assay, co-immunoprecipitation experiments were performed. DAT-G585A expression levels were relatively low in PAE cells, particularly when the two DAT fusion proteins were co-expressed. Therefore, for biochemical detection of oligomerization, we used HEK293 cells that allow high efficiency of DAT expression in a large fraction of the cell population. To this end, wild-type DAT was transiently co-expressed with YFP-ΔN-DAT-G585A, a mutant in which the entire amino-terminal portion was deleted and replaced by YFP. We have demonstrated previously that the YFP-ΔN-DAT traffics indistinguishably from the wild-type DAT (14). Cells expressing DAT or YFP-ΔN-DAT-G585A alone were used as controls. After 1 day of expression in HEK293 cells, YFP-ΔN-DAT-G585A was present exclusively in nonglycosylated form (~68 kDa) (Fig. 6C, 1st lane). To test for co-immunoprecipitation, DAT was precipitated from the lysates of cells co-expressing DAT and YFP-ΔN-DAT-G585A by using the antibody recognizing the DAT amino terminus, a region deleted in the YFP-ΔN-DAT-G585A mutant. The immunoprecipitates were then probed with the GFP antibody that recognizes the YFP-N-DAT association. Based on densitometric analysis of the intensity of chemiluminescence signals from GFP immunoreactivity in anti-DAT immunoprecipitates, ~15% of total cellular YFP-ΔN-DAT-G585A was co-immunoprecipitated with DAT. Together with FRET analysis, these data strongly suggest that G585A mutation does not prevent DAT oligomerization.
It has been shown previously (13, 14) that the formation of hetero-oligomers between the wild-type DAT and the ER export-defective DAT mutant may interfere with the normal delivery of wild-type DAT to the cell surface. Therefore, the effect of overexpression of YFP-DAT-G585A mutant on the plasma membrane levels of YFP-ΔN-DAT in HEK293 was examined using a surface biotinylation assay. As shown in Fig. 6D, coexpression of the 5-fold excess of YFP-DAT-G585A with YFP-ΔN-DAT completely abolished YFP-ΔN-DAT transport to the cell surface (Fig. 6D, top panel). Moreover, YFP-ΔN-DAT was retained intracellularly mainly as the nonglycosylated 68-kDa form as detected by blotting of supernatants with anti-GFP (Fig. 6D, bottom right panel). The bulk of YFP-DAT-G585A (75 kDa) was also not accessible to surface biotinylation and was therefore located intracellularly (Fig. 6D, bottom left panel). The dominant-negative effect of the ER export-deficient DAT mutant on the surface expression of DAT further suggested that proper DAT oligomerization is necessary for the efficient transport of DAT from the ER to the plasma membrane. Overall, the data in Fig. 6 are consistent with a model whereby homo-oligomers of DAT (or ΔN-DAT) are efficiently delivered to the cell surface, whereas the DAT/DAT-G585A hetero-oligomers and DAT-G585A homo-oligomers are mainly retained and subsequently degraded in the ER.

**G585A Mutation Does Not Result in the ER Retention of the EGFR-DAT Chimeric Protein**—Mutations in the carboxy-terminal tail of DAT could affect its delivery to the plasma membrane by creating an ER retention signal or unfolding of the tail leading to its recognition by the ER quality control machinery. In order to test for these possibilities, a chimeric EGF receptor-DAT protein (EGFR-DAT) was constructed, in which the carboxy-terminal tail of DAT was attached to the extracellular and transmembrane domains of the EGF receptor (Fig. 7A). The surface expression of EGFR-DAT, its G585A mutant, and a EGF-ΔC truncated mutant (an EGF receptor lacking the entire cytoplasmic domain) was compared by using the 125I-EGF binding assay and immunofluorescence staining with EGF receptor antibody in PAE cells lacking endogenous EGF receptors (17). As illustrated in Fig. 7B, cells expressing control EGFR-DAT and mutant EGFR-DAT(G585A) displayed equivalent 125I-EGF binding capacity, indicating that the levels of surface expression of these chimeric proteins are the same. Similarly, immunostaining of nonpermeabilized cells demonstrated that G585A mutation did not affect plasma membrane expression of the chimera (Fig. 7C). These data suggested that Gly-585 does not control the transport of the chimeric protein from the ER to the cell surface. Therefore, G585A mutation does not create an artificial ER retention signal or lead to the misfolding of this portion of the DAT molecule resulting in the ER retention. Likewise, K590A and D600A mutations did not affect surface expression of EGFR-DAT chimera (data not shown).

**Localization of DAT Mutants in Immortalized Neuronal 1RB₃AN₂₇ Cells and Rat Embryonic Midbrain Neurons**—Studies in PAE cells identified DAT mutants that do not exit or inefficiently exit the ER. To examine whether trafficking of these DAT mutants was affected similarly in neuronal cells, the immortalized cell line 1RB₃AN₂₇ (18) and primary embryonic midbrain neuronal cultures (19) were used as model expression systems. 1RB₃AN₂₇ cells were grown with dibutyryl cAMP to promote differentiation into dopaminergic neurons (18). Under these conditions many cells became extremely elongated, with long extensions reminiscent of forming neurites. In the original publication (18), the presence of tyrosine hydroxylase and endogenous DAT was demonstrated by reverse transcriptase-PCR. In our experiments the expression of tyrosine hydroxylase was detected by immunofluorescence microscopy in differentiated 1RB₃AN₂₇ cells. However, as reported by Torres et al. (12), no DAT protein immunoreactivity was detected in these cells.

The differentiated 1RB₃AN₂₇ cells were transfected with wild-type or mutant YFP-DATs and analyzed by fluorescence microscopy 2 and 3 days after transfection. Because of the low transfection efficiency of these cells, biochemical assays, such as biotinylation, were not performed. Therefore, only fluorescence microscopy was used to examine ER export and plasma membrane expression of these DATs. Wild-type YFP-DAT was normally delivered to the plasma membrane, as was clearly seen in the flattened regions of the cell extensions (Fig. 8). The YFP-DAT-G585A mutant did not exhibit any plasma membrane localization and was retained in the ER, predominantly in the perinuclear area of the cells. The fluorescence of K590A and D600A YFP-DAT mutants was limited mainly to an ER-like distribution, although these mutants extended outside of the cell bodies to a certain extent. After 3 days of expression K590A and D600A mutants were detected at the plasma membrane in 20–40% of cells in the population (Fig. 8). These results again are consistent with a slow delivery of DAT mutants K590A and D600A to the plasma membrane, and a complete ER retention of mutant G585A was observed in PAE cells. Trafficking of wild-type DAT and DAT mutants was also examined in primary cultures derived from E15 rat ventral
ER Export of Dopamine Transporter

Fig. 5. Localization of alanine and glycine substitution mutants of YFP-DAT. A, PAE cells were transiently transfected with YFP-tagged L583A, P584A, S588A, or F587A DAT mutants. After 2 days YFP images were acquired from living cells. Bar, 10 m. B, PAE cells were transiently transfected with YFP-tagged P584G/G585A, G585A/S586G, G585A/F587G, or L583G/G585A DAT double mutants. After 3 days YFP images were acquired from living cells. The inset shows high magnification image showing an example of plasma membrane localization of the G585A/S586G mutant. Bar, 10 m. C, PAE cells were transiently transfected with the same amount of YFP-DAT, YFP-G585A, or YFP-G585A/S586G plasmids. Cell surface biotinylation was performed after 3 days of expression as described in Fig. 3. NeutrAvidin precipitates (NeuAv) and immunoprecipitates from supernatants (SN) were resolved by SDS-PAGE, and DAT was detected by Western blotting using rat anti-DAT, YFP-DAT, monomeric mature form of YFP-DAT, YFP-ngDAT, nonglycosylated intracellular forms of YFP-DAT; WT, wild type. The ratio DAT/ngDAT (nonglycosylated DAT) was obtained by dividing the sum of the amounts of the mature YFP-DAT recovered from NeutrAvidin-conjugated beads and supernatants by the amount of nonglycosylated DAT in supernatants.

for α-Tau, the localization of this protein did not seem to be limited to axons but rather was spread throughout different cell compartments, including cell bodies and some dendrites (Fig. 9). Wild-type YFP-DAT was detected in the soma of α-Tau-positive cells and in neuronal processes of various shapes. The plasma membrane localization of YFP-DAT was especially pronounced in the flattened areas of the processes, presumably forming growth cones. The pattern of localization and the expression level of YFP-DAT were comparable with that of endogenous rat DAT, although higher amounts of DAT immunoreactivity were detected in cell bodies of transfected cells (Fig. 10). By contrast, DAT mutants accumulated exclusively in cell bodies and displayed a characteristic pattern of ER localization (Fig. 9). Very little, if any, specific YFP fluorescence was seen in the neuronal processes containing α-Tau. These data demonstrate that residues Gly-585, Lys-590, and Asp-600 are essential for efficient ER export of DAT in neurons and, therefore, normal targeting and functioning of the transporter at the synapse.

DISCUSSION

ER export is the first sorting step during the transport of newly synthesized DAT to the plasma membrane of the dendritic and axonal compartments in dopaminergic neurons. In this study, a large number of mutants were used to examine the mechanisms by which DAT trafficking from the ER is regulated by its carboxyl-terminal sequences. Early studies in COS-7 cells reported low affinity dopamine transport activity of the DAT mutant lacking a carboxyl terminus (22). However, a similar deletion mutant expressed in HEK293 cells was not detected on the plasma membrane by using the cell-surface biotinylation technique (23). Our experiments with DAT deletion mutants summarized in Fig. 1 were consistent with the essential role of the carboxyl terminus in DAT targeting to the plasma membrane.

What is the function of the carboxyl terminus in DAT anterograde trafficking? The first clue came from a study by Torres et al. (12) who reported that the direct interaction of the last three amino acids of DAT (E619LKV) with PICK1 increases expression of DAT at the cell surface. However, the mechanism by which the DAT-PICK1 complex controls plasma membrane expression of DAT is not understood. Our experiments in HEK293, HeLa, and PAE cells with DAT mutants lacking the last two or five residues confirmed the importance of Lys-619 and Val-620 for efficient ER exit (Fig. 1A) (14). However, whether the ER export function of these residues is mediated by PDZ domain interactions is not formally proven. For instance, in our experiments substitution of Lys-619 to alanine, which is not expected to interfere with the PDZ domain binding, nevertheless resulted in ER retention of DAT. By contrast, alanine substitution of Leu-618 did not affect ER export, indicating that the bulky hydrophobic residue at this position, which is thought to be important for efficient PDZ domain binding, is not essential for DAT trafficking.

Large deletions of the carboxy terminus revealed the importance of the juxtamembrane sequences for the ER export of DAT (Fig. 1) (13). This region appears to function upstream of the LKV motif, because the presence of the LKV residues is not sufficient to support efficient ER export of DAT mutants lacking residues 587–617 or 598–617 (Fig. 1B). Alanine mutagenesis of the carboxy-terminal residues proximal to the transmembrane domain revealed the key role of the amino acids Gly-585, Lys-590, and Asp-600 in DAT trafficking from the ER to the plasma membrane. Biochemical assays in PAE cells showed that the corresponding DAT mutants are predominantly present in an immature nonglycosylated form (Fig. 3), whereas fluorescence microscopy determined that these mu-
tants are localized in the ER (Figs. 2 and 4–6). Because ER quality control mechanisms and the capacity of ER export machinery may depend on the cell type, we showed that these mutants were also retained in the ER in immortalized neuronal cells (Fig. 8) and embryonic mesencephalic neurons (Fig. 9).

Complete understanding of the mechanisms by which Gly-585, Lys-590, and Asp-600 control ER export of DAT will be possible only with elucidation of the structure of the carboxyl terminus and identification of proteins binding to this part of the molecule. We can only speculate about such mechanisms. The retention of a specific cargo molecule in the ER can be due to at least three possibilities as follows: (i) inability of the cargo to be recognized by COPII sorting machinery; (ii) exposure of cryptic ER retention motifs; and (iii) incorrect folding of the protein detected by the ER quality control mechanisms. Regardless of mechanisms underlying the inability of cargo to exit ER, the accumulation of cargo proteins in the ER may lead to increased cargo degradation as observed in our experiments with DAT mutants (Fig. 3) (24).

Regarding the first possibility, known ER export motifs implicated in the interaction with Sec24 complexes and the COPII coat in the ER are apparently not involved in the ER export of DAT. For instance, our experiments ruled out the importance of di-acidic and di-hydrophobic motifs. The sequence Asp-600 to Lys-601 to Glu-602 of DAT confers the consensus of the ER export di-acidic motif (D/E)(X)(D/E), where X is any amino acid residue. This motif is present in the cytoplasmic tail of the vesicular stomatitis virus G protein and several other transmembrane proteins (8, 9, 25, 26). Mutations of aspartic or glutamic acid in this motif dramatically reduced the rate of ER export without affecting folding or oligomerization of the cargo molecules. However, in our experiments the E602A mutation...
Fig. 7. Surface expression of EGFR-DAT chimera is not affected by G585A mutation. EGFR-ΔC, EGFR-DAT chimera (schematically depicted in A), and EGFR-DAT-G585A mutant chimeric protein were transiently expressed in PAE cells. After 2 days the amount of 125I-EGF-binding sites at the cell surface was tested in mock- and chimera-transfected cells by incubating cells with labeled EGF at 4 °C as described under "Experimental Procedures" (B). C, transfected cells grown on coverslips were fixed and stained with EGF receptor antibodies without cell permeabilization, and the images were acquired through CY3 filter channel. Note that the expression efficiency of EGFR-ΔC construct was consistently lower compared with chimeric proteins. Bar, 10 μm.

Fig. 8. Localization of DAT mutants in immortalized neuronal 1RB3AN27 cells. 1RB3AN27 cells were transiently transfected with wild-type (WT) YFP-DAT or YFP-DAT-G585A, -K590A, or -D600A mutants. After 2 or 3 days YFP images were acquired from living cells. Insets represent high magnification images of cell lamellipodia showing an example of plasma membrane localization of wild-type YFP-DAT or ER localization of YFP-DAT mutants. Bars, 10 μm.

did not affect DAT exit from the ER (Fig. 1A). Furthermore, this di-acidic motif, particularly the negative charge corresponding to position 602 of DAT, is not conserved in other members of the monoamine transporter family (Fig. 1C). Thus, the requirement of Asp-600 for ER export is independent of the DXE (600–602) motif.

Another ER export signal, the di-phenylalanine (di-hydrophobic/aromatic) motif was found in the cytoplasmic domains of several transmembrane proteins (10, 27, 28). However, the di-phenylalanine motif is not present in the carboxyl terminus of DAT, and mutations F580A and F587A did not affect DAT trafficking (Figs. 1A and 5A). Also, alanine substitutions of all three carboxyl-terminal tyrosines (Tyr-575, -578, and -593), individually or in combination, as well as W617A mutation, did not influence the ER exit kinetics of DAT in PAE cells (data not shown).

A di-basic ER-export signal ((R/K)X(R/K)) has been identified recently for the Golgi resident glycosyltransferase. This putative ER export motif is located close to the transmembrane border and appears to interact with the small GTPase Sar1, a component of the Sec23-Sec24 complex (29). The DAT carboxyl terminus contains two di-basic sequences, 588REK590 and 599KDR601. Within the first motif, the alanine substitution at Lys-590 resulted in retarded trafficking of DAT to the plasma membrane (Figs. 2 and 3). Replacement of Lys-590 with arginine produced a mutant that traffics with the same kinetics as the wild-type DAT. In fact, Lys-590 of DAT is replaced by arginine in other monoamine transporters (Fig. 1C). Therefore, a positive charge at this position is important for proper trafficking of these transporters. However, alanine substitutions of Arg-588 and Glu-589, residues that are not conserved in other monoamine transporters (Fig. 1C), did not affect trafficking of DAT (Fig. 1A). Within the second di-basic motif, alanine substitutions of Lys-599 or Arg-601 had no effect on trafficking (Fig. 1A). These results indicate that trafficking of DAT from the ER is regulated by a mechanism that does not rely on di-basic motifs. Thus, if Gly-585, Lys-590, and Asp-600 are directly involved in the interaction with the COPII proteins, the molecular mechanisms of this interaction would be unconventional.

ER retention signals were found in several integral mem-
brane proteins. The best characterized signal is the RKR motif reported for the subunits of ATP-sensitive potassium channels (30, 31). This signal mediates retention of monomers and incomplete oligomers when it is not masked by the correct oligomerization. Such a motif, however, is not present in the DAT carboxyl terminus. Moreover, oligomerization, which is important for the plasma membrane delivery of different monoamine transporters including DAT (13, 14, 32, 33), was not affected by G585A mutation (Fig. 6). Moreover, G585A (Fig. 7) and other mutations (data not shown) in the EGFR-DAT chimera did not affect trafficking of this protein, suggesting that these mutations do not create an ER retention signal.

Do G585A or other mutations result in misfolding of DAT molecule? Since K590A and D600A mutants are capable of maturation and expression at the plasma membrane, it is likely that the quantitative alterations of trafficking of these mutants are because of inefficient interaction of these mutants with trafficking machinery rather than unfolding which typically leads to more severe consequences. G585A mutation resulted in the most dramatic effect on the ER plasma membrane expression of DAT. This finding was unexpected given the conserved nature of the small modification introduced in the DAT molecule by this mutation. This glycine is highly conserved among monoamine transporters (Fig. 1C). To our knowledge, glycine has not been previously assigned a key function in the ER export or other sorting motifs. Gly-585 is not part of a continuous sequence motif because alanine substitutions of the surrounding residues did not affect DAT trafficking (Fig. 5). Placement of a glycine residue at position 586 partially rescued ER export of the G585A mutant, suggesting that the presence of glycine in this region of DAT molecule is important for the ER exit of DAT. This experiment also provided indirect evidence that the G585A mutation per se does not result in misfolding of the DAT molecule. The ability of the G585A mutant of DAT to oligomerize with the wild-type DAT also indicates that the overall folding of this mutant is not affected. Finally, normal plasma membrane expression of the EGFR-DAT-G585A chimera suggests that at least in the context of the single membrane spanning protein the folding of the DAT tail with G585A mutation is normal. Given all considerations mentioned above, the most likely explanation of the effects of G585A mutation is its involvement in the intramolecular interactions, for instance with the intracellular loops of DAT.

Although much has been learned in recent years about trafficking, structure, and substrate-uptake mechanisms of monoamine transporters (34), most of this knowledge has been gained in experiments conducted in non-neuronal cell lines.

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**Fig. 9.** Localization of DAT mutants in primary cultures of midbrain neurons. Neuronal cultures were transiently transfected with wild-type YFP-DAT or YFP-DAT-G585A, -K590A, or -D600A mutants. The cells were then fixed, permeabilized, and stained with α-Tau antibody to identify neurons as described under “Experimental Procedures.” Images were acquired through fluorescein isothiocyanate (YFP, green) and CY3 (Tau, red) filter channels. Insets represent high magnification images of the regions marked by the white rectangles. Bars, 10 μm.

**Fig. 10.** Localization of endogenous DAT and YFP-DAT in primary cultures of midbrain neurons. Neuronal cultures were transiently transfected with wild-type YFP-DAT. The cells were then fixed, permeabilized, and stained with mouse monoclonal α-Tau antibody (anti-Tau) to identify neurons and rat monoclonal DAT antibody (anti-DAT) followed by the corresponding secondary antibodies conjugated with CY5 and CY3, respectively. Images of neurons expressing (A–D) or not expressing YFP-DAT (E–H) were acquired through fluorescein isothiocyanate (YFP, green), CY3 (DAT, red), and CY5 (α-Tau, blue) filter channels. Quantitative pseudocolor images of DAT immunostaining (anti-DAT) are presented. All settings of the RGB and pseudocolor CY3 images are identical. A.L.u.f.l., arbitrary linear units of fluorescence intensity. Bars, 10 μm.
Here we found that mutations in the carboxyl terminus of DAT have identical effects in PAE cells and in neuronal cells, such as the immortalized cell line 1RB\textsubscript{AN27} and primary cultures of embryonic rat midbrain neurons. In particular, the G585A mutant exhibited the strongest ER accumulation in neuronal cells. This mutant was absolutely restricted to the cell soma with the distribution pattern typical of ER. The elements of ER and Golgi have been detected in the dendrites of the hippocampal neurons (35). Because the G585A mutant was not detected in the forming dendrites and axonal processes in neurons, it is possible that the peripheral ER compartments are not formed in embryonic midbrain neurons. Alternatively, dendritic ER and Golgi elements may be specific for the processing of cargo destined for dendritic spines. In any case, our experiments suggest that the single amino acid mutations result in the inability of DAT to reach its site of action in neuronal cells and can, therefore, lead to the physiological consequences similar to those caused by the knockout of the transporter. This is reminiscent of the ER export defect caused by single mutation in the carboxyl terminus of the Cl\textsuperscript{−} channel cystic fibrosis transmembrane regulator, which underlies the molecular basis of cystic fibrosis (36, 37).

The data demonstrating the role of carboxyl-terminal residues of DAT in ER export are consistent with the critical role of the carboxyl terminus described for other monoamine transporters. Truncations of the carboxyl terminus of NET and SERT reduced ER export of these transporters (12, 38, 39). Most interesting, in polarized Madin-Darby canine kidney cells this interaction is proposed to be important for the retention of GAT at the plasma membrane, rather than for ER exit of GAT (40). Future studies are in progress to define the mechanism(s) by which the carboxyl terminus of DAT regulates trafficking and intracellular targeting of the transporter and to determine whether this is a unique or common mechanism among the monoamine transporter family.

Acknowledgments—We thank Cindy Hott for technical assistance, Dr. K. Prasad for 1RB\textsubscript{AN27} cells, and Dr. N. Zahniser and Davin Korstjens for critical reading of the manuscript.

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J. Biol. Chem. 2004, 279:30760-30770.
doi: 10.1074/jbc.M312774200 originally published online May 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312774200

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