Three Ubiquitin Conjugation Sites in the Amino Terminus of the Dopamine Transporter Mediate Protein Kinase C–dependent Endocytosis of the Transporter

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Dopamine levels in the brain are controlled by the plasma membrane dopamine transporter (DAT). The amount of DAT at the cell surface is determined by the relative rates of its internalization and recycling. Activation of protein kinase C (PKC) leads to acceleration of DAT endocytosis. We have recently demonstrated that PKC activation also results in ubiquitylation of DAT. To directly address the role of DAT ubiquitylation, lysine residues in DAT were mutated. Mutations of each lysine individually did not affect ubiquitylation and endocytosis of DAT. By contrast, ubiquitylation of mutants carrying multiple lysine substitutions was reduced in cells treated with phorbol ester to the levels detected in nonstimulated cells. Altogether, mutagenesis data suggested that Lys19, Lys27, and Lys35 clustered in the DAT amino-terminus are the major ubiquitin-conjugation sites. The data are consistent with the model whereby at any given time only one of the lysines in DAT is conjugated with a short ubiquitin chain. Importantly, cell surface biotinylation, immunofluorescence and down-regulation experiments revealed that PKC-dependent internalization of multilycine mutants was essentially abolished. These data provide the first evidence that the ubiquitin moieties conjugated to DAT may serve as a molecular interface of the transporter interaction with the endocytic machinery.

INTRODUCTION

The plasma membrane dopamine transporter (DAT) is responsible for the reuptake of extracellular dopamine (DA) back into presynaptic terminals of dopaminergic neurons by a Na^+/Cl^- -coupled cotransport mechanism (Giros and Caron, 1993). DAT is a member of the neurotransmitter transporter family (SLC6 gene family) that includes the serotonin, γ -aminobutyric acid, glycine, and the norepinephrine transporters (Torres *et al.*, 2003). As predicted by the hydropathy analysis, DAT consists of a single polypeptide embedded in the lipid bilayer by 12 transmembrane domains with intracellular amino- and carboxyl-terminal tails and a large highly glycosylated second extracellular loop (Shimada *et al.*, 1991; Androutsellis-Theotokis and Rudnick, 2002). Recently, a three-dimensional (3-D) structure of the homologous bacterial leucine transporter from *Aquifex aeolicus* (LeuT_{AA}) has been solved at 1.65-Å resolution, providing a valuable framework for further studies of the structure and transport mechanisms of the family of Na^+/Cl^- -dependent neurotransmitter transporters (Yamashita *et al.*, 2005). This structure confirmed the predicted topology of SLC6 family transporters; however, it did not provide structural information about the amino- and carboxyl-termini of the transporters.

DAT is synthesized in the soma of DA neurons located in the substantia nigra and ventral tegmental area and trafficked to axonal processes projecting mainly to the dorsal striatum and nucleus accumbens, where it resides in the plasma membrane near the active zones of dopaminergic synapses (Nirenberg *et al.*, 1997a, 1997b). The intensity and duration of dopaminergic neurotransmission depends on the level of the surface expression of DAT, which is regulated by trafficking of DAT between the plasma membrane and intracellular compartments. The rates of trafficking, particularly, rapid internalization and recycling, are thought to be controlled by signaling processes and DAT substrates (reviewed in Melikian, 2004; Zahniser and Sorkin, 2004). One of the most established modes of DAT regulation is through the activity of protein kinase C (PKC). Activation of PKC by phorbol 12-myristate 13-acetate (PMA) has been shown to cause down-regulation of DAT transport capacity and/or surface DAT protein in nonneuronal cells and in synaptosomal preparations from rat striatum (Vaughan *et al.*, 1997; Zhang *et al.*, 1997; Zhu *et al.*, 1997; Daniels and Amara, 1999; Blakely and Bauman, 2000; Chi and Reith, 2003). Activation of PKC results in phosphorylation of serine residues located in the amino-terminal tail of DAT (Huff *et al.*, 1997; Vaughan *et al.*, 1997; Foster *et al.*, 2002; Cervinski *et al.*, 2005). However, removal of the amino-terminal and other potential PKC phosphorylation sites did not prevent PKC-dependent internalization of DAT, suggesting that phosphorylation of DAT is not essential for DAT endocytosis (Granas *et al.*, 2003).

It has been demonstrated that both constitutive and PKC-dependent DAT internalization are mediated by clathrin-coated pits (Sorkina *et al.*, 2005). Although the molecular mechanisms of DAT internalization are not understood, two overlapping, unconventional motifs in the carboxyl-terminus of DAT have been proposed to mediate constitutive and PKC-induced DAT endocytosis (Holton *et al.*, 2005). Recently, we have demonstrated PKC-induced ubiquitylation

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Abbreviations used: EGF, epidermal growth factor; DA, dopamine; DAT, dopamine transporter; PAE, porcine aortic endothelial cells; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; YFP and CFP, yellow and cyan fluorescent protein, respectively.

Figure 1. PKC-induced ubiquitylation and endocytosis of single- and double-lysine mutants of DAT. (A) PAE cells transiently expressing wild-type or mutant FH-DAT were incubated with DMSO (vehicle) or 1 μ M PMA for 30 min at 37°C. FH-DAT was purified and subjected to SDS-PAGE and Western blotting (WB) with ubiquitin and DAT antibodies. (ng)-DAT, nonglycosylated DAT; DAT-Ub, ubiquitylated DAT. (B) The cells treated as in A were fixed and stained with rat DAT antibody followed by Cy3-conjugated secondary antibody. Single optical sections are shown. Scale bar, 10 μ m. (C) Cells transiently expressing wild-type or double-lysine DAT mutants were analyzed as in A.

of DAT (Miranda *et al.*, 2005). Ubiquitylation of various receptors and other transmembrane proteins has been proposed to serve as a sorting signal for endocytosis at the plasma membrane and for lysosomal degradation in endosomes (Hicke, 2001; Hicke *et al.*, 2005; Staub and Rotin, 2006). Therefore, we hypothesized that ubiquitin moieties appended to DAT may also serve as internalization and/or degradation signals. Moreover, we have recently demonstrated the importance of an E3 ubiquitin ligase, NEDD4-2, in DAT endocytosis (Sorkina *et al.*, 2006). However, the role of DAT ubiquitylation can be directly tested only by mapping and mutating ubiquitin-conjugation sites, and analyzing the functional consequences of these mutations. Typically, an obstacle of this type of analysis is the multiplicity and redundancy of ubiquitin-conjugation sites. Hence, in the present study we performed site-directed mutagenesis of

lysine residues at the amino- and carboxyl-terminal tails of human DAT and analyzed the role of individual or multiple lysine substitutions in DAT endocytosis. Surprisingly, mutagenesis guided by mass-spectrometry data revealed that simultaneous mutations of three lysine residues clustered in the amino-terminus of DAT was sufficient for dramatic inhibition of PKC-dependent ubiquitylation and endocytosis of DAT.

MATERIALS AND METHODS

Chemicals and Antibodies

PMA, *N*-ethylmaleimide, monensin, cycloheximide, anti-FLAG M2 affinity gel, and rabbit anti- α -actin antibody were purchased from Sigma (St. Louis, MO). Ni-NTA agarose was from Qiagen (Hilden, Germany). Monoclonal mouse antibody P4D1 to ubiquitin was from Santa Cruz Biotechnology (Santa Cruz, CA). Texas Red-conjugated transferrin (Tfn-TR) was purchased from

Figure 2. Ubiquitylation and endocytosis of multi-lysine DAT mutants. (A) PAE cells stably expressing wild-type or mutant DAT were treated with DMSO (vehicle) or PMA as in Figure 1A. DAT ubiquitylation was analyzed as in Figure 1A. WB, Western blotting; (ng)-DAT, nonglycosylated DAT; DAT-Ub, ubiquitylated DAT. (B) Quantification of the amount of ubiquitylated DAT normalized to total DAT from experiments performed as in A (mean $\pm$ SE; $n = 5$). (C) Cells stably expressing wild-type or multi-lysine mutants of DAT were treated as in A and stained with DAT antibodies as described in Figure 1B. Single optical sections are shown. Scale bar, 10 μ m.

Invitrogen (Carlsbad, CA). Monoclonal rat antibody against the amino terminus of DAT was from Chemicon International (Temecula, CA), mouse mAb HA.11 (HA11) from Covance (Berkley, CA), secondary antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA). mAb to EEA.1 was from BD Transduction Laboratories (San Jose, CA).

Plasmids and Mutations

The human DAT tagged with Flag epitope and 10 histidines at the N-termini (Flag, 10 $\times$ His-DAT/pcDNA3.1) was described previously (Miranda *et al.*, 2005) and here is referred to as FH-DAT. The plasmid YFP-HA-DAT was previously described (Sorkina *et al.*, 2006). Single and multiple amino acid substitutions were made using the FH-DAT or YFP-HA-DAT as templates and a QuickChange site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). CFP-Rab7 was kindly provided by Dr. E. Galperin (UCHSC). The mutations were verified by automatic dideoxynucleotide sequencing.

Cell Culture and Transfections

Human cervical carcinoma HeLa cells were grown in DMEM containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and antibiotics. Porcine aortic endothelial (PAE) cells were grown in Ham's F12 medium containing 10% FBS and antibiotics. HeLa and PAE cells were grown to 50–80% confluence

and transfected with appropriate plasmids using Effectene (Qiagen). The immortalized neuronal cell line 1RB₃AN₂₇ (Clarkson *et al.*, 1998) was kindly provided by Dr. K. Prasad (University of Colorado Health Sciences Center). These cells were maintained in RPMI medium supplemented with 10% FBS and antibiotics. HeLa and PAE cells stably expressing wild-type or mutant DAT were selected by growing them in the presence of G418 (400 μ g/ml). For microscopy, the cells were split 1 d after transfection onto glass coverslips and used for experiments on the second or third day.

Purification of FH-DAT Protein

FH-DAT purification was performed as described previously (Miranda *et al.*, 2005). Briefly, HeLa or PAE cells stably expressing wild-type or mutant FH-DAT were grown in 35-mm dishes to near 100% confluence and treated with vehicle (DMSO) or PMA. The cells were placed on ice and washed three times with Ca²⁺- and Mg²⁺-free cold phosphate-buffered saline (PBS), and the proteins were solubilized in lysis buffer (1% Triton X-100, 25 mM HEPES, pH 7.6, 10% glycerol, 100 mM NaCl, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 mM N-ethylmaleimide, 15 mM imidazole) for 10 min at 4°C. The lysate was then cleared by centrifugation at 16,000 $\times$ g for 15 min to remove insoluble material. After centrifugation, cleared lysate was incubated with Ni-NTA, previously equilibrated with the lysis buffer for 1 h at 4°C on the nutator. The

Figure 3. Localization of multilycine DAT mutants in 1RB₃AN₂₇ cells. 1RB₃AN₂₇ cells were transfected with wild-type, N3K, or N3KC4K DAT mutants. After 3 d, the cells were treated with DMSO or 1 μ M PMA for 30 min at 37°C, fixed, and stained with DAT antibodies as described in Figure 1B. Single optical sections are shown. Scale bar, 10 μ m.

beads were washed with lysis buffer five times, and the proteins were eluted with 250 mM imidazole in lysis buffer. The eluate was diluted 10 times with the FLAG binding buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 1% Triton) and incubated for 2 h with FLAG M2 affinity gel. The mixture was washed five times with 1 ml of FLAG binding buffer, and FH-DAT protein was eluted with 0.1 M glycine (pH 3.5). The eluted fraction was quickly mixed with an equal volume of 1 M Tris (pH 7.6). All procedures were performed at 4°C.

The purified proteins were analyzed by electrophoresis on 8.0% SDS-PAGE, and the proteins were transferred to the nitrocellulose membrane. Western blotting was performed with monoclonal mouse antibodies to ubiquitin and rat monoclonal antibodies to DAT, followed by corresponding secondary antibodies conjugated with horseradish peroxidase and detection using the

enhanced chemiluminescence kit from Pierce (Rockford, IL). Several x-ray films were analyzed to determine the linear range of the chemiluminescence signals, and the quantifications were performed using densitometry and Adobe Photoshop (San Jose, CA) and NIH Image J software.

Antibody Uptake Endocytosis Assay

HA11 antibody uptake experiments were performed as described previously (Sorkina *et al.*, 2006) with the exception that cells were permeabilized with saponin instead of Triton X-100. Briefly, PAE cells expressing wild-type or mutant YFP-HA-DAT grown on glass coverslips were incubated with 1 μ g/ml HA11 in medium for 60 min at 20°C, washed with binding medium (Ham's F12, 0.1% bovine serum albumin [BSA]), and incubated at 37°C with DMSO or PMA (1 μ M) with or without monensin for 30 min. The cells were then washed with ice-cold CMF-PBS and fixed with freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) for 15 min at room temperature. The cells were stained with the saturating concentration of secondary anti-mouse antibody conjugated with Cy5 (5 μ g/ml) to occupy surface HA11. After washing, the cells were permeabilized by 5-min incubation in CMF-PBS containing 0.1% saponin and 0.5% BSA at room temperature and then incubated with the same secondary antibody conjugated with Cy3 (1 μ g/ml) for 45 min in the same buffer to label internalized HA11. Both primary and secondary antibody solutions were precleared by centrifugation at 100,000 $\times$ g for 20 min. After staining, the coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA).

Surface Biotinylation

Cells expressing DAT proteins were grown in 35-mm dishes and biotinylated as described previously (Sorkina *et al.*, 2003). Briefly, the cells were washed with cold PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS) and incubated for 20 min on ice with 1.5 mg/ml sulfo-*N*-hydroxysuccinimidobiotin (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 supplemented with 10 mM Tris-HCl (pH 7.6) at 4°C. The lysates were cleared by centrifugation for 10 min at 16,000 $\times$ 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.

To precipitate nonbiotinylated proteins, supernatants from the NeutrAvidin precipitation were further subjected to Ni-NTA affinity chromatography. The precipitates were washed five times with lysis buffer and washed once without NaCl, the protein was eluted in lysis buffer containing 250 mM imidazole, and proteins were denatured by heating in sample buffer. The NeutrAvidin beads and Ni-NTA precipitates were subjected to SDS-PAGE and Western blotting with monoclonal rat antibodies to DAT. Quantifications were performed using densitometry and NIH Image J software.

Immunofluorescence Staining

The cells grown on glass coverslips were treated with DMSO or 1 μ M PMA for 30 min at 37°C. After treatment, the cells were washed with CMF-PBS,

Figure 4. Multilycine mutant of YFP-HA-DAT does not internalize HA11 antibody. PAE cells were transfected with wild-type YFP-HA-DAT or N3K mutant of YFP-HA-DAT. HA11 antibody uptake was carried out as described in *Materials and Methods*. After the chase incubation with vehicle (DMSO) or PMA (1 μ M) for 30 min at 37°C as in Figure 1A, the cells were fixed and mildly permeabilized. HA11:YFP-HA-DAT complexes remaining at the cell surface and internalized during chase incubation were detected using secondary anti-mouse antibody conjugated to Cy5 and Cy3, respectively. A 3-D image acquisition was performed through YFP, Cy3, and Cy5 filter channels. The sum projection images of two consecutive optical sections are presented. The fluorescence intensity settings for Cy5 (0–30 arbitrary units) and Cy3 fluorescence (0–150 arbitrary units) are the same in all images. Examples in colocalization of Cy5 and YFP (plasma membrane) are indicated by open arrowheads. Examples in colocalization of Cy3 and YFP (endosomes) are indicated by arrows. Scale bars, 10 μ m.

fixed with freshly prepared 4% paraformaldehyde 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% BSA at room temperature. The cells were then incubated in CMF-PBS containing 0.5% BSA at room temperature for 1 h with primary antibodies and subsequently incubated for 30 min with secondary antibodies labeled with CY3 or FITC (Jackson ImmunoResearch Laboratories). Both primary and secondary antibody solutions were pre-cleared by centrifugation at $100,000 \times g$ for 20 min. After staining, the coverslips were mounted in Mowiol (Calbiochem).

Microscopy

To obtain high-resolution 3-D images of cells, the fluorescence imaging Mariannas workstation (Intelligent Imaging Innovation, Denver, CO) consisting of a Zeiss inverted microscope equipped with a cooled CCD CoolSnap HQ (Roper, Tucson, AZ), z-step motor, dual filter wheels and a Xenon 175 W light source, all controlled by SlideBook 4.1 software (Olympus, Melville, NY), was used. Typically, 10–30 serial 2-D images were recorded at 300-nm intervals. A Z-stack of images obtained was deconvoluted using either a nearest neighbors method or a modification of the constrained iteration method. Final arrangement of all images was performed using Adobe Photoshop.

In HA11 antibody uptake experiments, a Z-stack of 34 2-D images were acquired at 300-nm intervals through Cy5, Cy3, and YFP filter channels. All image acquisition settings were identical in each experiment. The Z-stack of images was deconvoluted using a modification of the constrained iteration method (Gaussian noise smoothing). Quantification of the relative amount of YFP, Cy5, and Cy3 fluorescence in the cell was performed using the statistics module of the SlideBook4.1. The background-subtracted 3-D images were segmented using a minimal intensity of YFP as a low threshold. The integrated voxel intensity of YFP, Cy5, and Cy3 in each cell was then quantified.

RESULTS

Single Mutations of Lysine Residues Do Not Affect DAT Ubiquitylation and Endocytosis

Mass-spectrometry analysis revealed that that amino-terminal residues Lys19 and Lys35 as well as carboxyl-terminal residue Lys599 can be conjugated to ubiquitin in human DAT expressed in HeLa cells (Miranda *et al.*, 2005). To analyze the role of these lysines in DAT ubiquitylation and trafficking, lysine residues 19 and 599 were replaced with arginine, and Lys35 was mutated to methionine in the full-length FH-DAT. Lys35 was substituted for by methionine because lysine and methionine are both found in the same position in the human DAT gene, probably due to genetic polymorphism (Giros *et al.*, 1992; Vandenbergh *et al.*, 1992; Giros and Caron, 1993; Pristupa *et al.*, 1994). In addition, Lys27 was also mutated because it is located in proximity to and between two identified ubiquitin-conjugation sites and because ubiquitylation sites are known to be highly redundant. The single-site mutants were expressed transiently in HeLa and PAE cells. Western blot analysis demonstrated that constitutive and PMA-induced ubiquitylation was not significantly affected by single mutations of DAT ubiquitylation sites (Figure 1A).

To test whether mutations of single lysine residues affect endocytosis of DAT induced by PMA, transiently expressed wild type and mutants of FH-DAT were analyzed by fluorescence microscopy. PAE cells were used as our preferred expression system for trafficking studies because these cells are flattened and allow clear visualization of plasma membrane and endosomal organelles by fluorescence microscopy. Also, transiently expressed DAT is efficiently targeted to the plasma membrane in PAE cells (Sorkina *et al.*, 2003; Miranda *et al.*, 2004). As expected, when DAT localization was analyzed 2–3 d after transfection, wild-type DAT was located mainly at the plasma membrane and accumulated in filopodia, ruffles, and membrane protrusions in untreated or vehicle-treated cells. Similar localization was observed for all of the single mutants, suggesting that these mutations do not impair DAT trafficking to the plasma membrane (Figure 1B). Activation of PKC by PMA caused accumulation of

Figure 5. Multilycine DAT mutants are not down-regulated by PMA. (A) PAE cells expressing wild-type or mutant DAT were incubated with DMSO (vehicle) or PMA for 30 min at 37°C. The cells were subjected to cell surface biotinylation, and biotinylated proteins were pulled down with Neutravidin beads (NeuAv). Nonbiotinylation proteins were purified from NeuAv supernatants (SN) using Ni-NTA agarose. NeuAv and Ni-NTA precipitates were separated on SDS-PAGE and transferred to nitrocellulose, and the blots were probed with DAT antibodies. WB, Western blot; (ng)-DAT, nonglycosylated DAT. (B) Quantification of the amount biotinylated DAT relative to the amount of total DAT from experiments presented in A (mean ± SE; n = 3). The data are presented as percent of the amount of biotinylated DAT in DMSO-treated cells.

FH-DAT in the vesicular compartments that have been previously shown to contain markers of early (EEA1, Rab5, transferrin), sorting (Hrs), and late recycling endosomes (Rab11, transferrin; Sorkina *et al.*, 2003, 2005; Miranda *et al.*, 2005). As shown in Figure 1B, PKC-dependent accumulation of DAT in endosomes was not affected by single lysine mutations. These data suggested that no single ubiquitylation site is solely responsible for PKC-dependent ubiquitylation and endocytosis of DAT.

Multiple Lysine Substitutions Abolish DAT Ubiquitylation and Endocytosis

The experiments in Figure 1, A and B, suggested that there is redundancy in ubiquitin-conjugation sites of DAT. Therefore, we prepared double mutants of FH-DAT, in which several combinations of two lysine residues in the amino-

Figure 6. Localization of wild-type and mutant DAT in early and late endosomes. (A) PAE cells stably expressing wild-type and mutant FH-DATs were serum-starved for 15 min and incubated with PMA and TfR-TR (5 μ g/ml) for 30 min at 37°C. The cells were fixed and immunostained with anti-DAT followed by fluorescein-conjugated secondary antibody. Images were acquired through FITC (DAT) and Cy3 (TfR-TR) filter channels. Individual optical sections are shown. Insets, high-magnification images of the cell regions indicated by white rectangles. Scale bars, 10 μ m. (B) PAE cells stably expressing wild-type and mutant FH-DATs were transiently transfected with CFP-Rab7. After 2 d, the cells were incubated with PMA for 1 h at 37°C. The cells were then fixed and stained with anti-DAT followed by Cy3-conjugated secondary antibody. Images were acquired through Cy3 (DAT) and CFP (Rab7) filter channels. Individual optical sections are shown. Insets, high-magnification images of the cell regions indicated by white rectangles. Scale bars, 10 μ m.

terminal tail were mutated (K19R/K27R, K19R/K35M, and K27R/K35M). As illustrated in Figure 1C, the levels of PKC-dependent DAT ubiquitylation were either unchanged or, in some experiments, slightly reduced (e.g., K19/27R mutant). However, no visible defect in PKC-dependent endocytosis of these DAT mutants was observed (data not shown). This prompted us to generate multi-lysine mutants of DAT. Two mutants named N3K (K19R/K27R/K35M) and N3KC4K (K19R/K27R/K35M/K579R/K590R/K599R/K619R) were made and initially tested in transient transfection experiments. Affinity purification and Western blot analysis of transiently expressed DAT mutants demonstrated that ubiquitylation of these mutants was severely impaired (data not shown). To analyze the effects of these mutations in more detail, both mutants were stably expressed in PAE cells. Wild-type or mutant FH-DAT-expressing cells were incubated with vehicle or PMA, and FH-DAT was purified by tandem affinity chromatography. As shown in Figure 2, A and B, PMA induced a 10-fold increase in ubiquitylation of wild-type DAT cells. By contrast, ubiquitylation of N3K and N3KC4K was only slightly increased in PMA-treated cells (Figure 2, A

and B). The constitutive ubiquitylation of DAT was modestly reduced in the same mutants.

Availability of mutants that are minimally ubiquitylated in response to PKC activation allowed us to analyze PKC-dependent endocytosis of these mutants. First, localization of DAT in PAE cells stably expressing wild-type or mutant FH-DAT was examined by immunofluorescence. As shown in Figure 2C, PMA treatment caused substantial accumulation of wild-type FH-DAT in endosomes. In contrast, PKC activation did not lead to significant accumulation of N3K and N3KC4K mutants in endosomes. In these cells most DATs remained at the cell surface after PMA incubation and only few small endosomes could be seen occasionally in some cells. These data strongly suggested that N3K and N3KC4K mutants are endocytosis-impaired.

To examine whether trafficking of these DAT mutants was affected similarly in neuronal cells, the immortalized neuronal cell line 1RB₃AN₂₇ was used as a model expression system. We have previously used these cells to demonstrate trafficking defects of other DAT mutants (Miranda *et al.*, 2004). 1RB₃AN₂₇ cells were transfected with wild-type or

mutant FH-DAT and analyzed by fluorescence microscopy 3 d after transfection. As shown in Figure 3, wild-type FH-DAT was effectively accumulated in endosomes upon PMA treatment. No detectable accumulation of N3K and N3KC4K DAT mutants was observed, suggesting that DAT ubiquitylation is required for its endocytosis in 1RB₃AN₂₇ cells.

To provide the direct evidence that the endocytosis of nonubiquitylated DAT mutants is inhibited, the N3K mutant was prepared using the template of full-length DAT tagged with YFP at the N-terminus and an HA-epitope in the second extracellular loop (YFP-HA-DAT) recently characterized in our laboratory (Sorkina *et al.*, 2006). Wild-type and mutant YFP-HA-DAT were transiently expressed in PAE cells, and the HA11 antibody uptake experiments were performed. To this end, the cells were incubated with the HA11 antibody at room temperature (~20°C) to label surface YFP-HA-DAT. Subsequently, the cells were incubated with or without PMA for 30 min at 37°C, followed by the detection of surface and internalized HA11:YFP-HA-DAT complexes by Cy5- and Cy3-conjugated secondary antibodies, respectively. As shown in Figure 4, HA11-marked wild-type and N3K mutant YFP-HA-DAT were localized mainly at the plasma membrane in vehicle-treated cells as evident from the intense Cy5 fluorescence colocalized with YFP fluorescence at the cell edges and membrane ruffles and a minimal amount of Cy3 fluorescence. When the cells were treated with PMA, wild-type HA11:YFP-HA-DAT complexes were internalized into endosomes as revealed by colocalization of YFP and Cy3 fluorescence in the vesicular structures. In contrast, no significant Cy3 staining was detected in PMA-treated N3K mutant-expressing cells, whereas most HA11:YFP-HA-DAT complexes were labeled by Cy5 secondary antibody in plasma membrane ruffles, protrusions, and filopodia.

Together, the data of immunofluorescence microscopy presented in Figures 2–4 indicate that DAT ubiquitylation is necessary for PKC-dependent redistribution of DAT from the cell surface to endosomes. The defect in PMA-induced endocytosis of multilycine DAT mutants was confirmed using a surface biotinylation assay. As shown in Figure 5, PMA caused 40–50% down-regulation of surface-exposed wild-type FH-DAT. By contrast, the same PMA treatment only slightly, by 5–15%, reduced the amount of multilycine mutants of DAT at the plasma membrane. In summary, single-cell microscopy and biochemical analysis demonstrated that the amino-terminal Lys19, Lys27, and Lys35 are the major ubiquitylation sites and play a major role in regulation of endocytosis of the full-length DAT, whereas Lys599 and other carboxyl-terminal lysines have only a minor, if any, contribution to DAT ubiquitylation and endocytosis.

Nonubiquitylated DAT Mutants Are Defective in Internalization and Lysosomal Targeting Steps of PKC-induced Trafficking

The data presented in Figures 2–5 established a strong correlation between PKC-dependent DAT ubiquitylation, accumulation of DAT in endosomes, and down-regulation of DAT surface expression. Our previous studies demonstrated that fluorescent-protein-tagged DAT is located mainly in early/sorting/recycling endosomes after exposing cells to PMA for 15–30 min (Sorkina *et al.*, 2003, 2005). Likewise, FH-DAT displayed substantial colocalization with Tfn-TR, a marker of early and recycling endosomes (Figure 6A) and early endosomal marker EEA.1 (see Figure 8A below) in cells treated with PMA.

Figure 7. PKC-induced degradation of DAT is impaired by lysine mutations. PAE cells stably expressing wild-type (A) or mutant (B and C) FH-DAT were incubated with 50 μ g/ml cycloheximide for 2 h. The cells were then treated with PMA for indicated times and lysed. DAT was detected in lysates using rat DAT antibodies. Actin immunoreactivity is shown as a control for protein loading. WB, Western blotting. Bar graphs show quantification of total cellular DAT from three independent experiments (mean $\pm$ SE).

We have previously demonstrated that PKC activation accelerates the turnover of DAT protein (Miranda *et al.*, 2005). Therefore, to examine whether DAT is targeted to late endosomes and lysosomes in a PKC-dependent manner, the localization of DAT was compared with that of late endosome marker CFP-Rab7, transiently expressed in PAE/FH-DAT cells. As shown in Figure 6B, wild-type FH-DAT was highly colocalized with CFP-Rab7 in cells incubated with PMA for 1 h. This indicates that PKC activation results in elevated degradation of DAT by efficiently targeting internalized DAT to late endosomes/lysosomes.

As described in Figure 2, very few vesicular compartments were seen in cells expressing N3K and N3KC4K DAT mutants. Most, if not all, of these vesicular compartments containing FH-DAT mutants were Tfn-TR positive after 30-min incubation with PMA (Figure 6A). After prolonged activation of PKC (1 h) very little colocalization of mutant FH-DAT with CFP-Rab7 was observed (Figure 6B). These results suggest that the internalized nonubiquitylated mu-

Figure 8. Inhibition of recycling by monensin does not result in accumulation of N3K DAT mutant in endosomes. (A) PAE cells stably expressing wild-type DAT or N3K DAT mutant were incubated with 1 μ M PMA or 1 μ M PMA plus 25 μ M monensin for 30 min at 37°C. The cells were fixed and stained with rat anti-DAT followed by secondary anti-rat Cy3-conjugated antibody as described in Figure 1A. Subsequently, the cells were stained with antibody to EEA.1 followed by secondary anti-mouse FITC-conjugated antibody. Individual optical sections are shown. Insets represent high magnification of the regions indicated by rectangles. Scale bars, 10 μ m. (B) Quantification of the Cy3/Cy5 ratio of internalized/surface YFP-HA-DAT in PAE cells transiently expressing wild-type or N3K mutant YFP-HA-DAT. The HA11 antibody uptake assay was performed exactly as in Figure 4. The cells preincubated with HA11 were incubated with PMA or PMA plus monensin as in A. The values of Cy3/Cy5 ratio represent averaged values ($\pm$ SD) obtained from analysis of 3-D images of 5–8 cells under each experimental condition. *** p < 0.005 compared with cells untreated with monensin.

tant of DAT is not efficiently trafficked to late compartments. However, because nonubiquitylated mutants of DAT did not internalize to a significant extent, statistically reliable quantification of the amount of mutant DAT in Rab7-positive endosomes and the comparison of this amount with that of wild-type DAT was not possible.

Because mutations in DAT leading to abolished ubiquitylation dramatically reduce accumulation of DAT in early endosomes and slow down the passage of internalized DAT to late endosomes, we tested whether elimination of ubiquitylation sites would also impair PKC-induced degradation of DAT. As shown in Figure 7, wild-type FH-DAT was degraded with the half-life of ~1–1.5 h in PMA-treated cells, in agreement with previous observations (Daniels and Amara, 1999; Miranda *et al.*, 2005). By contrast, N3K and N3KC4K mutants were degraded at a very slow rate. The expression levels of conventional PKCs were identical in PAE cells lines expressing wild-type and mutant DATs (data not shown). Altogether, the experiments presented in Figures 6 and 7 demonstrated that DAT ubiquitylation is nec-

essary for PKC-dependent acceleration of DAT protein turnover.

The decreased accumulation of nonubiquitylated DAT mutants in endosomes could be due to either 1) rapid degradation of internalized DAT mutants; 2) slow internalization; 3) elevated recycling of mutant DAT from endosomes back to plasma membrane; or 4) both slow internalization and elevated recycling. The first possibility is obviously unlikely because the degradation of mutant DATs is very slow (Figure 7). An elevated recycling can be the consequence of the inability of cargo destined for degradation in the lysosomes to escape recycling (recycling is a default pathway from early/sorting endosomes) and properly sorted in the multivesicular bodies. Therefore, we tested whether the reduced accumulation of mutant DAT in endosomes can be observed in the absence of recycling. To this end, DAT recycling was inhibited by the proton ionophore, monensin, that blocks DAT trafficking through acidic endosomes (Sorkina *et al.*, 2005). As expected, monensin treatment amplified the PMA-induced redistribution of wild-

type FH-DAT from the cell surface to endosomes (Figure 8A). Plasma membrane FH-DAT was barely detectable. In PMA-stimulated cells that were treated or not treated with monensin, FH-DAT was highly colocalized with the marker of early/intermediate endosomes EEA.1 (Figure 8A) and transferrin (data not shown). In contrast, nonubiquitylated N3K DAT mutant was mainly located at the cell surface in cells treated with PMA (Figure 8A). In the presence of both PMA and monensin, the N3K mutant was accumulated in endosome-like compartments. However, most of DAT staining was still seen at the plasma membrane. The amount of mutant FH-DAT in endosomes was significantly lower than that of wild-type FH-DAT under the same conditions. These endosomes containing mutant FH-DAT were well colocalized with EEA.1 (Figure 8A) and transferrin (data not shown).

To quantify the extent of internalization in the presence or absence of monensin, HA11 antibody uptake experiments were performed in PAE cells transiently transfected with wild-type YFP-HA-DAT and N3K mutant of YFP-HA-DAT as described in Figure 4. To this end, the ratio of Cy3/Cy5 fluorescence (internalized/surface YFP-HA-DAT) was calculated as described (Sorkina *et al.*, 2006; see *Materials and Methods*). As shown in Figure 8B, monensin increased the relative amount of internalized YFP-HA-DAT in PMA-treated cells expressing wild-type or mutant YFP-HA-DAT. However, internalized/surface HA11 ratio remains very low in monensin-treated cells expressing N3K YFP-HA-DAT as compared with that in cells expressing wild-type YFP-HA-DAT. The small amount of mutant DAT in endosomes of monensin-treated cells can be attributed to the constitutive internalization of mutant DAT that is possibly ubiquitylation-independent. Thus, the data in Figure 8 demonstrated that the effects of lysine mutations on DAT localization could be observed in the absence of recycling. Therefore, these mutations directly affect the internalization step of PKC-dependent DAT trafficking.

DISCUSSION

In the present study, the essential role of DAT ubiquitylation in PKC-dependent endocytosis of DAT is demonstrated. Ubiquitylation is a highly regulated process that is important for many cellular functions, including endocytic trafficking of membrane proteins and targeting of cytosolic proteins for degradation by the proteasome. Ubiquitylation has been implicated in endocytosis and lysosomal targeting of various transmembrane proteins, such as receptor tyrosine kinases, G-protein-coupled receptors, and epithelium sodium channels (Levkowitz *et al.*, 1999; Marchese and Benovic, 2001; Staub and Rotin, 2006). However, there are only a few examples of studies where the specific role of individual ubiquitylation sites of these cargo proteins has been directly demonstrated. Typically, such demonstration is technically difficult because of the redundancy of ubiquitylation sites, which implies that multiple lysines must be mutated. Multiple mutations often result in the generation of nonfunctional or mistargeted proteins. Hence, mutations of lysines in the amino- and carboxyl-terminal tails of DAT resulted in mutant DATs that were normally delivered to the cell surface (Figures 2–4, 6, and 8) and displayed normal characteristics of dopamine uptake (data not shown). Despite the presence of numerous lysines in the tails and intracellular loops of DAT, our analysis that was guided by our mass-spectrometry data (Miranda *et al.*, 2005) revealed the importance of three major sites of DAT ubiquitylation, all clustered in the middle of the amino-terminal tail. In the

context of the full-length transporter molecule, elimination of this lysine cluster led to a dramatic reduction of PKC-dependent ubiquitylation of the transporter. Interestingly, these lysine residues are highly conserved in the other transporters of the SLC6 family.

Altogether, the data of mutagenesis suggest that each one of three lysine residues (Lys19, Lys27, Lys35) can be ubiquitylated. Furthermore, analysis of numerous Western blots of wild-type and single- or double-lysine mutant DATs tagged with different epitopes and recovered from various types of cells revealed that the ubiquitylated DAT is present as a single band that migrates slower than the nonubiquitylated DAT by ~25–30 kDa (Figures 1 and 2; Miranda *et al.*, 2005; Sorkina *et al.*, 2006). The simplest explanation of this observation is that at any given time only one lysine is conjugated per DAT molecule. Based on the molecular-weight difference between ubiquitylated and nonubiquitylated DAT, such ubiquitin moiety may represent a di-ubiquitin chain. The same molecular mass difference was interpreted as a K63-linked di-ubiquitin modification in a recent study of Ras ubiquitylation (Jura *et al.*, 2006). In fact, the presence of polyubiquitin, in particular Lys63-linked, was detected in purified DAT recovered from PMA-treated cells by mass spectrometry (Miranda *et al.*, 2005). Importantly, K63-linked chains have been proposed to serve as endocytosis signals as opposed to K48-linked chains that typically target proteins to the proteasome (Pickart, 2000; d'Azzo *et al.*, 2005; Geetha *et al.*, 2005; Huang *et al.*, 2006).

Generation of DAT mutants with minimal ubiquitylation allowed us to establish the strong correlation of PKC-dependent endocytosis and DAT ubiquitylation. It has been proposed that activation of PKC results in the acceleration of DAT internalization and the reduction of recycling of DAT, both leading to redistribution of DAT from the plasma membrane to endosomes (Loder and Melikian, 2003). Because internalized cargo can be rapidly recycled, precise and independent measurements of specific internalization and recycling rates of DAT are currently technically difficult. Our endocytosis assays performed in the presence of monensin, an inhibitor of recycling and degradation, suggested that DAT ubiquitylation is important for the internalization step (Figure 8). That DAT ubiquitylation is required for the PKC-dependent and clathrin-mediated internalization is also supported by the involvement of epsin, Eps15, and Eps15R, proteins capable of binding the ubiquitin moieties and located in plasma membrane clathrin-coated pits (Sorkina *et al.*, 2005, 2006).

Demonstration of ubiquitylation of a transmembrane protein and/or an involvement of ubiquitin ligases in regulation of endocytosis of this protein often leads to the conclusion that internalization of such endocytic cargo is mediated by its ubiquitylation. However, these two properties do not necessarily imply that the internalization is mediated by cargo ubiquitylation. For instance, ubiquitylation of G-protein-coupled receptors is required for their endosomal sorting rather than for the internalization step of trafficking (Marchese and Benovic, 2001; Huang *et al.*, 2006). In a well-studied model of endocytosis of the epidermal growth factor (EGF) receptor, receptor ubiquitylation appears to be not essential for its internalization despite the fact that Cbl, an E3 ubiquitin ligase, has been shown to be responsible for ubiquitylation and internalization of this receptor (Marchese and Benovic, 2001; Huang *et al.*, 2006). Overall, generating direct experimental evidence for the role of cargo ubiquitylation in internalization in mammalian cells has been difficult because it requires mapping multiple ubiquitylation sites and measurements of specific internalization rates. Thus,

DAT represents a unique example of a cargo for which the importance of ubiquitylation in internalization was shown through mapping and mutating the ubiquitin-conjugation sites. Our data are consistent with the view that cargo ubiquitylation can serve as a molecular signal for clathrin-dependent endocytosis. This view has been recently challenged in studies of the EGF receptor (Sigismund *et al.*, 2005).

PKC activation leads to a dramatic acceleration of the turnover of wild-type DAT in PAE (Figure 7) and HeLa cells (Miranda *et al.*, 2005). Sorting of wild-type DAT to late endosomal compartments is evident by substantial colocalization of DAT with Rab7 (Figure 6) and LysoTrackerRed (data not shown). PKC-dependent acceleration of lysosomal degradation of DAT expressed in MDCK cells was reported in earlier studies (Daniels and Amara, 1999). Degradation of DAT was severely delayed by elimination of DAT ubiquitylation (Figure 7); however, this delay is mostly due to slow internalization. It should be noted that whereas nonubiquitylated DAT mutants could be occasionally seen in the compartments containing early endosomal markers, it was difficult to find an example of localization of mutant DAT in late endosomes containing Rab7 (Figure 6). This suggests that not only internalization but also sorting of mutant DAT from early to late endosomes is impaired. In general, studies of many types of cargo showed that cargo ubiquitylation mediates their lysosomal targeting (Marchese and Benovic, 2001; Huang *et al.*, 2006). Thus, we predict that nonubiquitylated DAT in PKC-activated cells is not efficiently sorted in multivesicular bodies to the lysosomal degradation pathway and instead recycled back to the cell surface.

The mechanisms by which PKC activation leads to DAT ubiquitylation and ubiquitylation-dependent endocytosis and degradation remain to be elucidated. Although PKC-dependent phosphorylation of DAT has been demonstrated, this phosphorylation does not appear to be required for PKC-induced endocytosis of DAT (Chang *et al.*, 2001; Granas *et al.*, 2003; Cervinski *et al.*, 2005). According to our working model, PKC activity leads to ubiquitylation of amino- and carboxyl-terminal (Lys599) lysine residues, which is directly or indirectly mediated by an E3 ubiquitin ligase NEDD4-2. Amino-terminal lysines appear to be responsible for DAT endocytosis and, therefore, could be recognized by ubiquitin-binding proteins located in clathrin-coated pits, such as epsin (Sorkina *et al.*, 2006), and in multivesicular endosomes, such as ESCRT proteins (Raiborg *et al.*, 2003; Babst, 2005). The function of PKC in DAT ubiquitylation and its interactions with endocytic machineries could involve phosphorylation of proteins associated with DAT or regulating DAT, such as NEDD4-2, rather than phosphorylation of DAT itself. The elucidation of the sequence of events leading to PKC-induced DAT endocytosis would require identification of PKC targets that mediate DAT ubiquitylation.

The role of PKC-dependent DAT ubiquitylation and endocytosis in dopamine neurons has been so far difficult to address because of the low efficiency of transfection of primary dissociated rat embryonic dopaminergic neurons. Several classes of G-protein-coupled and other types of receptors present in dopaminergic neurons are capable of activating PKC (Gulley and Zahniser, 2003). Activation of PKC by phorbol esters caused down-regulation of DAT in striatal synaptosomes (Chi and Reith, 2003) and primary embryonic mesostriatal neuronal cultures (B. R. Hoover and N. R. Zahniser, unpublished observations). On the other hand, ubiquitylation has been shown to regulate trafficking of several classes of receptors expressed in neurons (Buttner *et al.*, 2001; Kato *et al.*, 2005; Makkerh *et al.*, 2005; Almeida *et al.*, 2006; Arevalo *et al.*, 2006; Cottrell *et al.*, 2006). The com-

ponents of ESCRT complexes responsible for recruitment of the ubiquitylated cargo into internal vesicles of multivesicular bodies have been shown to be expressed throughout the brain and are found in both the axonal and somatodendritic parts of neurons (Almeida *et al.*, 2006). Thus, accumulating evidence suggests that PKC-dependent ubiquitylation and trafficking may play an important role in the regulation of endogenous DAT. Development of new methodologies in the future studies will be necessary in order to dissect these signaling pathways in dopamine neurons.

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