ENHANCED UBIQUITYLATION AND ACCELERATED DEGRADATION OF THE DOPAMINE TRANSPORTER MEDIATED BY PROTEIN KINASE C

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Running Title: Ubiquitylation of dopamine transporter

Summary
Dopamine transporter (DAT) localization in dopaminergic neurons plays an important role in regulating dopamine signaling. However, the mechanisms of DAT trafficking that control DAT localization are still poorly understood. To gain insight into these mechanisms, human DAT was purified in large amounts using a two-step affinity chromatography procedure from untreated HeLa cells or cells treated with phorbol 12-myristate 13-acetate (PMA). Mass spectrometric analysis of purified DAT complexes revealed the presence of several proteins, among which ubiquitin was particularly abundant in the PMA-treated sample. Western blotting of highly-purified DAT protein confirmed constitutive ubiquitylation of DAT and the dramatic increase in DAT ubiquitylation in cells treated with PMA. This increase was blocked by pre-treatment with the protein kinase C (PKC) inhibitor bis-indolylmaleimide. DAT ubiquitylation by ectopically-expressed ubiquitin was demonstrated in cells transiently transfected with yellow fluorescent protein (YFP)-tagged ubiquitin. In addition, fluorescence resonance energy transfer (FRET) was detected between cyan fluorescent protein (CFP) tagged-DAT and YFP-ubiquitin, indicative of DAT-ubiquitin conjugation. Interestingly, the largest FRET signals were observed in endosomes. Ubiquitylated DAT was detected in the plasma membrane using cell surface biotinylation as well as in intracellular compartments, suggesting that ubiquitylation begins at the plasma membrane and is maintained in endosomes. In both porcine aortic endothelial and HeLa cells, where PKC-dependent DAT ubiquitylation was observed, PKC activation resulted in rapid degradation of DAT (t_{1/2} = 1-2 hrs). Altogether, these data suggest that PKC-induced DAT ubiquitylation may target DAT to lysosomal degradation.

Introduction
The dopamine transporter (DAT) is a member of the family of Na+/Cl- dependent plasma membrane transporters (SLC6 gene family), which are responsible for rapid clearance of neurotransmitters from the extracellular space. This family also includes norepinephrine (NET), serotonin (SERT), γ-aminobutyric acid (GAT) and glycine (GLYT) transporters (1). Members of the SLC6 family share similar predicted topology of a single polypeptide that contains twelve transmembrane segments, a large second extracellular glycosylated loop, and cytoplasmic amino- and carboxyl-terminal tails (2,3). DAT and several other transporters of this family have been found to be constitutively oligomerized in vitro and in cells (4-8).

The amount of DAT at the plasma membrane and, therefore, dopamine (DA) uptake capacity are determined by trafficking of the DAT protein, which is regulated by several signaling cascades including signaling through protein kinase C (PKC). Activation of PKC by 4α-phorbol 12-myristate 13-acetate (PMA) leads to a reduction in the V_max of DA transport without a change in the substrate affinity (K_m), as well as in down-regulation of surface DAT protein (9-15). This down-regulation of DAT activity and levels has been shown to be due to accelerated internalization of the transporter through clathrin-coated pits and possibly reduced recycling of DAT (16,17). Similar effects of phorbol esters on SERT, NET, GAT1 and GLYT1 have also been reported (reviewed in ref. 18). In addition to down-regulation of the plasma membrane pool of DAT, PKC activation caused degradation of DAT in MDCK cells (14). In contrast, it has been suggested that internalized DAT is mostly sorted to the recycling pathway in PC12 cells stimulated with PMA (15).
The mechanisms of PKC effects on DAT endocytosis and DA uptake are not fully understood. Phosphorylation of the amino-terminal tail of DAT by PKC has been observed in rat striatal synaptosomal preparations and transfected mammalian cell lines (10,19,20). However, truncations or point mutations of the phosphorylation sites in DAT did not inhibit PKC-induced down-regulation of DAT, suggesting that this PKC effect is not mediated by DAT phosphorylation (17,21,22). On the other hand, amino-terminal phosphorylation has been shown to be important for reversed DA transport by DAT (23). In contrast, PKC-dependent phosphorylation of the second intracellular loop of GLYT1 has been implicated in the phorbol ester mediated down-regulation of this transporter (24), thus illustrating differences in the regulation of endocytosis of different members of this transporter family (25).

Mutations of the amino acid motifs that correspond to consensus sequences for conventional internalization and sorting signals, such as tyrosine- and di-leucine based signals, did not establish any role of these motifs in PKC-induced DAT down-regulation (17,21). Recently, monoubiquitylation has been proposed to serve as sorting signal for internalization and lysosomal targeting of membrane proteins (26). Unlike polyubiquitylation that typically targets proteins for proteosomal degradation, monoubiquitin moieties can be recognized by the components of plasma membrane internalization and endosomal sorting machineries. Hence, we performed mass-spectrometry and immunoblot analyses of DAT and demonstrated constitutive ubiquitylation of DAT and a dramatic increase of DAT ubiquitylation upon PKC activation. Since PMA caused a substantial decrease in the DAT half-life in our model expression systems, our data imply that DAT ubiquitylation may be responsible for the accelerated degradation of DAT caused by PKC activation.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies

PMA, N-ethylmaleimide (NEM), cyclohexamide (CHX), and anti-Flag M2 affinity gel were purchased from Sigma (St. Louis, MO). Bisindolylmaleimide I (BIM) was from Calbiochem (Darmstadt, Germany). Polyclonal rabbit antibody ab290 to GFP was purchased from Abcam Ltd. (Cambridge, UK); monoclonal mouse antibody to GFP was from Zymed Laboratories (South San Francisco, CA); monoclonal rat antibody against the amino terminus of DAT was from Chemicon, Inc. (Temecula, CA); and monoclonal mouse antibody P4D1 to ubiquitin was from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids construction

The full-length human DAT (hDAT) in pcDNA3.1 was kindly provided by Dr. G. W. Miller (Emory University, GA). To generate the Flag-epitope and His-tagged DAT, a set of forward primers containing the Nhe I site and sequences encoding the Flag and His tags (six or ten histidines) and reverse primers carrying an EcoR I site after the stop codon were used to amplify the hDAT sequence by PCR using Pfu polymerase (Stratagene, Cloning Systems, La Jolla, CA). The DNA fragment was cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). To generate wild type and mutant YFP-Ubiquitin (YFP-Ub and YFP-UbAA, respectively), a fragment BsrGI-HindIII containing the ubiquitin sequence was transferred from the plasmid GFP2-UbiWT or GFP2-UbiAA kindly donated by Dr. M. Bouvier (University of Montreal, Quebec, Canada) (27) into pEYFP-C1 vector (Clontech, Palo Alto, CA). All constructs and point mutations were verified by automatic dideoxynucleotide sequencing. The plasmid CFP-DAT was previously described (6). YFP-Hrs (hepatocyte-growth-factor receptor substrate) was provided by Dr. H. Stenmark (Radium Institute, Oslo, Norway).

Cell culture and transfections

Human cervical carcinoma HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and antibiotics. Porcine aortic endothelial (PAE) cells were grown in F12 medium containing 10% fetal bovine serum (FBS) and antibiotics. Cells were grown to 50–80% confluency and transfected with appropriate plasmids using Effectene (Qiagen, Hilden, Germany). The constructs Flag/6XHis-DAT or Flag/10XHis-DAT (FH-DAT) were transfected
into HeLa and PAE cells using Effectene, and the cells stably expressing DAT were selected by growing them in the presence of G418 (400 μg/ml). For DAT purification, the cells were plated into 20 cm square dishes for large scale purification or 35 mm dishes for small scale purification and used at near 100% confluency. For microscopy, the cells were split 1 day after transfection onto glass coverslips and used for experiments on the second or third day.

**Purification of FH-DAT by Ni-NTA agarose and Flag M2 affinity chromatography**

To purify FH-DAT, HeLa cells stably expressing FH-DAT were grown to near 100% confluency and treated with vehicle (DMSO) or PMA in the absence or presence of BIM. The cells were placed on ice and washed three times with Ca²⁺- and Mg²⁺-free cold phosphate-buffered saline (CMF-PBS), and the proteins were solubilized in lysis buffer (25 mM HEPES, pH 7.6, 10% glycerol, 100 mM NaCl, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1% Triton X-100, 10 mM NEM, 15 mM Imidazole) for 20 min at 4°C. The lysate was then centrifuged at 100,000 xg for 45 min to remove insoluble material. After centrifugation, cleared lysate was incubated with Ni-NTA, previously equilibrated with the same lysis buffer, for 1 h at 4°C on the nutator. The mixture was then transferred to a plastic column (Poly-Prep Chromatography columns, Bio-Rad), the flow-through was discarded, and the column washed with lysis buffer. FH-DAT was then eluted with 250 mM imidazole in lysis buffer. The eluate was diluted ten times with the Flag binding buffer (50 mM Tris, 150 mM NaCl, 10% Glycerol, 1% Triton) and incubated for 2 hs with Flag M2 affinity gel. The mixture was washed five times with 1 ml of Flag binding buffer, and FH-DAT was eluted with 0.1 M glycine (pH 3.5). The eluted fraction was quickly mixed with an equal volume of 1M Tris (pH 7.6). All procedures were performed at 4°C.

**Mass spectrometry and data processing**

Affinity-purified hDAT samples were analyzed using mass spectrometry. Samples were either first resolved by gel electrophoresis followed by gel excision, digestion, and extraction of both tryptic and non-tryptic (elastase digests) peptides (28,29) or directly digested in solution to produce both tryptic and non-tryptic peptides (30). Peptides prepared using both methods were analyzed by Multidimensional Protein Identification Technology (MudPIT) (31) with a Finnigan LTQ linear ion trap (ThermoElectron, San Jose, CA) using an in-house constructed microspray ion source. Briefly, peptide samples were desalted and loaded onto a biphasic chromatography column constructed from 100-μm i.d. 365-μm o.d. fused silica capillary tubing and packed with 8 cm of 5 μm Aqua C18 reverse phase packing material (Phenomenex) followed by 4 cm of 5 μm Partisphere strong cation exchanger (Whatman, Clifton, NJ). Peptide digests were loaded using a pressure bomb and an automated MudPIT analysis was performed with 6 salt elution steps as previously described (30). All MS/MS spectra were searched against an NCBI human-mouse-rat database concatenated onto a randomized “shuffled” database using the Sequest algorithm (32). Ubiquitylations were identified by searching for mass shifts localized to lysine residues (+114 and +383) from the samples digested with trypsin (33). Positive protein identifications required normalized cross-correlation scores (XCorr) ≥ 0.3 for any charge state (29) and delta correlation score (ΔCn) ≥ 0.1. False discovery rates were ≤ 5%, as determined by hits to the randomized “shuffled” database. Partial tryptic status was required for ubiquitination identifications. Polyubiquitin sites were identified on ubiquitin lysine residues (34,35).

**Surface Biotinylation**

Cells expressing FH-DAT proteins were grown in 35-mm dishes and treated with vehicle (DMSO) or PMA for the indicated times. Cell surface biotinylation was performed as previously described (36). Briefly, the cells were washed with cold phosphate-buffered saline containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS) and incubated for 20 min on ice with 1 mg/ml sulfo-N-hydroxy succinimidobiotin (EZ-LinkTM sulfo-NHS-biotin, Pierce, Inc., Rockford, IL) 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 at 4 °C in lysis buffer (described above) supplemented with...
10 mM Tris-HCl (pH 7.6). The lysates were cleared by centrifugation for 10 min at 16,000 × g, and the FH-DAT was precipitated with 50 μl of Ni-NTA, as described above. After elution of FH-DAT from the Ni-NTA agarose, the eluted fraction was diluted with lysis buffer, and the biotinylated proteins were precipitated with NeutrAvidinTM beads (Pierce), washed five times with lysis buffer, and denatured by heating the beads in sample buffer at 95 °C for 5 min. The supernatant from the NeutrAvidinTM bead precipitation was incubated with 50 μl of Flag M2 affinity gel and non-biotinylated DAT was purified as described above. Western blotting was performed with mouse or rat monoclonal antibodies to ubiquitin or DAT, respectively, followed by secondary antibodies conjugated with horseradish peroxidase. Detection was accomplished using the enhanced chemiluminescence (Pierce).

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. Immunostaining with DAT antibodies and image acquisition using epifluorescence Mariannas workstation (Intelligent Imaging Innovation, Denver, CO) were performed essentially as described previously (36).

Live Cell Microscopy and FRET measurements
PAE stably expressing CFP-DAT cells, PAE or Hela cells transiently expressing CFP-DAT and YFP-tagged ubiquitin or YFP-Hrs were grown on 25 mm glass coverslips. After treatments, the coverslips were mounted in a microscope chamber and placed on a microscope stage. In experiments with YFP-Hrs a z-stack of 30 images were acquired through CFP and YFP channels and deconvoluted using a nearest neighbor algorithm.

The method of sensitized FRET measurements used to examine FRET between CFP and YFP has been described previously (37,38). Briefly, images through YFP, CFP and FRET filter channels were acquired using a Mariannas™ fluorescence imaging workstation consisting of a Zeiss inverted microscope equipped with a cooled CCD CoolSnap HQ (Roper, CA), dual filter wheels and a Xenon 175 W light source, all controlled by SlideBook software. Images were background-subtracted, and the corrected FRET (FRETc) was calculated. FRETc images were presented in a quantitative pseudocolor. The apparent FRET efficiency (Ed) was calculated as described (38). All calculations were performed using the FRET statistic module of SlideBook 4.0 or 4.1.

RESULTS
To isolate DAT in amounts sufficient for mass-spectrometry analysis, a two-step affinity purification procedure was developed. Two sequences corresponding to the Flag-epitope and 6xHis or 10xHis were introduced in tandem at the amino-terminus of DAT. Although we initially used a hexahistidine tag, 10xHis allowed tighter binding of tagged DAT to Ni-NTA agarose and therefore more stringent washing conditions, thus eliminating a significant amount of non-specific interactions. Therefore, in most of the experiments Flag-10xHis-DAT (FH-DAT) was used (Fig. 1A). FH-DAT was stably expressed in HeLa and PAE cells. FH-DAT migrated on SDS-PAGE as a smeared band of 85-90 kD (Fig. 1). Localization of FH-DAT at steady-state growth conditions or in cells stimulated with 1 μM PMA (condition of increased DAT endocytosis, Fig 1A) was essentially similar to that observed in numerous studies of untagged DAT or various DAT fusion proteins (Fig 1B) (6,14,21). It has been demonstrated in several studies that attachment of small epitope tags or GFP to DAT amino-terminus does not affect DAT function and trafficking (6,14,21,39).

Purification of FH-DAT from HeLa cells by consecutive binding and elution from Ni-NTA agarose and FlagAbM2 gel typically yielded several micrograms of FH-DAT readily detectable as 85-90 kD (monomer) and ~150-170 kD (SDS-resistant dimer) bands by Coumassie staining (Fig. 1C). As shown in Fig. 1B, the second purification step dramatically enriched DAT and eliminated most of the contaminating proteins from the preparation. Several proteins that are specifically or non-specifically associated with DAT were also detected in the final samples (Fig. 1B).
Surprisingly, LC/MS/MS analysis of the 85-kD FH-DAT band or total eluates from the FlagAbM2 gel revealed the presence of the large number of peptides corresponding to human ubiquitin. Furthermore, Lys11-, Lys48- and Lys63-linked ubiquitin chains were detected in PMA-treated and untreated FH-DAT (Table). Moreover, the number of ubiquitylated DAT peptides hits was ~3 fold higher by spectral count in DAT purified from HeLa cells treated with PMA than from untreated cells. Lysines 19, 35 and 599 of DAT were found to be ubiquitylated (Table), although additional experiments are necessary to complete mapping of DAT ubiquitylation sites.

Western blotting of an aliquot of the eluate from the Flag-agarose confirmed the presence of ubiquitin immunoreactivity in FH-DAT recovered from untreated and PMA treated-cells (Fig. 1C). To ensure that ubiquitin is conjugated to DAT, rather than to a DAT-associated protein, FH-DAT was purified in the presence of SDS to minimize protein-protein interactions. Staining of the specific eluate demonstrated substantially cleaner preparations than those obtained in the absence of SDS (Fig. 1C). Mass-spectrometry as well as Western blotting of these DAT preparations detected, respectively, ubiquitin peptides and a characteristic smear of the ubiquitylated signal with the maximum ubiquitin immunoreactivity at ~125-130 kD.

DAT ubiquitylation in cells treated with 1 μM PMA reached a maximum at approximately 15-30 min at 37°C and then decreased with continued incubation of cells with PMA (data not shown). PMA-induced ubiquitylation was blocked by the inhibitor of conventional PKCs, BIM (1 μM), confirming that this PMA effect is PKC-dependent (Fig. 2). To test whether DAT can be ubiquitylated by ectopically-expressed ubiquitin, HeLa/FH-DAT cells were transiently transfected with YFP-ubiquitin, and FH-DAT was then affinity-purified on Ni-NTA agarose. Blotting with GFP antibodies revealed the presence of high-molecular weight GFP immunoreactivity in purified FH-DAT, indicative of the conjugation of YFP-ubiquitin to FH-DAT (Fig. 3). There appear to be discrete YFP-ubiquitin bands, suggesting that these bands may correspond to mono- and di-ubiquitylated FH-DAT (Fig. 3A). The specificity of ubiquitylation was confirmed by the absence of incorporation of YFP-ubiquitin mutant incapable of conjugation to lysines (YFP-UbAA). The amount of YFP-ubiquitin conjugated to DAT was relatively small, probably due to the very high concentration of endogenous ubiquitin that competed with a YFP-ubiquitin for conjugation to DAT. Importantly, while there was a significant increase of YFP-ubiquitin incorporation induced by PMA, the overall incorporation of YFP-ubiquitin in cellular proteins was not dependent on PKC activity (Fig. 3A, supernatants). This suggests that the PKC effect on DAT is specific.

To examine where in the cell FH-DAT is ubiquitylated, CFP-DAT was co-expressed with YFP-ubiquitin. Fluorescence microscopy revealed that a bulk of YFP-ubiquitin was distributed in the cytosol and nucleus (Fig. 4A). In addition, YFP-ubiquitin was accumulated in endosomes in untreated cells and cells stimulated with PMA, although CFP-DAT could be detected in YFP-ubiquitin decorated endosomes only in cells treated with PMA. Co-localization of CFP-DAT and YFP-ubiquitin in the plasma membrane ruffles and cell edges was also detected, although this co-localization was much less pronounced. The YFP-UbAA mutant was diffusely distributed throughout the cytosol and nucleus, was not detected in endosomes or plasma membrane, and was not co-localized with CFP-DAT (Fig. 4A). These data suggested that the plasma membrane and endosomal YFP-ubiquitin represents a pool of YFP-ubiquitin conjugated to membrane-associated proteins, including DAT.

Measurements of fluorescence energy transfer (FRET) between CFP-DAT and YFP-ubiquitin revealed specific FRET signals in endosomes of cells treated with PMA, indicative of the close proximity of CFP-DAT and YFP-ubiquitin (Fig. 4B). This result is consistent with conjugation of YFP-ubiquitin to CFP-DAT. The relatively low apparent efficiency of energy transfer (Ed) could be due to a very low extent of DAT ubiquitylation by YFP-ubiquitin as evident from immunoprecipitation experiments in Fig. 3A. Altogether, the experiments presented in Figs. 3 and 4 suggest that endosomes represent the major localization site of ubiquitylated DAT.

Surface biotinylation of cells incubated with PMA revealed the presence of ubiquitylated DAT in the extracellular-exposed pool of proteins, suggesting that DAT ubiquitylation may begin at
the cell surface (Fig. 5). The amount of ubiquitylated DAT was, however, higher in the non-biotinylated fraction of cells (Fig. 5). This suggests that a large pool of mature DAT protein is ubiquitylated in intracellular membranes, presumably endosomes.

Because ubiquitylation of integral membrane proteins has been implicated in lysosomal targeting of these proteins, we examined the rate of degradation of DAT in cells treated with PMA. In these experiments protein synthesis was inhibited by CHX to eliminate the contribution of newly-synthesized DAT in the total pool of cellular DAT. In both HeLa (Fig. 6A) and PAE cells (data not shown), PMA treatment resulted in dramatic down-regulation of the FH-DAT protein with a half-life time of approximately 1-2 hrs. These experiments suggest that DAT ubiquitylation correlates with PKC-dependent degradation and may, therefore, underlie the PKC-dependent acceleration of DAT turnover.

To test whether internalized DAT is endocytosed in the endosomes containing ubiquitin-recognition sorting machinery, the localization of CFP-DAT was compared with the localization of overexpressed YFP-Hrs. The Hrs-STAM complex is directly involved in the recognition of ubiquitylated cargo in sorting endosomes (40). Live-cell three-dimensional imaging and deconvolution revealed significant accumulation of DAT in large endosomes decorated by YFP-Hrs (Fig. 6B). Overexpression of Hrs is known to result in dramatic enlargement of endosomes and blockade of trafficking through multivesicular endosomes (MVBs) of various cargos, in particular, ubiquitylated membrane proteins destined for the lysosomal degradation pathway (41,42). Fig. 6B shows that overexpression of YFP-Hrs did result in accumulation of intracellular CFP-DAT. CFP-DAT was partially co-localized with YFP-Hrs on the limiting membranes of endosomes. In addition, CFP-DAT could be seen in the lumen of many large endosomes. This pool of CFP-DAT probably represented transporters that were incorporated into internal vesicles of MVBs.

**DISCUSSION**

Ubiquitylation of transmembrane proteins, such as various receptors and channels, has been implicated in the regulation of their endocytosis, post-endocytic trafficking and turnover (26,43). In our study we detected constitutive and PKC-dependent ubiquitylation of DAT by endogenous ubiquitin in HeLa and PAE cells. We provide several lines of evidence that ubiquitin is conjugated to the DAT molecule, rather than to DAT-associated proteins. First, endogenous ubiquitin and polyubiquitin were detected in highly purified DAT preparations by mass-spectrometry. Second, several ubiquitylated peptides of DAT were identified. Third, a high-molecular weight band was detected by immunoblotting with the ubiquitin antibody in highly purified DAT preparations. Fourth, PKC-induced ubiquitylation of DAT by heterologously expressed YFP-ubiquitin was demonstrated.

Ubiquitylation of immunoprecipitated DAT with epitope-tagged ubiquitin has been recently reported in cells overexpressing Parkin, a RING-containing E3-ubiquitin ligase (44). The latter study implicated Parkin-induced DAT ubiquitylation in the quality control of newly-synthesized DAT processing in the endoplasmic reticulum. Our data show that the mature DAT protein is constitutively ubiquitylated by endogenous ubiquitin at the plasma membrane and that ubiquitylation of the mature DAT is markedly enhanced by PKC activity (Figs. 1, 2 and 5). Furthermore, we have not detected ubiquitylation of immature non-glycosylated DAT. Therefore, our observations imply that DAT ubiquitylation may have role in endocytic trafficking of DAT.

The effects of PKC on DAT endocytosis and post-endocytic trafficking have been observed in various experimental systems, but the molecular mechanisms underlying these effects are unknown. Recently, it has been demonstrated that the simultaneous replacement of the carboxyl-terminal residues 587-591 with alanines in the Tac-DAT chimera or the full-length human DAT inhibits PKC-dependent endocytosis of the chimera or the DA uptake capacity of DAT, respectively (45). Based on these data the latter report suggested that residues 587-591 constitute an internalization signal. However, the crystal structure of the homologous bacterial leucine transporter revealed that residues 587 and 588 represent a part of the twelve’s transmembrane α-helix (46). Therefore, multiple alanine substitutions of these two and neighboring residues may lead to misfolding of
this part of the DAT molecule. In fact, our previous studies revealed that mutation of Lys590 to alanine resulted in the retention of the newly-synthesized DAT in the endoplasmic reticulum, presumably due to transporter misfolding (36). Thus, residues 587-591 may not represent an endocytosis signal per se but rather be indirectly required for PKC-dependent endocytosis.

Demonstration of DAT ubiquitylation in response to PKC activation may shed light on some of the mechanisms of PKC-induced DAT trafficking. A growing literature supports the model by which ubiquitylation of various transmembrane endocytic cargos facilitates their internalization and subsequent degradation (26,43). Whereas the mechanisms of internalization of ubiquitylated cargo remain obscure, the involvement of ubiquitylation in the endosomal sorting step of trafficking and the mechanisms of this step involving recognition of ubiquitylated cargo by endosomes HRS-STAM and ESCRT complexes are well established (47). Since PKC activation led to accelerated degradation of DAT in both HeLa and PAE cells in our experiments (Fig. 6A), such a PKC effect on DAT turnover is likely a common phenomena in different cells. Thus, it is possible that DAT ubiquitylation could be responsible for DAT interactions with Hrs and ESCRT complexes at the limiting membrane of MVB followed by DAT incorporation into internal vesicles of MVBs. In fact, we have frequently observed DAT in the lumen of large endosomes containing Hrs at the limiting membrane (Fig. 6B).

Typically, degradation of ubiquitylated proteins is blocked by inhibitors of lysosomes suggesting that these cargos are degraded through lysosomal, rather than proteosomal, pathways. Although in several cases degradation of transmembrane cargo is also blocked by proteosomal inhibitors, the mechanisms of these effects are not understood (48,49). Degradation of DAT induced by PMA in MDCK cells was shown to be blocked by lysosomal but not proteosomal inhibitors (14). Sorting of transmembrane cargo in endosomes is thought to be mediated by monoubiquitylation rather than polyubiquitylation, which is responsible for targeting of proteins to the proteosome (Lys48-linked ubiquitin chains) (26). However, recent reports suggested a role of polyubiquitylation in lysosomal targeting (48,50). Likewise, polyubiquitylation of DAT may be involved in PKC-induced DAT degradation. In particular, detection of Lys63-linked chains in DAT in our experiments is especially interesting since Lys63-mediated di-ubiquitylation has been suggested to serve as a trafficking signal (51).

The mechanisms by which PKC activation leads to DAT ubiquitylation are unknown. PKC-dependent ubiquitylation must be mediated by either direct or indirect interaction of DAT with E3-ubiquitin ligase(s). In dopaminergic neurons, the RING domain containing E3 ligase Parkin and α-synuclein may mediate DAT ubiquitylation (44,52). However, these proteins are not expressed in HeLa and PAE cells. Yeast membrane transporters and several other proteins are ubiquitylated by the HECT-domain-containing E3 ubiquitin ligase Rsp5p/Npl1 (26). Therefore, it is also possible that the mammalian homolog of Rsp5, NEDD4 E3-ligase, that has a Ca2+ and phospholipid-binding C2 domain, could mediate PKC effects on DAT ubiquitylation (53). Interestingly, activation of PKC by phorbol esters leads to polyubiquitylation and degradation of PKC itself (54). This may suggest that there is an E3 ubiquitin ligase that is associated with PKC and that can participate in PKC-dependent ubiquitylation of transmembrane proteins like DAT.

**FOOTNOTES**

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**Abbreviations used:** DA, dopamine; DAT, dopamine transporter; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; FH-DAT, Flag/6XHis-DAT or Flag/10XHis-dopamine transporter; NET, norepinephrine, SERT, serotonin transporter; GAT, γ-aminobutyric acid transporter; GLYT, glycine transporters; NEM, N-ethylmaleimide; CHX, cyclohexamide; BIM, Bisindolylmaleimide I; YFP and CFP, yellow and cyan fluorescent protein; FRET, fluorescence resonance energy transfer; FRET C,
corrected FRET; PAE, porcine aortic endothelial; YFP-Hrs, hepatocyte-growth-factor receptor substrate; MudPIT, Multidimensional Protein Identification Technology; MVB, multivesicular endosomes.

REFERENCES

1. Chen, N. H., Reith, M. E., and Quick, M. W. (2004) Pflugers Arch 447, 519-531
2. Giros, B., and Caron, M. G. (1993) Trends in Pharmacological Sciences 14, 43-49
3. Torres, G. E., Gainetdinov, R. R., and Caron, M. G. (2003) Nat Rev Neurosci 4, 13-25
4. Kilic, F., and Rudnick, G. (2000) Proc Natl Acad Sci U S A 97, 3106-3111
5. Schmid, J. A., Scholze, P., Kudlacek, O., Freissmuth, M., Singer, E. A., and Sitte, H. H. (2001) J Biol Chem 276, 3805-3810
6. Sorkina, T., Doolen, S., Galperin, E., Zahniser, N. R., and Sorkin, A. (2003) J. Biol. Chem. 278, 28274-28283
7. Torres, G. E., Carneiro, A., Seamans, K., Fiorentini, C., Sweeney, A., Yao, W. D., and Caron, M. G. (2003) J Biol Chem 278, 2737-2739
8. Hastrup, H., Karlin, A., and Javitch, J. A. (2001) Proc Natl Acad Sci U S A 98, 10055-10060
9. Kitayama, S., Dohi, T., and Uhl, G. R. (1994) European Journal of Pharmacology 268, 115-119
10. Huff, R. A., Vaughan, R. A., Kuhr, M. J., and Uhl, G. R. (1997) Journal of Neurochemistry 68, 225-232
11. Copeland, B. J., Vogelsberg, V., Neff, N. H., and Hadjiconstantinou, M. (1996) Journal of Pharmacology and Experimental Therapeutics 277, 1527-1532
12. Zhang, L., Coffey, L. L., and Reith, M. E. A. (1997) Biochemical Pharmacology 53, 677-688
13. Zhu, S.-J., Kavanaugh, M. P., Sonders, M. S., Amara, S. G., and Zahniser, N. R. (1997) Journal of Pharmacology and Experimental Therapeutics 282, 1358-1365
14. Daniels, G. M., and Amara, S. G. (1999) Journal of Biological Chemistry 274, 35794-35801
15. Melikian, H. E., and Buckley, K. M. (1999) Journal of Neuroscience 19, 7699-7710
16. Loder, M. K., and Melikian, H. E. (2003) J. Biol. Chem. 278, 22168-22174
17. Sorkina, T., Hoover, B. R., Zahniser, N. R., and Sorkin, A. (2005) Traffic 6, 157-170
18. Melikian, H. E. (2004) Pharmacol Ther 104, 17-27
19. Vaughan, R. A., Huff, R. A., Uhl, G. R., and Kuhr, M. J. (1997) Journal of Biological Chemistry 272, 15541-15546
20. Foster, J. D., Pananusorn, B., and Vaughan, R. A. (2002) J Biol Chem 277, 25178-25186
21. Granas, C., Ferrer, J., Loland, C. J., Javitch, J. A., and Gether, U. (2003) J. Biol. Chem. 278, 4990-5000
22. Lin, Z., Zhang, P. W., Zhu, X., Melgari, J. M., Huff, R., Spieldoch, R. L., and Uhl, G. R. (2003) J Biol Chem 278, 20162-20170
23. Khoshbouei, H., Sen, N., Gubaroy, B., Johnson, L., Lund, D., Gnegy, M. E., Galli, A., and Javitch, J. A. (2004) PLoS Biol 2, E78
24. Fornes, A., Nunez, E., Aragon, C., and Lopez-Corcuera, B. (2004) J Biol Chem 279, 22934-22943
25. Robinson, M. B. (2002) J Neurochem 80, 1-11
26. Hicke, L., and Dunn, R. (2003) Annu Rev Cell Dev Biol 19, 141-172
27. Perroy, J., Pontier, S., Charest, P. G., Aubry, M., and Bouvier, M. (2004) Nature Methods 1, 203-208
28. Wu, C. C., Yates, J. R., 3rd, Neville, M. C., and Howell, K. E. (2000) Traffic 1, 769-782
29. MacCoss, M. J., McDonald, W. H., Saraf, A., Sadygov, R., Clark, J. M., Tasto, J. J., Gould, K. L., Wolters, D., Washburn, M., Weiss, A., Clark, J. I., and Yates, J. R., 3rd. (2002) Proc Natl Acad Sci U S A 99, 7900-7905
30. Wu, C. C., MacCoss, M. J., Howell, K. E., and Yates, J. R., 3rd. (2003) Nat Biotechnol 21, 532-538
31. Washburn, M. P., Wolters, D., and Yates, J. R., 3rd. (2001) *Nat Biotechnol* **19**, 242-247
32. Eng, J. K., McCormack, A. L., and Yates, J. R. I. (1994) *J. Amer. Soc. Mass Spectrom* **5**, 976-989
33. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003) *Nat Biotechnol* **21**, 921-926
34. Kirkpatrick, D. S., Gerber, S. A., and Gygi, S. P. (2005) *Methods* **35**, 265-273
35. Cooper, H. J., Heath, J. K., Jaffray, E., Hay, R. T., Lam, T. T., and Marshall, A. G. (2004) *Anal Chem* **76**, 6982-6988
36. Miranda, M., Sorkina, T., Grammatopoulos, T. N., Zawada, W. M., and Sorkin, A. (2004) *J Biol Chem* **279**, 30760-30770
37. Sorkin, A., McClure, M., Huang, F., and Carter, R. (2000) *Curr Biol* **10**, 1395-1398.
38. Galperin, E., Verkhusha, V. V., and Sorkin, A. (2004) *Nature Methods* **1**, 209-217
39. Saunders, C., Ferrer, J. V., Shi, L., Chen, J., Merrill, G., Lamb, M. E., Leeb-Lundberg, L. M., Carvello, L., Javitch, J. A., and Galli, A. (2000) *Proc Natl Acad Sci USA* **97**, 6850-6855
40. Raiborg, C., and Stenmark, H. (2002) *Cell Struct Funct* **27**, 403-408
41. Raiborg, C., Bache, K. G., Mehlum, A., Stang, E., and Stenmark, H. (2001) *Embo J* **20**, 5008-5021
42. Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E., and Stenmark, H. (2002) *Nat Cell Biol* **4**, 394-398
43. Hicke, L. (2001) *Nat Rev Mol Cell Biol* **2**, 195-201
44. Jiang, H., Jiang, Q., and Feng, J. (2004) *J Biol Chem*
45. Holton, K. L., Loder, M. K., and Melikian, H. E. (2005) *Nat Neurosci*
46. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) *Nature*
47. Raiborg, C., Rusten, T. E., and Stenmark, H. (2003) *Curr Opin Cell Biol* **15**, 446-455
48. Carter, S., Urbe, S., and Clague, M. J. (2004) *J Biol Chem* **279**, 52835-52839
49. Hammond, D. E., Carter, S., McCullough, J., Urbe, S., Vande Woude, G., and Clague, M. J. (2003) *Mol Biol Cell* **14**, 1346-1354
50. McCullough, J., Clague, M. J., and Urbe, S. (2004) *J Cell Biol* **166**, 487-492
51. Galan, J. M., and Hagenauer-Tsapis, R. (1997) *Embo J* **16**, 5847-5854
52. Doss-Pepe, E. W., Chen, L., and Madura, K. (2005) *J Biol Chem* **280**, 16619-16624
53. d'Azzo, A., Bongiovanni, A., and Nastasi, T. (2005) *Traffic* **6**, 429-441
54. Leontieva, O. V., and Black, J. D. (2004) *J Biol Chem* **279**, 5788-5801
Table. Ubiquitylated peptides of DAT and ubiquitin.

| Identification | Peptides | Charge |
|----------------|----------|--------|
| gi|4507041|ref|NP_00103 | 5.1| dopamine transporter | |
| + PMA | M.SSVVAPAK\(^{19}\)*EPNAVGPK.E | +2 |
| A.PAK\(^{19}\)*EPNAVGPKKEVELILVKEQNGVQLTSSTLTNPR.Q | +3 |
| I.LV\(^{35}\)*EQNGVQLTSSTLTNPR.Q | +3 |
| K.EVELILV\(^{35}\)*EQNGVQLTSSTLTNPR.Q | +3 |
| L.AYAIAPEK\(^{399}\)*DRELVDRGEVR.Q | +2 |
| vehicle | I.LV\(^{35}\)*EQNGVQLTSSTLTNPR.Q | +3 |
| gi|11024714|ref|NP_0618 | 28.1| ubiquitin B precursor | |
| + PMA | K.TLTGK\(^{11}\)*TITLEVEPSDTIENVK.A | +3 |
| R.TLSDYNIQK\(^{63}\)*ESTLHLVLR.L | +2 |
| R.TLSDYNIQK\(^{63}\)*ESTLHLVLR.L | +3 |
| Y.NIQK\(^{63}\)*ESTLHLVLR.L | +3 |
| vehicle | R.LIFAGK\(^{48}\)*QLEDGR.T | +2 |
| Y.NIQK\(^{63}\)*ESTLHLVLR.L | +3 |

* denotes Gly-Gly modification of the ε-amino group of the lysine side chain.
**FIGURE LEGENDS**

**Figure 1. Detection of DAT ubiquitylation in purified Flag- and His-tagged DAT.**

*A*, Schematic representation of DAT tagged with the Flag-epitope and 10xHis sequences at the amino-terminus (FH-DAT). HeLa cells stably expressing FH-DAT were incubated with vehicle (DMSO) or 1 μM PMA for 30 min at 37°C, and the cells were immunostained using anti-DAT followed by secondary anti-rat labeled with CY3. Bar, 10 μm.

*B*, HeLa/FH-DAT cells grown in 20 cm trays were treated with vehicle or PMA as in *A*, and then solubilized in 20 ml lysis buffer/tray. The cleared lysate (~30 mg protein) was affinity-purified using Ni-NTA agarose and FlagM2 gel as described in the “Experimental Procedures”. Coomassie-stained gels of the eluates from the Ni-NTA (left) and FlagM2 (right) are shown. dDAT, DAT dimers; mDAT, mature DAT monomer; ngDAT, non-glycosylated DAT.

*C*, FH-DAT was purified as described in *B* with the exception that 0.1% SDS was included throughout the purification procedure. A 10% aliquot of purified FH-DAT preparation was used for Western blotting with ubiquitin and DAT antibodies.

**Figure 2. PMA enhancement of DAT ubiquitylation is mediated by PKC.**

HeLa/FH-DAT cells were treated with vehicle or PMA as in Fig. 1. BIM (1 μM) was added 20 min before the incubation and was present during incubation of the cells with PMA. FH-DAT was pulled-down using Ni-NTA agarose, and ubiquitin immunoreactivity was detected in FH-DAT pull-downs. DAT-Ub, ubiquitylated DAT.

**Figure 3. Conjugation of YFP-ubiquitin to DAT.**

HeLa/FH-DAT cells were transiently mock-transfected (YFP), or transfected with wild-type YFP-Ub or a YFP-UbAA mutant incapable of conjugations to lysines. The cells were treated with PMA as in Fig. 1, and FH-DAT was pulled-down using Ni-NTA. YFP-containing proteins were then immunoprecipitated with GFP antibodies. FH-DAT pull-downs were probed with GFP, ubiquitin and DAT antibodies. Asterisks indicate non-specific bands detected by GFP antibodies.

**Figure 4. FRET between CFP-DAT and YFP-ubiquitin.**

PAE cells stably expressing CFP-DAT were transiently transfected with YFP-Ub or YFP-UbAA, and stimulated with PMA as described in Fig. 1. FRET measurements were performed as described in the “Experimental Procedures”. FRET is presented as quantitative pseudocolor image. The apparent FRET efficiency (Ed) was measured for individual endosomes. The error bars represent SEM (n=20). *p<0.05 compared with cells expressing YFP-UbAA. Bar, 10 μm.

**Figure 5. DAT ubiquitylation on the cell surface.**

HeLa/FH-DAT cells were treated with vehicle or PMA (1 μM) for 15 or 30 min at 37°C. The cells were surface-biotinylated, DAT was pulled down using Ni-NTA agarose and biotinylated FH-DAT was pulled-down from imidazole eluate using NeutrAvidin agarose. Non-biotinylated FH-DAT was pulled down from NeutrAvidin supernatants using FlagM2 gel. Both NeutrAvidin and FlagM2 pull-downs were immunoblotted with ubiquitin and DAT antibodies.

**Figure 6. Down-regulation of DAT by PMA and accumulation of endosomal DAT in cells overexpressing Hrs.**

*A*, HeLa/FH-DAT cells were incubated with 50 μg/ml CHX for 2 hr to inhibit protein synthesis before the addition of PMA. Vehicle or PMA (1 μM) were added for indicated times. The cells were lysed in the lysis buffer in which NEM was omitted to minimize DAT ubiquitylation and smearing of the
DAT immunoreactivity. FH-DAT was then detected in cell lysates using DAT antibody. Actin immunoreactivity is shown as a control for protein loading.

B, PAE cells stably expressing CFP-DAT were transiently transfected with YFP-Hrs. After 2 days the cells were treated with PMA for 30 min at 37°C. A z-stack of 30 images was acquired through the YFP and CFP channels from living cells at room temperature. Higher magnification images of the groups of individual endosomes indicated by white rectangles are shown below. Yellow in the merged images signifies co-localization of YFP and CFP fluorescence. Bar, 10 μm.
Figure 1

(A) FH-DAT Flag-His

(B) 

Parental HeLa FH-DAT FH-DAT+PMA
Parental HeLa FH-DAT FH-DAT+PMA

(B) 

250 |
150 |
100 |
75 |
50 |
37 |

Eluate from Ni-NTA
Eluate from Flag-M2

(C) 

Parental HeLa FH-DAT FH-DAT+PMA
Parental HeLa FH-DAT FH-DAT+PMA

(C) 

250 |
150 |
100 |
75 |
50 |
37 |

Eluate from Flag-M2

WB: Ubiquitin
WB: DAT

+SDS

dDAT
mDAT
ngDAT

DMSO
PMA
Figure 2

|            | HeLa | HeLa/FH-DAT | HeLa | HeLa/FH-DAT |
|------------|------|-------------|------|-------------|
| BIM        | -    | -           | +    | -           |
| PMA        | +    | +           | +    | +           |

```
250
150
100
75
ng-DAT
50
```

![WB:DAT](image1)

![WB:Ubiquitin](image2)

- DAT-Ub
- IgG
Figure 3

[Diagram showing protein expression and gel electrophoresis results with labels such as FH-DAT, YFP-Ub, and Blot: anti-GFP, anti-UBiquitin, and anti-DAT.]
Figure 4

(A)

(B)

$Ed$ (endosomes)

YFP-Ub  YFP-UbAA

0 0.04 0.08 0.12

0 15 A.Lu.f.L.

***

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Figure 6

A

| Time (hs) | PMA | Veh |
|----------|-----|-----|
| 0        |     |     |
| 0.5      |     |     |
| 1        |     |     |
| 2        |     |     |
| 4        |     |     |
| 6        |     |     |

WB: DAT

WB: Actin

B

CFP-DAT

YFP-Hrs

Merge
Enhanced ubiquitylation and accelerated degradation of the dopamine transporter mediated by protein kinase C
Manuel Miranda, Christine C. Wu, Tatiana Sorkina, Davin Korstjens and Alexander Sorkin

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