Oligomerization of Dopamine Transporters Visualized in Living Cells by Fluorescence Resonance Energy Transfer Microscopy*

Tatiana Sorkina, Suzanne Doolen, Emilia Galperin, Nancy R. Zahniser, and Alexander Sorkin‡

From the Department of Pharmacology and Neuroscience Program, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received for publication, October 18, 2002, and in revised form, May 5, 2003
Published, JBC Papers in Press, May 13, 2003, DOI 10.1074/jbc.M210652200

To examine the oligomeric state and trafficking of the dopamine transporter (DAT) in different compartments of living cells, human DAT was fused to yellow (YFP) or cyan fluorescent protein (CFP). YFP-DAT and CFP-DAT were transiently and stably expressed in porcine aortic endothelial (PAE) cells, human embryonic kidney (HEK) 293 cells, and an immortalized dopaminergic cell line 1RB/ANpr. Fluorescence microscopic imaging of cells co-expressing YFP-DAT and CFP-DAT revealed fluorescence resonance energy transfer (FRET) between CFP and YFP, which is consistent with an intermolecular interaction of DAT fusion proteins. FRET signals were detected between CFP- and YFP-DAT located at the plasma membrane and in intracellular membrane compartments. Phorbol esters or amphetamine induced the endocytosis of YFP/CFP-DAT to early and recycling endosomes, identified by Rab5, Rab11, Hrs and EEA1 proteins. Interestingly, however, DAT was mainly excluded from Rab5- and Hrs-containing microdomains within the endosomes. The strongest FRET signals were measured in endosomes, indicative of efficient oligomerization of internalized DAT. The intermolecular DAT interactions were confirmed by co-immunoprecipitation. A DAT mutant that was retained in the endoplasmic reticulum (ER) after biosynthesis was used to show that DAT is oligomeric in the ER. Moreover, co-expression of an ER-retained DAT mutant and wild-type DAT resulted in the retention of wild-type DAT in the ER. These data suggest that DAT oligomers are formed in the ER and then are constitutively maintained both at the cell surface and during trafficking between the plasma membrane and endosomes.

Plasma membrane transporters belonging to the family of Na⁺/Cl⁻-dependent neurotransmitter transporters play an important role in terminating the activity of the monoamine neurotransmitters and of γ-aminobutyric acid (see Ref. 1). Thus, reuptake of dopamine (DA)¹ from the synaptic cleft by the dopamine transporter (DAT) serves as the major mechanism for terminating dopaminergic neurotransmission in the brain (2). Because the efficiency of DA removal depends on the number of DAT molecules expressed on the plasma membrane, trafficking processes that control transporter distribution in the cell represent a potentially important mechanism by which neurotransmission could be regulated.

Newly synthesized DAT acquires glycosylation in the endoplasmic reticulum (ER) and Golgi complex and is then trafficked to the plasma membrane. Typically, a large pool of mature DAT molecules are found at the cell surface of dopaminergic neurons and when DAT is heterologously expressed in tissue culture cells. However, DAT localization can be altered rapidly. Acute exposure of cells to either phorbol esters or substrates reduces the number of plasma membrane DATs and thus DAT function, and this reduction is due to acceleration of DAT endocytosis through a dynamin-dependent mechanism (3–6). Recently, it has been shown that DAT can interact with the anchoring protein PICK 1 and the adaptor protein Hic-5 (7, 8). However, in general, the molecular mechanisms controlling DAT trafficking are not yet well understood.

The DAT molecule is predicted to have 12 membrane-spanning sequences with both amino and carboxyl termini oriented intracellularly. The specific function of DAT transmembrane (TM) motifs and termini are not well established. TM domains may play a role in intra- and intermolecular interactions. Many membrane receptors and other integral membrane proteins require dimerization or higher oligomerization for their activity. Several lines of evidence have suggested that monoamine transporters are also dimers or oligomers. Results with dominant negative forms of the serotonin transporter (SERT) and norepinephrine transporter were consistent with this idea (9, 10). Oligomerization of SERT was demonstrated directly by co-immunoprecipitation (11). The results of fluorescence resonance energy transfer (FRET) studies further confirmed that SERT and a γ-aminobutyric acid transporter (GAT-1) are homo-oligomers (12, 13). Early radiation inactivation studies also suggested that DAT exists as a dimer or oligomer (14, 15). Recently, the potential for DAT to exist as dimer or higher-order oligomer in the plasma membrane has been demonstrated using chemical cross-linking (16). More recently, Torres and co-workers (17) reported detection of DAT oligomerization by co-immunoprecipitation. In general, the mechanisms and functional roles of oligomerization of monoamine transporters remain to be defined. However, it has been proposed that SERT oligomerization may be important for its transport activity (9). Oligomerization may also be required for proper trafficking of

*This work was supported by Grants DA14204 (to A. S. and N. R. Z.) and DA1505 from National Institute on Drug Abuse (to N. R. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Dept. of Pharmacology, University of Colorado Health Science Center, 4200 E. Ninth Ave., Denver, CO 80262. Tel.: 303-315-7252; Fax: 303-315-7097; E-mail: alexander.sorkin@uchsc.edu.

¹The abbreviations used are: DA, dopamine; DAT, dopamine transporter; GFP, YFP, and CFP, green, yellow, and cyan fluorescent proteins; FRET, fluorescence resonance energy transfer; FRET**, corrected FRET; PAE, porcine aortic endothelial; ER, endoplasmic reticulum; PMA, phorbol 12-myristate 13-acetate; TM, predicted transmembrane domains; sulfo-NHS-S-S-biotin, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; SERT, serotonin transporter; GAT-1, γ-aminobutyric acid transporter; EGFR, epidermal growth factor receptor; CMF-PBS, Ca²⁺-Mg²⁺-free phosphate-buffered saline; DTT, dithiothreitol; FRETN, normalized sensitized FRET.
the newly synthesized transporter to the plasma membrane (13, 17).

Hence, we generated fluorescent human DAT fusion proteins, which allow visualization of DAT interactions during trafficking in living cells, and then used these proteins in conjunction with high resolution FRET microscopy to examine DAT oligomerization in various cellular compartments. The effective energy transfer between cyan (CFP) and yellow fluorescent protein (YFP) occurs when two proteins are 1–5 Å apart. This distance permits unusually resolved direct observation of proteins tagged with YFP and CFP. Measurements of sensitized FRET signals (fluorescence of the acceptor due to energy transfer from excited donor) on a pixel-by-pixel basis are designed to visualize protein–protein interactions in individual compartments of single living cells. Using this approach, we found that DAT is oligomerized in all cellular compartments in which it is localized, including post-synaptic compartments, plasma membrane, and endosomes, where DAT is accumulated when endocytosis is stimulated by phorbol esters or the DAT substrate amphetamine.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—Phorbol 12-myristate 13-acetate (PMA), p-amphetamine sulfate, DA, pargyline, and catechol were purchased from Sigma (St. Louis, MO). (-)-Cocaine HCl was obtained from National Institute on Drug Abuse/RTI International (Research Triangle Park, NC). Polyclonal rabbit antibody to GFP a.k.290 was purchased from Abcam Ltd. (Cambridge, UK); monoclonal mouse antibody to GFP was from Zymed Laboratories (South San Francisco, CA); monoclonal rat antibody to the amino terminus of DAT was from Chemicon, Inc. (Temecula, CA); and monoclonal mouse antibody to ERA 1 was from Transduction Laboratories, Inc. (San Diego, CA). [3H]DA (3,4-dihydroxyphenylethylamine hydrochloride; specific activity, 50 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA).

Plasmids—To generate the fusion proteins of human DAT (18) with YFP (YFP-DAT) or CFP (CFP-DAT), a DNA fragment encoding the full-length DAT was obtained by PCR using Pfu polymerase (Stratagene) and a DNA fragment corresponding to amino acid residues 66–620 was obtained by PCR using Pfu polymerase (Stratagene) and a KpnI restriction site into the 5’-end and a Smal site into the 3’-end, and cloned into the pEYFP-C1 vector or pECFP-C1 vector (Clontech, Palo Alto, CA), respectively. To generate YFP-DAT/C615, a stop codon was created at the position corresponding to amino acid residue 616 in the full-length DATpEYFP-C1 using a QuickChange site-directed mutagenesis kit according to the manufacturer’s protocol (Stratagene). To generate YFP-AN-DAT lacking amino-terminal cytoplasmic portion, a DNA fragment encoding the DAT sequence corresponding to amino acid residues 66–620 was obtained by PCR using Pfu polymerase (Stratagene) and a I site into the 3’/H11032 Sma I restriction site. The PCR products were then digested with KpnI and Smal and blunt-ended with T4 DNA ligase (Stratagene). The fragments were cloned into the pSUMS-Hygro vector (Stratagene Cloning Systems, La Jolla, CA), with a XhoI site between the KpnI and Smal sites, and the constructs were digested with XhoI and then ligated with the XhoI fragment of the pEYFP-C1 plasmid. The resulting plasmid was named pSUMS+Hygro-YFP-DAT. All plasmids were purified using a gigaMax-spin DNA purification kit (Gibco BRL). At least 10 pmol of the plasmid DNA was used for transformation of E. coli DH5α, and about 90% cotransformation efficiency was obtained. The concentration of the plasmid DNA was determined by UV absorption.

Western blotting was performed with monoclonal antibodies to GFP or CFP, respectively. The proteins were transferred to the membrane. Western blotting was performed with monoclonal antibodies to GFP or DAT followed by species-specific secondary antibodies conjugated with horse-radish peroxidase. The enhanced chemiluminescence kit was purchased from Pierce (Rockford, IL).

Surface Biotinylation—PAE or HEK293T cells were grown in 35-mm dishes and grown to 50–80% confluency and transfected with appropriate plasmids using Effectene (Qiagen, Hilden, Germany). In transient transfection experiments, the cells were split 1 day after transfection onto glass coverslips and used for experiments on the second or third day at about 50% confluency. For biochemical immunoprecipitation and biotinylation, cells were plated to about 100- or 35-mm dishes, respectively, and used for experiments on the second day at about 90% confluency. Enactin-Collagen IV-Laminin coating (Upstate Biotechnology, Lake Placid, NY) was applied to 35-mm dishes to increase HEK293 cell adherence. The immobilized dopaminergic cell line 1RB3AN27 was kindly provided by Dr. H. Stenmark (Radium Institute, Oslo, Norway).

Buffers—Phosphate-buffered saline containing 0.1 mM CaCl2 and 1 mM MgCl2 (PBS) and incubated for 20 min on ice with 1 mg/ml sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (EZ-LinkTM sulfo-NHS-S-S-biotin). After biotinylation, the cells were washed twice with cold phosphate-buffered saline containing 0.1 mM CaCl2 and 1 mM MgCl2 (CMF-PBS). The cells were then solubilized by scraping with a rubber policeman in lysis buffer (50 mM NaCl, 2 mM MgCl2, 0.5 mM MgSO4, 12 mM KCl, 10 mM HEPES, pH 7.4) supplemented with 10 μM pargyline, 10 μM ascorbic acid, and 10 μM catechol. Assays (1 ml) included 50 nM [3H]DA plus increasing amounts of unlabeled DA (final concentrations 50 nM to 50 μM). Non-specific [3H]DA accumulation was determined in the presence of 1 mM cocaine and averaged 2.0 ± 0.2% of total. After 10 min of incubation at 37 °C, uptake was terminated by quickly washing the cells three times with 1 ml of ice-cold KRH. Cells were then solubilized in 0.5 ml of 3% trichloroacetic acid for 30 min with gentle shaking. Accumulated [3H]DA was determined by liquid scintillation counting. Data were analyzed using GraphPad Prism (San Diego, CA).

Immunoprecipitation—PAE cells stably expressing DAT and/or YFP-DAT were grown on 100-mm dishes to about 90% confluency. The cells were then washed three times with Ca2+-, Mg2+-free phosphate-buffered saline (CMF-PBS). The cells were then solubilized by scraping with a rubber policeman in lysis buffer (50 mM NaCl, 2 mM MgCl2, 20 mM HEPES, pH 7.2, 10% glycerol, 10 mM dithiothreitol (DTT), 1 mM EGTA, 1 mM EDTA, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 544 μg/ml aprotinin, 1% Triton X-100, 1% sodium deoxycholate) and by further incubating for 10 min at 4 °C. The lysates were then cleared by centrifugation for 10 min at 16,000 × g and incubated with polyclonal antibodies to GFP (18 μg per dish) overnight at 4 °C. Under these conditions, at least 95% of [3H]DAT was immunoprecipitated. The precipitates were washed twice with lysis buffer supplemented with 10% fetal bovine serum and 0.1% sodium deoxycholate and then denatured by heating in sample buffer for 5 min at 95 °C. The immunoprecipitates and the aliquots of cell lysates were resolved on 7.5% SDS-PAGE, and the proteins were transferred to the membrane. Western blotting was performed with monoclonal antibodies to GFP or DAT followed by species-specific secondary antibodies conjugated with horseradish peroxidase. The enhanced chemiluminescence kit was purchased from Pierce (Rockford, IL).

Orientation Imaging Microscopy—PAE cells expressing hDAT, CFP-DAT, or YFP-DAT were grown in 12-well plates for 2–3 days. The cells were rinsed and then assayed in Krebs-Ringer HEPES buffer (KRH; 120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 10 mM glucose, 10 mM HEPES, pH 7.4) supplemented with 10 μM pargyline, 10 μM ascorbic acid, and 10 μM catechol. Assays (1 ml) included 50 nM [3H]DA plus increasing amounts of unlabeled DA (final concentrations 50 nM to 50 μM). Non-specific [3H]DA accumulation was determined in the presence of 1 mM cocaine and averaged 2.0 ± 0.2% of total. After 10 min of incubation at 37 °C, uptake was terminated by quickly washing the cells three times with 1 ml of ice-cold KRH. Cells were then solubilized in 0.5 ml of 3% trichloroacetic acid for 30 min with gentle shaking. Accumulated [3H]DA was determined by liquid scintillation counting. Data were analyzed using GraphPad Prism (San Diego, CA).

Immunofluorescence Staining—PAE cells expressing CFP-DAT were grown on glass coverslips and treated with MeSO, PMA, or amphetamine at 37 °C. The cells were then washed with CMF-PBS and fixed for 10 min by preparing 4% formaldehyde (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. Cells were then incubated in CMF-PBS containing 0.5% bovine serum albumin at room temperature for 1 h with a monoclonal antibody to GFP (1:100 in CMF-PBS) and then incubated with an Alexa 488-conjugated secondary antibody (Jackson Laboratory, West Grove, PA). For secondary antibody solutions were preclarified by centrifugation at 100,000 × g for 10 min. After staining, the coverslips were mounted in Fluoromount-G (Southern Biotech Inc., Birmingham, Alabama) and dried for at least 24 h before viewing.
AL) containing 1 mg/ml p-phenylendiamine. To obtain high resolution three-dimensional images of cells, the fluorescence imaging workstation consisted of a Nikon inverted microscope equipped with a 100× oil immersion objective lens, cooled charge-coupled device SensiCam QE 16 MHz (Cooke, Germany), z-step motor, dual filter wheels, and a xenon 175-watt light source, all controlled by SlideBook 3.0 software (Intelligent Imaging Innovation, Denver, CO). Typically, 25–40 serial two-dimensional images were recorded at 100–200-nm intervals. A Z-stack of images obtained was deconvoluted using a modification of the constrained iteration method. Final arrangement of all images was performed using Adobe Photoshop.

Live Cell Microscopy and FRET Imaging—PAE or HEK293 cells transiently or stably co-expressing various YFP/CFP-tagged proteins were grown on glass coverslips. The coverslips were mounted in a microscope chamber and placed on a microscope stage. To visualize CFP and YFP, consecutive images were acquired through corresponding filter channels.

The method of sensitized FRET measurement has been described previously (19, 21). We previously validated this technique by measuring FRET intensities in various control interaction pairs and by using the donor fluorescence recovery method (21). Briefly, images were acquired sequentially through YFP, CFP, and FRET filter channels. Filter sets used were: YFP (excitation, 500/20 nm; emission, 520/30 nm), CFP (excitation, 436/10 nm; emission, 470/30 nm), and FRET (excitation, 436/10 nm; emission, 535/30 nm). An 86004S dichroic mirror (Chroma, Inc.) was utilized. Running 2 × 2, 100- to 250-nm integration times were used. The background images were subtracted from the raw images prior to carrying out FRET calculations. Corrected FRET (FRET<sup>c</sup>) was calculated on a pixel-by-pixel basis for the entire image using Equation 1: FRET<sup>c</sup> = FRET − [0.50 × CFP] − [0.02 × YFP], where FRET, CFP, and YFP correspond to background-subtracted intensities of cells co-expressing CFP and YFP acquired through the FRET, CFP, and YFP filter channels, respectively. 0.50 and 0.02 are the fractions of bleed-through of CFP and YFP fluorescence, respectively, through the FRET filter channel. The negative FRET<sup>c</sup> values obtained in some control experiments are due to slight overestimation of the bleed-through coefficients.

FRET<sup>c</sup> values were calculated from the mean fluorescence intensities for each selected sub-region of the image containing individual ruffles, phalloidin and lamellipodia (plasma membrane), diffuse fluorescence of the plasma membrane, endosomes, and tubular-vesicular ER structures according to Equation 1. Normalized sensitized FRET (FRET<sup>N</sup>) values for individual cellular compartments were calculated according to Equation 2: FRET<sup>N</sup> = FRET<sup>c</sup>/YFP × CFP, where FRET<sup>c</sup>, CFP, and YFP are the mean intensities of FRET<sup>c</sup>, CFP, and YFP fluorescence in the selected sub-region. In these calculations, the fluorescence intensity of CFP was underestimated because of donor fluorescence quenching due to FRET. The decrease in donor fluorescence was calculated from FRET<sup>c</sup> values using the conversion coefficient G (3.2 in all experiments) as described (22) and was found to be about 5–10% of CFP fluorescence intensities. Therefore, the underestimation did not significantly affect FRET<sup>N</sup> values. Because FRET<sup>N</sup> displays a non-linear dependence when donor or acceptor are in a significant molar excess over each other, FRET<sup>N</sup> values were calculated in the sub-regions of the cell in which the amount of the donor did not exceed the amount of the acceptor more than 2-fold and vice versa.

FRET<sup>c</sup> images are presented in pseudocolor mode. FRET<sup>c</sup> intensity is displayed stretched between the low and high renormalization values, according to a temperature-based lookup table with blue (cold) indicating low values and red (hot) indicating high values. To eliminate the distracting data from regions outside of cells, the YFP channel was used as a saturation channel, and the FRET<sup>c</sup> images are displayed as YFP intensity-modulated images. In these images, data with YFP values greater than the high threshold of the saturation channel are displayed at full saturation, whereas data values below the low threshold are displayed with no saturation (i.e. black). All calculations were performed using the Channel Math and FRET modules of the SlideBook software.

RESULTS

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).

Characterization of CFP-DAT and YFP-DAT—To visualize DAT in living cells, fusion proteins of full-length DAT with YFP or CFP were generated, in which YFP or CFP was fused to the amino terminus of DAT (Fig. 1A). Previous studies reported that the fusion of GFP or YFP to the amino terminus of DAT did not affect functional properties of the transporter (3, 23). YFP-DAT and CFP-DAT were transiently or stably expressed in PAE cells. PAE cells have been our preferred cell line for oligomerization of Dopamine Transporter (24).
tagged DAT and YFP-DAT migrated on SDS-PAGE as smeared bands of ~73–75 kDa and 103–105 kDa (the difference corresponds to ~26 kDa of YFP), respectively (Fig. 1C). Minor biotinylated bands of ~180–190 kDa for DAT and ~250 kDa for YFP-DAT were also detected. These forms may represent SDS-resistant dimers of DAT and YFP-DAT, correspondingly. Several species of DAT and YFP-DAT were also detected in the supernatants from the NeutrAvidin affinity column. The non-biotinylated forms, which migrated similar to the biotinylated forms, are likely to correspond to surface-exposed DAT (especially, in the case of YFP-DAT) that were not biotinylated due to the limited efficiency of biotinylation or internalized, endosomal DAT. Other forms with molecular weights different from the surface forms probably represent monomeric and dimeric species of DAT and YFP-DAT located intracellularly. A 75- to 80-kDa band of YFP-DAT (IC-1) recognized by antibodies to DAT (Fig. 1C) and GFP (data not shown) represents non-glycosylated YFP-DAT. The corresponding form of DAT (50 kDa) was poorly detected because of the overlap with IgG, but was clearly detectable in cell lysates (data not shown). A 180- to 190-kDa form (IC-2) may correspond to SDS-resistant dimers of IC-1 (Fig. 1C).

That YFP- and CFP-DAT were efficiently transported along the biosynthetic pathway to the cell surface was also demonstrated by fluorescence microscopy. Fig. 2 shows that CFP-DAT and YFP-DAT co-expressed in PAE cells were mainly localized in the plasma membrane, often concentrating in ruffle- and phyllopodia-like structures (Fig. 2). In addition, a pool of fluo-
FRET between CFP-DAT and YFP-DAT—DA uptake and cell surface biotinylation experiments validated the use of YFP-DAT and CFP-DAT expressed in PAE cells to examine the oligomeric state of these proteins in various cellular compartments. In previous studies we described a three-filter method of FRET microscopic analysis in living cells (19). This method allows rapid measurement of sensitized FRET signals with high spatial resolution. Fig. 2A shows that positive CFP → YFP FRET signals were detected in the diffuse fluorescence areas and phyllodopa/ruffle structures of the plasma membrane when YFP-DAT and CFP-DAT were co-expressed in PAE cells. These data suggest that the amino termini of the two proteins are in close proximity to each other and that DAT forms dimers or higher oligomers at the cell surface.

As a control for FRET analysis of the CFP/YFP-DAT pair, we measured FRET signals in cells co-expressing YFP-DAT with an unrelated integral membrane protein, the receptor for epidermal growth factor (EGFR) tagged with CFP (EGFR-CFP). Co-expressed YFP-DAT and EGFR-CFP were highly co-localized in the plasma membrane (Fig. 2B). However, no FRET signals were detected in these cells, suggesting that the proteins did not form complexes.

Because the efficiency of energy transfer depends on the amount of donor and acceptor capable of interaction, FRET values were divided by the product of YFP and CFP intensities on a pixel-by-pixel basis to obtain normalized FRET (FRET N) values for various regions of the cell (see “Experimental Procedures”). FRET N values correlate with the equilibrium binding constant for the measured interaction and may, therefore, serve as a measure of the relative affinity of interaction (22). FRET N values in cells expressing CFP-DAT/YFP-DAT were similar in the diffuse fluorescence areas and ruffle/phyllodopa structures of the plasma membrane (Table I). These values were substantially higher than in control cells expressing two co-localized but non-interacting proteins, EGFR-CFP and YFP-DAT (Table I).

DAT has been shown to be capable of rapid endocytosis when heterologously expressed (3, 5, 6, 25). To test whether DAT remains oligomerized during endocytic trafficking, the endocytosis of CFP- and YFP-DAT in PAE cells was induced by PMA. Exposure to PMA at 37 °C for 5–30 min (1 μM) caused accumulation of YFP- and CFP-DAT in vesicular structures in the perinuclear area in most of the cells, and this occurred concomitantly with a decrease in the plasma membrane fluorescence (Fig. 2). Down-regulation of DAT has also been shown to be triggered by amphetamine in HEK293 cells, although the endosomal localization of DAT in amphetamine-treated cells was not confirmed (6). Using fluorescent DAT proteins we show here that YFP- and CFP-DAT accumulated in vesicular organelles after 2-h treatment of PAE cells at 37 °C with 4 μM amphetamine (Fig. 2A). In contrast to PMA-treated cells, visible accumulation of DAT in vesicular organelles was observed in a limited fraction of the amphetamine-treated cell population, and the extent of endosomal accumulation of DATs was smaller than in cells treated with PMA. Importantly, positive FRET signals were detected in vesicular organelles containing YFP- and CFP-DAT in cells treated with either PMA or amphetamine (Fig. 2A). FRET N values varied significantly in different cells but were consistently higher in endosomes (see Table I).

Phorbol esters are known to cause transient down-regulation and endosomal accumulation of EGFR (26). Interestingly, PMA treatment of PAE cells co-expressing YFP-DAT and EGFR-CFP resulted in translocation of both cargoes into the same vesicular organelles (Fig. 2B). As in the case of untreated cells, no FRET signals between CFP-DAT and EGFR-YFP were detected in PMA-treated cells, suggesting that the proteins did not form complexes (Table I).

Having shown FRET between co-expressed CFP- and YFP-DAT in endothelial cells, we examined whether similar oligomerization can be observed in dopaminergic cells. To this end, we performed FRET imaging experiments in neurons grown in culture as described in Table I. Mean FRET N values calculated for various subcellular regions of the cells

<table>
<thead>
<tr>
<th>FRET pair</th>
<th>Cellular compartment</th>
<th>Cells</th>
<th>FRET N (10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP-DAT/CFP-DAT</td>
<td>Plasma membrane</td>
<td>PAE</td>
<td>18.77 ± 4.03 (n = 40)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1RB, AN</td>
<td>16.98 ± 4.52 (n = 21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HEK</td>
<td>16.98 ± 4.52 (n = 21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endosomes</td>
<td>58.65 ± 29.6 (n = 78)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1RB, AN</td>
<td>43.33 ± 30.16 (n = 10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HEK</td>
<td>9.14 ± 3.00 (n = 27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER</td>
<td>10.99 ± 4.05 (n = 16)</td>
</tr>
<tr>
<td>YFP-DAT/EGFR-CFP</td>
<td>Plasma membrane</td>
<td>HEK</td>
<td>0.00 ± 4.57 (n = 11)</td>
</tr>
<tr>
<td>YFP-DAT/EGFR-CFP</td>
<td>Endosomes</td>
<td>HEK</td>
<td>20.55 ± 4.05 (n = 16)</td>
</tr>
<tr>
<td>YFP-DAT-C615/EGFR-CFP</td>
<td>Endosomes</td>
<td>ER</td>
<td>-0.11 ± 4.86 (n = 18)</td>
</tr>
</tbody>
</table>

* Measured in cells co-expressing CFP-DAT and YFP-DAT in the regions of diffuse fluorescence, ruffles, phlloypodia, lamellipodia, and cell contacts.
* Mean value ± S.D.
* Measured in cells co-expressing CFP-DAT and YFP-DAT treated with PMA or amphetamine.
* Measured in cells co-expressing CFP-DAT and the YFP-DAT-C615 mutant.
* Negative control. Cells expressing proteins that are co-localized but that do not interact.
CFP images were acquired from living cells at room temperature. In Bor YFP-Hrs (B). After 2 days the cells were treated as in Fig. 2. YFP and CFP images were acquired from living cells at room temperature. In A, higher magnification images of small regions of the cell (white rectangle) are shown. In B, higher magnification images of individual endosomes indicated by the arrows are shown. Yellow in the merged images signifies co-localization of YFP and CFP fluorescence. Bar, 10 μm.

end, we used an immortalized dopaminergic cell line 1RB3AN27 (20). These cells begin to express tyrosine hydroxylase and differentiate under conditions of growth in the presence of dibutyryl cAMP (20). Oligomerization of CFP/YFP-DAT was also observed in 1RB3AN27 cells expressing CFP-DAT and YFP-DAT (Fig. 3). CFP- and YFP-DAT displayed localization similar to that observed in PAE cells. FRET c signals were readily detectable at the cell surface in control cells, in endosomes in cells treated with PMA (Fig. 3 and Table I), and in the ER (data not shown).

Accumulation of CFP-DAT in Early Endosomes and Late Recycling Compartment in Cells Treated with PMA and Amphetamine—To confirm that vesicular structures containing DAT in cells treated with PMA or amphetamine represent endosomal compartments, the localization of DAT was compared with that of endosomal markers. PAE cells stably expressing CFP-DAT (PAE/CFP-DAT) were transiently transfected with YFP-Rab5 (A) or YFP-Hrs (B). After 2 days the cells were treated as in Fig. 2. YFP and CFP images were acquired from living cells at room temperature. In A, higher magnification images of small regions of the cell (white rectangle) are shown. In B, higher magnification images of individual endosomes indicated by the arrows are shown. Yellow in the merged images signifies co-localization of YFP and CFP fluorescence. Bar, 10 μm.

Fig. 4. PMA- and amphetamine-induced endocytosis of CFP-DAT into Rab5- and Hrs-containing endosomes. PAE cells stably expressing CFP-DAT were transiently transfected with YFP-Rab5 (A) or YFP-Hrs (B). After 2 days the cells were treated as in Fig. 2. YFP and CFP images were acquired from living cells at room temperature. Arrows point to examples of co-localization of CFP and YFP fluorescence, signified by yellow. Bar, 10 μm.

DAT was also well co-localized with another endosomal protein, Hrs (Fig. 4B). This protein is involved in targeting of ubiquitylated cargo to the internal membranes of multivesicular bodies and lysosomal degradation pathway (30). Hrs overexpression results in the blockade of endosomal trafficking, recruitment of clathrin to endosomes, and enlargement of endosomes (31). Interestingly, however, DAT was mainly excluded from the Hrs-containing microdomains/coats within individual endosomes (Fig. 4B), suggesting that the endosomal sorting of DAT is not mediated by the Hrs pathway.

YFP-Rab11 was mainly localized in tubular-vesicular and vesicular compartments in the Golgi area and cell periphery (Fig. 5). As in the case of Rab5, a pool of CFP-DAT was co-localized with YFP-Rab11 in endosomes upon treatment with PMA or amphetamine. A small amount of CFP-DAT was seen co-localized with Rab11 in untreated cells, presumably as the result of constitutive internalization and recycling of CFP-DAT. These data demonstrate in living cells that DAT is internalized in response to PMA and amphetamine treatment into various types endosomal compartments that are involved in major sorting and recycling pathways.

Confocal microscopy was used to confirm the accumulation of DAT in early endosomes using an endogenous marker of these compartments. To this end, CFP-DAT localization was compared with localization of the early endosomal marker EEA1 in fixed cells using image reconstitution by deconvolution of three-dimensional images (Fig. 6). The optical sectioning revealed significant co-localization of CFP-DAT and EEA1 in endosomes (Fig. 5). Together, Figs. 4–6 demonstrate the PMA- or amphetamine-induced endocytosis of tagged DAT into early-recycling endosomal compartments, in which, according to FRET analysis, DAT molecules are oligomerized (Figs. 2–3).

Co-immunoprecipitation of Wild-type Untagged and Mutant YFP-tagged DAT—FRET microscopic analysis provided strong evidence for the constitutive oligomerization of DAT in living PAE cells. To confirm the oligomeric status of DAT using a different assay, co-immunoprecipitation experiments were performed. To this end, wild-type DAT or CFP-DAT was constitutively or transiently co-expressed with YFP-ΔN-DAT, a mutant in which the entire amino-terminal portion (65 amino acid treated in one or several poles of the endosome, in so-called “Rab5 domains” (29).
residues) was deleted and replaced by YFP. PAE cells constitutively expressing DAT or YFP-ΔN-DAT alone were used as controls. YFP-ΔN-DAT was efficiently transported to the surface in PAE cells under all expression conditions based on surface biotinylation (Fig. 7A) and fluorescence imaging experiments (Fig. 7B). A similar, but untagged, DAT mutant was retained in the ER (17). It is possible that the attachment of YFP to the truncated DAT in our experiments had a stabilizing effect on the transporter molecule, thus allowing proper folding of YFP-ΔN-DAT in the ER and effective ER export. Transient co-expression of YFP-ΔN-DAT with CFP-DAT in PAE cells resulted in co-localization of the two proteins in the plasma membrane and positive FRETC signals (Fig. 7B), similar to what was observed with wild-type CFP- and YFP-DAT proteins (Fig. 2). Despite expression at the cell surface, however, no [3H]DA uptake by YFP-ΔN-DAT was detected (data not shown). Interestingly, a DAT mutant, in which the 59 amino terminal residues of DAT were deleted, is delivered to the plasma membrane and displayed normal dopamine transport in Madin-Darby canine kidney cells (32). This may suggest a critical role of residues 59–65 in DAT transport activity. Alternatively, the discrepancies between trafficking and transport activities of amino-terminally truncated DAT mutants observed by Gu and co-workers (32), Torres and co-workers (17), and in our experiments may be due to different cellular backgrounds.

In co-immunoprecipitation experiments, YFP-ΔN-DAT was precipitated from the lysates of PAE cells stably co-expressing DAT and YFP-ΔN-DAT using a GFP antibody. The immunoprecipitates were probed with rat anti-DAT antibody that recognizes the full-length DAT but not the YFP-ΔN-DAT mutant. The wild-type DAT migrating on SDS-PAGE at the position corresponding to monomeric plasma membrane DAT (73–75 kDa, see also Fig. 1) was detected in GFP immunoprecipitates, indicative of YFP-ΔN-DAT/DAT association (Fig. 7C). Other forms of DAT were not detected in significant amounts in immunoprecipitates and cell lysates in these experiments. Based on densitometric analysis of the intensity of chemiluminescence signals from DAT immunoreactivity in anti-GFP immunoprecipitates and aliquots of lysates (11% of the amount used for immunoprecipitation) were probed with antibodies to DAT or GFP. DAT-M and YFP-ΔN-DAT, monomeric forms of DAT and YFP-ΔN-DAT, respectively. Intracellular, presumably, immature forms of 75 kDa and ~180–190 kDa of YFP-ΔN-DAT were not detected in significant amounts in these cells.

**Fig. 7. Interaction of amino-terminal deleted YFP-ΔN-DAT fusion protein with full-length DAT detected by FRET and co-immunoprecipitation.** A, PAE cells stably expressing YFP-ΔN-DAT were subjected to surface biotinylation as described in Fig. 1C. NeutrAvidin-conjugated beads (NeuAv) were resolved by SDS-PAGE, and DAT was detected by Western analysis using rat anti-DAT. The two lanes are duplicates. B, CFP-DAT and YFP-ΔN-DAT were transiently co-expressed in PAE cells, and FRET was measured as described in Fig. 2. FRETC is presented as pseudocolor intensity-modulated images (FRETC/YFP), A.I.u.f.i., arbitrary linear units of fluorescence intensity. Bar, 10 μm. C, PAE cells stably expressing wild-type DAT and YFP-ΔN-DAT or co-expressing both DAT and YFP-ΔN-DAT were lysed, and YFP-ΔN-DAT was precipitated using anti-GFP antibody. Immunoprecipitates and aliquots of lysates (11% of the amount used for immunoprecipitation) were probed with antibodies to DAT or GFP. DAT-M and YFP-ΔN-DAT-M, monomeric forms of DAT and YFP-ΔN-DAT, respectively. Intracellular, presumably, immature forms of 75 kDa and ~180–190 kDa of YFP-ΔN-DAT were not detected in significant amounts in these cells.

**Oligomerization of YFP-DAT and CFP-DAT in the Biosynthetic Compartments**—To test whether newly synthesized DAT exists as an oligomer during its anterograde transport through the ER and Golgi, a mutant of DAT with impaired transport to the plasma membrane was constructed. In this mutant the last five amino acid residues of the carboxyl terminus that include a PDZ (PSD/Discs large/zona occludens-1) domain-binding sequence important for plasma membrane transport (7) were deleted (YFP-DAT-C′615). When transiently expressed in PAE or other cells for 1–2 days, this mutant was primarily localized in the tubular-vesicular network seen throughout the entire cell, often in the nuclear envelope membrane and concentrated in the Golgi area (Fig. 8). This pattern of localization is typical
of the distribution of newly synthesized proteins in the ER, intermediate compartment, and Golgi complex (further referred as “ER localization”). A small pool of YFP-DAT-C615 could be detected at the cell surface after 3 days of expression, in contrast to DAT mutants lacking 35 or 42 amino acid residues of the carboxyl terminus, which were not transported to the plasma membrane under any conditions (data not shown).

When YFP-DAT-C615 was co-expressed with CFP-DAT, both proteins were co-localized in the ER in some cells. In other cells CFP-DAT displayed strong plasma membrane fluorescence, whereas YFP-DAT-C615 was located in the ER. Analysis of the relative expression of the two transporters in individual cells revealed that in cells with a relatively high CFP/YFP ratio (for example, 3:1 in Fig. 8A), most of the CFP-DAT reached the cell surface. In contrast, in cells expressing relatively higher levels of YFP-DAT-C615 (for example, 1:1 CFP/YFP ratio in Fig. 8A), both wild-type and mutant DAT were retained in the ER. In the latter case, positive FRET signals were detected, suggesting CFP-DAT/YFP-DAT-C615 oligomerization in the ER (Fig. 8A and Table I). To control for the specificity of FRET signals in the ER, YFP-DAT-C615 was co-expressed with EGFR-CFP for 16–18 h. At this time after transfection, a large pool of EGFR-CFP was moving through the biosynthetic pathway and was highly co-localized with YFP-DAT-C615 (Fig. 8B). However, no positive FRET signal was detected (Fig. 8B). The FRETN values of ER-localized YFP/CFP-DAT in PAE cells were on average lower than these values in other cellular compartments, although they were significantly higher than FRETN of the negative control, EGFR-CFP/YFP-DAT-C615 (Table I). The data presented in Fig. 8A suggest 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.

To further substantiate this conclusion, the effect of overexpression of DAT-C615 on plasma membrane expression of wild-type DAT was examined using a surface biotinylation assay. DAT expression levels were relatively low in PAE cells, particularly, when the two DAT fusion proteins were co-expressed. Therefore, for these biochemical experiments we used HEK293 cells, which allow high efficiency of DAT expression in a large fraction of the cell population. These cells have previously been well characterized as a DAT expression system (6). Transiently co-expressed CFP-DAT and YFP-DAT were located at the plasma membrane in many HEK293 cells (Fig. 9A), although in cells with very high levels of expression, the two proteins were often retained in the ER. As observed in PAE cells, CFP-DAT was retained together with the traffic-defective mutant YFP-DAT-C615 in the ER of HEK293T cells when the mutant was expressed at near equimolar or higher levels compared with CFP-DAT (Fig. 9A).

Co-expression of YFP-DAT-C615 with untagged DAT significantly and concentration-dependently reduced the amount of DAT detectable by surface biotinylation (Fig. 9B). Only small amounts of a 105-kDa form of YFP-DAT-C615 (corresponding to the monomeric plasma membrane form of YFP-DAT, see Fig. 1) was detected by blotting with anti-GFP in lysates (Fig. 9B, right panel) and the biotinylated fraction (data not shown). The major forms of the mutant (75–80 kDa and 180–190 kDa bands) were not accessible to surface biotinylation and were, therefore, located intracellularly (Fig. 9B). These data are consistent with the imaging analysis demonstrating an impaired transport of YFP-DAT-C615 to the cell surface. The dominant negative effect of the ER export-deficient DAT mutant on surface expression of DAT further suggested that oligomerization of the newly synthesized DAT occurs in the ER and that proper DAT oligomerization is necessary for the efficient transport of DAT from the ER to the plasma membrane.

**DISCUSSION**

In this study we used fluorescence microscopy to visualize the compartmentalization and oligomerization state of DAT in living cells. We chose to use the PAE endothelial cells as the main expression system for YFP- or CFP-DAT. In addition to the usefulness of these cells for fluorescence microscopy studies (19), we found that PAE cells were the best model in which to perform experiments with transiently expressed DAT fusion constructs. Unlike HEK293, COS-1, and HeLa cells, in which transient expressions resulted in the retention of DAT in the ER in a large fraction of the cell population, wild-type DAT was...
Moreover, using PAE/DAT cells we were able to demonstrate endocytosis of DAT induced by 4 μM amphetamine into the same early/recycling endosomal compartments. This observation provides direct support for the hypothesis that DAT down-regulation observed upon similar exposure to amphetamine in HEK293 cells is the result of DAT internalization to endosomes (6). In all of these studies, including our own, relatively long exposure times were used. However, evidence from experiments following in vitro exposure to methamphetamine and DA supports the relevance of substrate-induced down-regulation in brain (4, 33, 34). Furthermore, protein kinase C inhibitors block substrate-induced DAT down-regulation in in vitro models, suggesting that protein kinase C-induced endocytosis is involved in the regulation mediated by substrates (4, 35, 36).

The capability to visualize trafficking of functional YFP/CFP-DAT in PAE cells prompted us to use FRET to analyze DAT oligomerization. Biochemical methods of detection of dimerization, such as immunoprecipitation and cross-linking, require cell disruption and cannot guarantee that dimers are not formed during cell lysis. In contrast, the FRET technique allows detection of dimerization in intact living cells. Modification of the three-filter method to measure sensitized FRET was previously employed in our studies of proteins involved in trafficking and signaling of EGFR (19). The high resolution of this technique allows analysis of FRET efficiencies in individual organelles in living cells. The three-filter method also allows normalization of FRET signals to the amount of donor and acceptor on a pixel-by-pixel basis. The resulting FRETN values obtained for each individual pixel or group of pixels corresponding to a cellular organelle can be then compared.

FRET analysis of interactions between YFP-DAT and CFP-DAT revealed positive FRET and FRETN values in all cellular compartments, indicative of close proximity of two proteins and consistent with dimerization or higher oligomerization. The stringent quality control in the ER could be the reason for relatively homogeneous but low level expression of DAT in PAE cells and, therefore, relatively weak FRET signals. It should be pointed out that even under conditions of an equimolar ratio of YFP- and CFP-DAT, the probability of heterodimerization of YFP- and CFP-DAT is only 50%, which reduces the maximal FRET and FRETN values by 2-fold compared with the values that would be observed for the same affinity interaction of two different proteins. In general, FRETN values in Table I have relatively high standard deviations, as have been observed in all previous studies using this technique (22, 37). Furthermore, the biological significance of the differences among FRETN values obtained in different cellular compartments is uncertain. The ER in PAE cells tended to yield the lowest FRETN values, presumably due to low local concentrations of YFP- and CFP-DAT in this organelle in these cells. The highest FRETN values were obtained in endosomes, possibly because of the high local concentrations of DAT fusion proteins in these compartments. Overall, the results of the FRET analysis suggest that DAT is oligomerized constitutively and that it is unlikely that oligomerization is involved in the regulation of endocytic trafficking.

Dimerization or higher oligomerization may be, however, important for DAT trafficking from the ER to the plasma membrane. Figs. 8 and 9 show that overexpression of an ER export-defective mutant has a dominant-negative effect on the plasma membrane transport of wild-type DAT. The same effects were convincingly documented in a report by Torres and co-workers (17). These observations are consistent with the model whereby ER export-competent DAT molecules must oligomerize to traffic through the ER-Golgi system to the cell surface. The impor-
Oligomerization of Dopamine Transporter

28283

tance of oligomerization of GAT-1 for transport to the plasma membrane has been directly demonstrated by Scholze and co-workers (13).

The mechanisms responsible for DAT-DAT intermolecular interactions have recently begun to be elucidated. It is likely that TM domains are responsible for oligomerization. For example, in our experiments deletion of the amino terminus of DAT did not prevent DAT-DAT oligomerization. The predicted leucine zipper motif in TM2 appears to be involved in the intermolecular interactions (17), similar to what was demonstrated for GAT-1 oligomerization (13). TM6 has also been proposed to be one of the interaction interfaces (16). Studies of DAT molecular size using radiation inactivation predicted that DAT is a tetramer (14, 15). The tetrameric state of SERT was also predicted based on co-immunoprecipitation experiments (11). However, our FRET microscopic analysis does not provide a reliable way to distinguish tetramerization from dimerization.

Although live-cell FRET analysis requires expression of transporters in heterologous systems, DAT oligomerization has been demonstrated in an immortalized dopaminergic cell line (Fig. 3) and rat striatum by both cross-linking and radiation inactivation techniques (14, 16), suggesting that this property of the DAT molecule may be important for its function in dopaminergic neurons. The exact functional role of DAT oligomerization in the regulation of DA uptake remains to be elucidated. However, expression of SERT concatenations consisting of four SERT molecules resulted in chimeric proteins with increased affinities for serotonin uptake (9). Oligomerization of SERT has also been suggested to be important for efficient serotonin transport (11). Because TM domains are critical for both proper folding and oligomerization of DAT, mutations in TM domains can affect the transport capacity of DAT at the plasma membrane indirectly through the poor delivery of the newly synthesized transporter to the plasma membrane, as well as directly. Therefore, careful analysis of subcellular localization of TM mutants of DAT will be necessary to elucidate the role of oligomerization in the transport function of DAT.

Acknowledgments—We thank Dr. B. Hoover for help with dopamine uptake experiments, Dr. H. Stenmark for YFP-Hrs plasmid, and Dr. K. Prasad for IRB, ANP cells.

REFERENCES
Oligomerization of Dopamine Transporters Visualized in Living Cells by Fluorescence Resonance Energy Transfer Microscopy

Tatiana Sorkina, Suzanne Doolen, Emilia Galperin, Nancy R. Zahniser and Alexander Sorkin

doi: 10.1074/jbc.M210652200 originally published online May 13, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M210652200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 23 of which can be accessed free at http://www.jbc.org/content/278/30/28274.full.html#ref-list-1