The dopamine transporter (DAT) is a presynaptic plasma membrane protein responsible for the termination of dopaminergic neurotransmission in the central nervous system. While most studies have focused on structure/function analysis, much less information is available regarding the assembly and the trafficking of this protein. To address this problem, we performed a mutational analysis of the DAT protein, combined with biochemical, immunological, and functional approaches. In mammalian cells co-expressing differentially tagged DAT molecules, HA-tagged DAT co-purified with 6His-tagged DAT demonstrating a physical interaction between transporter proteins. Evidence for the functional oligomerization of DAT was obtained using dominant-negative mutants of DAT. Two loss-of-function mutant transporters (Y335A and D79G) that were targeted to the cell surface inhibited wild-type DAT uptake activity without affecting the membrane targeting of the wild-type transporter. Moreover, non-functional amino and carboxyl termini-truncated mutants of DAT inhibited wild-type DAT function by interfering with the normal processing of the wild-type transporter to the cell membrane. Mutations in the leucine repeat of the second transmembrane domain of the transporter could eliminate the dominant-negative effect of all these mutants. In addition, a small fragment comprising the first two transmembrane domains of DAT inhibited wild-type transporter function but not when the leucine repeat motif was mutated. Taken together, our results suggest that the assembly of DAT monomers plays a critical role in the expression and function of the transporter.

The dopamine transporter (DAT) belongs to a large family of Na\(^+\)/Cl\(^-\)-dependent plasma membrane transporters that also includes the closely related norepinephrine and serotonin transporters (NET and SERT, respectively), and carriers for GABA, glycine, proline, taurine, and betaine. In the central nervous system, DAT mediates the re-uptake of released dopamine (DA) from the synaptic cleft back into the nerve terminal for subsequent storage and release. Pharmacological and genetic studies highlight the DAT-mediated re-uptake process as the main mechanism for the termination of dopamine neurotransmission (1). In addition, DAT represents the main target site for commonly abused drugs such as cocaine and amphetamine as well as some therapeutic agents used in the management of affective disorders (2).

Hydropathicity analysis of their deduced amino acid sequence reveals that Na\(^+\)/Cl\(^-\)-dependent plasma membrane neurotransmitter transporters are proteins containing twelve transmembrane domains (TMs) with both the amino and the carboxyl termini located on the intracellular side of the membrane. This topological arrangement has been confirmed for several members of the family, including DAT (3). Since the molecular cloning of this transporter gene family, a great deal of information has been accumulated concerning the relationship between the structure and function of this class of proteins (4). Studies using mutagenesis and heterologous expression systems have identified several amino acid residues and domains involved in substrate and inhibitor binding. Moreover, there is growing evidence suggesting that the subcellular distribution of monoamine transporters is regulated by second messenger systems (5). Activation of protein kinase C in cells expressing DAT, SERT, or NET results in decreased transporter activity (6–8). This effect is believed to result from a rapid redistribution of transporter proteins from the cell surface to intracellular compartments (9–10). In the case of DAT, there is also evidence indicating that substrates and inhibitors appear to regulate the cellular distribution of this transporter (11–12).

Despite the increasing numbers of studies reporting DAT function and regulation, much less information is available regarding the mechanisms involved in the cellular processing of this transporter. Recent biochemical evidence suggests that neurotransmitter transporters exist as oligomeric complexes in cells, but the relevance of this process to transporter function as well as the molecular determinants involved in transporter assembly are unclear. Oligomers of SERT have been detected by co-immunoprecipitation of differentially tagged monomers (13). Fluorescence resonance energy transfer analysis has also provided evidence for the oligomerization of GABA transporters (14). In addition, electrophysiological and freeze fracture electron microscopic studies revealed a pentameric structure.
for the unrelated neuronal glutamate transporter EAAT3 in the plasma membrane of Xenopus oocytes (15). More recently, Hastrup et al. (16) in an elegant series of experiments, demonstrated that the human DAT could be cross-linked as a homodimer at the plasma membrane of HEK-293 cells and provided evidence for a role of TM6 as an oligomerization domain (16). The only exception to the transporter oligomerization rule so far appears to be the glycine transporter (GlyT), another member of the Na+/Cl- dependent transporter family. Biochemical analysis of the size of GlyT expressed in Xenopus oocytes is not consistent with an oligomeric structure for this transporter protein (17). This seemingly contradictory finding suggests that oligomerization might not be a common feature in transporter biology, or alternatively it is possible that biochemical approaches used to study oligomerization might disrupt the protein-protein interactions required to maintain oligomeric complexes. In any case, these results point out to the need for functional approaches to establish the oligomeric nature of a given transporter complex and its functional relevance.

Given the importance of DAT in normal and abnormal brain function, it becomes important to understand how these proteins are regulated at the cellular level. In this report, we used mutational analysis combined with biochemical, immunological, and functional approaches to examine the assembly and trafficking properties of the human DAT expressed in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]DIA (31.6 Ci/mmol) was supplied by PerkinElmer Life Sciences, Taq polymerase was from Fisher Scientific, restriction enzymes were from Takara Biomedicals, and DNA purification kits were from Qiagen, anti-HA antibody was from Roche, and the anti-His antibody was from Sigma. Secondary antibodies conjugated with HRP, FITC, or Texas Red were from Jackson Immunoresearch. Sulfo-NHS-SS-biotin and ultralink avidin beads were from Pierce.

**DNA Constructs and Mutagenesis**—The full-length cdna encoding the human DAT was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen). PCR-based mutagenesis (36 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min) was used to incorporate the His6 (HHHHHHH) or the HA (YPYDYVDPYD) epitopes into the amino terminus of DAT. Mutations in the coding region of DAT including Y435A, D796, and the substitution of leucine residues from TM2 or TM9 to alanine were performed by site-directed mutagenesis and combined with overlapping PCR. Amino-terminal deletions of DAT were generated by introducing initiator methionine residues at amino acid positions 11, 20, 48, and 60. Carboxyl-terminal transporter truncations were created by introducing stop codons in the coding region of DAT at amino acid positions 611, 601, 591, 582, 293, and 141. Asparagine residues from N-linked glycosylation consensus sequences were replaced by glutamine residues by site-directed mutagenesis. After PCR mutagenesis, restriction fragments containing mutated sequences were digested with appropriate restriction enzymes, subcloned into pcDNA3.1 and verified by automated sequencing.

**Cell Culture and Transfections**—HEK-293 cells were grown to 60% confluence in 100-mm tissue culture dishes and transiently transfected using the CaPO4 precipitation method with 5 μg of total DNA. Cells were incubated with the CaPO4-DNA mixture at 37 °C for 16 h, followed by 48 h recovery in minimal essential medium supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, and 50 units/ml of gentamycin. Subsequent experiments were performed 48–72 h after transfections.

**Transport and Binding Measurements**—The conditions for dopamine uptake in HEK-293 cells have been adopted from Giros et al. (18). Briefly, 48–72 h after transfections, medium was removed, and uptake was measured following incubation of cells for 5 min with 250 μl of uptake buffer (in mm: Tris base, 7.5 Hepes, 120 NaCl, 5.4 KCl, 1.2 CaCl2, 1.8 MgCl2, 1.2 Na2HPO4, pH 7.4) containing 50 nM [3H]DIA (31.6 Ci/mmol) and increasing concentrations of cold DA ranging from 100 nm to 30 μM. After rinsing with 1 ml of NaCl-free uptake buffer, cells were solubilized in 0.5 ml of 1% SDS, and the radioactivity incorporated into the cells was measured by liquid scintillation counting. Nonspecific uptake was determined in the presence of 2 μM mazindol or 10 μM cocaine. The protein concentration was measured using the BCA protein assay kit (Pierce). Data are presented as the mean ± S.E. For assessment of whole cell DAT levels, transiently transfected HEK-293 cells were grown in 150-mm dishes. The medium was removed, and cells were washed twice with PBS. Cells were lysed at 4 °C for 20 min with 2 mM HEPES and 1 mM EDTA buffer and then scraped and centrifuged at 31,000 × g for 20 min. The resultant pellet was homogenized in binding buffer (0.25 M sucrose and 10 mM Na2HPO4, pH 7.4) using a polytron homogenizer. For saturation experiments, 100 μg of protein aliquots were incubated for 1 h at room temperature with 4 nM [3H]CFT (83.9 Ci/mmol) and increasing concentrations of cocaine ranging from 1 μM to 100 μM. The reaction was terminated by filtering the samples through Whatman GF/C glass fiber filters with a Brandel cell harvester. Nonspecific binding was determined in the presence of 2 μM mazindol. The protein concentration was measured using the BCA protein assay kit (Pierce). Data are presented as means ± S.E.

**Immunocytochemistry and Confocal Microscopy**—For immunostaining experiments, transiently transfected HEK-293 cells grown on glass coverslips were placed in 6-well dishes at a density of 5 × 105 cells/well, followed by fixation in 4% paraformaldehyde. After three washes with PBS, cells were permeabilized in PBS containing 0.1% Triton X-100 for 10 min and incubated in blocking solution (1% bovine serum albumin, 5% goat serum in PBS) for 1 h. Cells were incubated with rat anti-DAT (1:1000) or a rabbit anti-DAT (1:1000) antibodies for 1 h at room temperature followed by incubation with Texas red-conjugated anti-rat or FITC-conjugated anti-rabbit secondary antibodies. Cells were then washed three times in PBS, and the coverslips were mounted on glass slides using Vectashield (Vector Laboratories). Immunofluorescent images were generated using a Zeiss laser scanning confocal microscope at 855 nm for Texas Red and 488 nm for FITC.

**Cell Surface Biotinylation**—Transiently transfected monolayers of HEK-293 cells were washed three times with PBS and then incubated with gentle agitation for 30 min at 4 °C with 1 ml of 1 mg/ml sulfo-NHS-biotin prepared in 150 mM NaCl, 2 mM CaCl2, 10 mM triethanolamine, pH 7.6. The reaction was quenched by incubating the cells for an additional 10 min at room temperature. The cells were then washed three times in PBS and incubated in radiolabelled precipitation assay buffer (RIPA) (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate, pH 7.4) for 1 h. Each sample was divided into two aliquots. One aliquot was used for isolation of biotinylated proteins with ultralink-immobilized neutravidin beads. The second aliquot was used to determine total DAT levels. Samples were analyzed by Western blotting with the rat anti-DAT antibody and an HRP-conjugated secondary antibody.

**His6-DAT Purification and Western Blotting**—HEK-293 cells expressing His6-DAT with or without HA-DAT were lysed in PBS containing 1% Triton X-100, followed by fixation in 4% paraformaldehyde. The lysates were run through a nickel-based purification assay in transfected HEK-293 cells. The lysates were then washed three times in PBS and incubated in radiolabelled precipitation assay buffer (RIPA) (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate, pH 7.4) for 1 h. Each sample was divided into two aliquots. One aliquot was used for isolation of biotinylated proteins with ultralink-immobilized neutravidin beads. The second aliquot was used to determine total DAT levels. Samples were analyzed by Western blotting with the rat anti-DAT antibody and an HRP-conjugated secondary antibody.

**RESULTS**

**Physical Association between Differentially Tagged DAT Proteins**—As a first step to understand the cellular regulation of the human DAT, we investigated whether DAT proteins could form oligomeric complexes in intact cells. DAT proteins were differentially tagged with either the His6 (His6-DAT) or the HA (HA-DAT) epitopes and assayed for protein-protein interaction by a nickel-based purification assay in transiently transfected HEK-293 cells. To ensure that these epitope-tagged transporter molecules retained their structural and functional integrity, uptake assays were first performed in HEK-293 cells transfected with the individual constructs. As seen in Fig. 1A the tagged transporters were functional and displayed kinetic and pharmacological properties similar to the wild-type transporter. Purification of His6-DAT on nickel columns under non-denaturing conditions allowed the detection of HA-DAT when both proteins are expressed simultaneously in the same cell, demonstrating that DAT proteins form tight complexes in living cells (Fig. 1B, third lane). No interaction was detected when lysates from cells expressing individual constructs were mixed prior to
purification on nickel columns (Fig. 1B, second lane) indicating that the interaction occurs only when both tagged proteins are co-expressed in the same cell and is not promoted during protein solubilization.

Non-functional DAT Mutants Inhibit Wild-type DAT Function—A physical interaction between DAT proteins strongly suggest the formation of an oligomeric complex. To provide direct functional evidence for DAT oligomerization, we reasoned that mutant transporter molecules devoid of uptake activity might still associate with the wild-type DAT and confer a dominant-negative effect on wild-type transporter function when co-expressed in cells. In searching for critical amino acids involved in transporter function and expression, we identified several residues that when mutated produced transporter proteins that exhibited little or no uptake activity. One of these residues, a tyrosine at amino acid position 335 located on the third intracellular loop between TMs 6 and 7, is conserved in all members of the Na⁺/Cl⁻ transporter family and forms part of a putative consensus sequence for tyrosine-based internalization motif (19). We replaced this residue to alanine by site-directed mutagenesis and examined transporter activity in HEK-293 cells. When expressed in cells, the DATY335A mutant does not exhibit detectable uptake activity (Fig. 2A), despite the fact that the protein is properly targeted to the cell membrane as evidenced by cell surface biotinylation experiments (Fig. 2B) and confocal microscopy images of immunostained transfected cells (Fig. 2C). Recently, Loland et al. (20) have also shown that the Y335A mutation in DAT results in a transporter protein exhibiting less than 1% of uptake activity. In addition, these authors provide evidence suggesting that this tyrosine is critical in mediating conformational changes in DAT during activation-inactivation steps.

To test the possibility that this non-functional mutant might exhibit a dominant-negative effect, mutant and wild-type transporters were co-expressed in cells, and uptake activity was assayed. Co-expression of DATY335A with wild-type DAT results in a significant reduction of [³H]DA uptake as compared with that observed in cells expressing wild-type DAT alone (Fig. 3A). In contrast, no decrease in uptake activity of the wild-type DAT was observed when co-expressed either with an empty vector or with the unrelated β₂-adrenergic receptor protein (Fig. 3C). The reduction of wild-type DAT activity upon DATY335A co-expression was not due to a decrease in the total expression levels of the wild-type DAT or the amount of the wild-type transporter expressed at the cell membrane as revealed by biotinylation of cell surface proteins using the HA-tagged transporter (Fig. 3B). Kinetic analysis revealed an approximate 60% decrease in $V_{\text{max}}$ with a small alteration in $K_m$ (2.2 μM in cells expressing wild-type DAT versus 3.6 μM in cells co-expressing wild-type DAT and Y335A). Thus, these findings provide evidence for the formation of an oligomeric complex between wild-type DAT and mutant Y335A transporter proteins at the cell membrane.

In the second mutant examined, an aspartate residue at position 79 in the first TM of DAT was replaced with glycine (D79G). This residue is conserved only in monoamine transporters, whereas in the rest of the members of the Na⁺/Cl⁻-dependent transporter family, this position is occupied by a glycine residue. When expressed in HEK-293 cells, the D79G mutant did not exhibit detectable uptake activity (Fig. 4A). Preliminary studies suggest that Asp-79 in DAT is critical in determining substrate selectivity.² Cell surface biotinylation and immunostaining revealed that the mutant protein was efficiently delivered to the plasma membrane (Fig. 4A, inset). Thus, as in the case of the Y335A mutant, D79G lacks intrinsic

²G. Torres, unpublished results.
transport activity. We then explored the possibility that the D79G mutant might exhibit dominant-negative effect when co-expressed with the wild-type transporter. As shown in Fig. 4B, at a constant level of wild-type DAT, co-expression of D79G caused a significant reduction of [3H]DA uptake. The dominant-negative effect exhibited by the mutated transporter occurs when both wild-type and mutated DAT are co-expressed at the cell membrane. Thus, these results provide another example of a transporter complex at the cell membrane formed by a functional and a non-functional transporter.

Role of the Intracellular Carboxyl-terminal Domain in DAT Assembly—Next, we sought to determine which domains are involved in transporter oligomerization. We first turned our attention to the intracellular carboxyl-terminal of DAT. This domain has been shown to interact with intracellular proteins such as PICK1 and synuclein (21, 22) and thus represent a candidate domain for monomer-monomer interaction. We made a series of deletions in the carboxyl-terminus of DAT at amino acid positions 611, 601, 591, and 582 (designated S582* through Q611*, Fig. 5A) by introducing stop codons at the respective positions. All carboxyl-terminal-truncated proteins expressed well in HEK-293 cells and gave proteins of the appropriate molecular size (data not shown). Uptake experiments from HEK-293 cells expressing each of these mutants revealed that progressive deletions of the carboxyl terminus of DAT (Q611*, R601*, and L591*) produced a progressive decrease in transport activity (Fig. 5B). L591* exhibited less than 1% of wild-type uptake function. A further deletion in the tail of DAT (S582*) completely abolished transporter function (Fig. 5B), suggesting that S582* lacks transport activity and/or is not expressed at the plasma membrane. Confocal microscopy analysis of transfected cells stained with the anti-DAT antibody demonstrates that the lack of function of S582* is due to the impaired targeting of this mutant to the plasma membrane (Fig. 5C).

To examine the possibility that the S582* mutant might inhibit the function of the wild-type DAT, we expressed both constructs simultaneously in HEK-293 cells and measured uptake activity. No detectable uptake activity was observed in cells expressing the S582* mutant (Fig. 6A). However, the uptake activity of DAT is decreased in the presence of the S582* mutant as compared with that of DAT alone. Under conditions in which the whole cell levels of wild-type DAT remains constant, there is a dramatic decrease in the amount of cell surface wild-type DAT when co-expressed with S582* (Fig. 6B, inset). Thus, the decreased uptake of the wild-type DAT when co-expressed with the deletion mutant was apparently due to a physical trapping of the wild-type and mutant DAT proteins in the cytoplasm. We further explored this possibility by examining the subcellular localization of wild-type and mutant transporters when expressed individually or in combination in HEK-293 cells. The wild-type transporter was tagged with GFP at the amino terminus whereas the deletion mutant was tagged with the HA epitope also at the amino terminus. This strategy allows for the differential visualization of wild-type and mutant proteins when co-expressed in the same cell. When expressed alone, the HA-tagged truncated transporter showed an intracellular staining pattern contrasting the clear plasma membrane distribution observed in cells expressing the wild-type GFP-tagged transporter (Fig. 6C, upper panels). When wild-type and mutant transporters were co-expressed, the distribution of the wild-type transporter
changed dramatically. Immunofluorescent experiments demonstrate that wild-type DAT colocalizes with the mutant transporter intracellularly (Fig. 6, middle panels). As a control, we co-transfected the GFP-tagged α1B-adrenergic receptor along with the SS582* mutant transporter. As shown in Fig. 6C (lower panels) the membrane localization of the receptor is not altered in the presence of the deletion mutant demonstrating that the mutant transporter does not cause a general nonspecific effect on protein processing. Taken together, our results indicate that the intracellular tail of the transporter is essential for the trafficking of this protein to the plasma membrane, and the inhibitory effect of the truncated transporter on wild-type DAT function results from the formation of oligomeric complexes unable to undergo normal processing to the cell surface. Because the truncated mutant is still able to associate with the wild-type transporter, these findings also suggest that the intracellular tail of DAT does not appear to be essential for DAT oligomerization.

**Functional Expression of DAT Amino-terminal-truncated Mutants**—Amino-terminal deletions of DAT were generated to examine the role of this domain in the functional expression of the transporter. We created DAT deletion mutants lacking the first 10, 20, 48, and 60 amino acids (designated Δ10, Δ20, Δ48, and Δ60, respectively). In each case an initial methionine was engineered into the truncated sequence to ensure correct translation. When expressed in HEK-293 cells Δ10, Δ20, and Δ48 displayed transport activity similar to the wild-type transporter. In all cases only $V_{\text{max}}$ was reduced whereas $K_m$ values were not significantly altered ($K_m$ values in μM; wild-type DAT, 2.6 ± 0.7; Δ10, 2.8 ± 0.6; Δ20, 2.1 ± 0.5; and Δ48, 1.91 ± 0.6, n = 4). In contrast, cells expressing the Δ60 transporter mutant did not show any detectable uptake activity (Fig. 7A). Immunofluorescence analysis with an anti-DAT antibody against the second extracellular loop of DAT, revealed an intracellular distribution of the Δ60 deletion mutant in transfected HEK-293 cells when compared with cells expressing the wild-type DAT (Fig. 7B). These results demonstrate that the lack of transport displayed by the Δ60 mutant is due to the improper processing and/or sorting of this protein leading to the absence of transporter molecules on the plasma membrane.

Next, we examined the ability of the amino-terminal deletion Δ60 mutant to co-associate with the full-length transporter by testing for dominant-negative effect when co-expressed in HEK-293 cells. Similar to the dominant-negative effect showed by the SS582* mutant, the Δ60 mutant caused a significant reduction of wild-type transport activity (Fig. 7C). Immunostaining of cells transfected with the GFP-DAT in the presence of the Δ60 mutant revealed an increase in the intracellular distribution of the full-length transporter when compared with cells expressing the GFP-tagged wild-type DAT alone (Fig. 7D). Hence, the dominant-negative effect of Δ60 on the wild-type transporter results as a consequence of the association of mutant and wild-type transporters inside the cells. These results also rule out the involvement of the amino terminus of DAT as an essential domain in oligomerization.

**A Leucine-repeat Motif in TM2 Is Required for DAT Oligomerization and Trafficking**—We next investigated the role of two leucine zipper-like motifs present within the second and the ninth TMs of DAT. This class of motif, which consists of four leucine residues periodically spaced by six amino acids and arranged in an α helix-like structure, was originally described in DNA-binding proteins and believed to mediate protein-protein interactions (23). Using site-directed mutagenesis, we replaced the three leucine residues and one methionine from TM2 at amino acid positions 99, 106, 113, and 120 by alanine...
residues (TM24LA). Cells expressing this mutant transporter did not exhibit detectable uptake activity (Fig. 8A) despite the fact that the protein was synthesized at similar levels compared with the wild-type transporter (Fig. 8B). Interestingly, the size of this mutated transporter was much smaller than the size of the wild-type transporter. Cell surface biotinylation revealed that TM24LA was not expressed at the plasma membrane (data not shown). The affinity of cocaine for the transporter mutant was slightly decreased (IC50 = 333.2 nM in cells expressing DAT versus 832.5 nM in cells expressing TM24LA, Fig. 8C) suggesting that the mutant transporter is still capable of forming functional binding sites. In contrast, replacement of the four leucine residues in TM9 at amino acid positions 440, 447, 454, and 461 by alanine residues (TM9LA) resulted in a functional transporter with similar kinetic and pharmacological properties compared with the wild-type transporter (Fig. 8A). Despite the similarities between these two domains, our results are consistent with a critical role for the leucine repeat from TM2, but not for that from TM9, in the functional expression of the transporter.

Having demonstrated that the substitution of the leucine repeat from TM2 results in a non-functional transporter, we next explored whether this mutant might still interact with the wild-type transporter. To examine this possibility, we co-transfected wild-type DAT with TM24LA and assayed uptake activity. As seen in Fig. 8D, [3H]DA uptake was similar in cells expressing DAT and TM24LA compared with cells expressing DAT alone. In addition, we failed to detect a protein-protein interaction between the HA-tagged TM24LA and the HIS-tagged DAT (data not shown). Thus, these results suggest that the inability of TM24LA to inhibit the function of the wild-type DAT is due to the lack of interaction between this mutant transporter and the wild-type DAT. To support this possibility, we performed immunofluorescence analysis in cells co-expressing the GFP-tagged full-length DAT and the HA-tagged TM24LA mutant transporter. As shown in Fig. 8E, the HA-tagged TM24LA mutant is not expressed at the cell membrane as compared with the full-length GFP-tagged DAT. In addition, the trafficking of the GFP-tagged DAT to the cell surface was not altered in the presence of the TM24LA mutant suggesting that the TM24LA transporter mutant is unable to associate with the wild-type DAT. Furthermore, the dominant negative effect exhibited by the Y355A, D79G, S582T, and Δ60 mutants upon co-expression with the wild-type transporter could be eliminated when the leucine repeat was mutated in these mutants (Table I).

Role of N-linked Glycosylation in Transporter Oligomerization and Trafficking—As shown above, elimination of the leucine repeat from TM2 in DAT results in a non-functional transporter devoid of dominant-negative effect. However, the observation that the size of this mutant protein was much smaller than that of the wild type and similar to the expected size for the non-glycosylated form, raised the possibility that lack of glycosylation might explain the lack of function in the mutant transporter. To examine this possibility, we investigated the role of N-linked glycosylation in the functional expression of DAT. The human DAT contains three putative N-linked glycosylation sites in the second extracellular loop at positions 181, 188, and 205. We generated a series of mutants by replacing each of the asparagines to alanine residues by site-directed mutagenesis. These glycosylation mutants were transiently transfected in HEK-293 cells and analyzed for transporter activity, protein size, and subcellular distribution. Individual substitution of asparagine residues from N-glycosylation consensus sequences resulted in a reduction in the size of the protein (Fig. 9B), suggesting that the three consensus sequences are glycosylated. Analysis of transporter activity in cells transfected with the single mutants revealed uptake activity similar to that observed for the wild-type transporter (Fig. 9A), indicating that single glycosylation sites are not essential for transporter function. Cell surface biotinylation experiments and immunostaining demonstrated that these mutant transporters are properly targeted to the plasma membrane (Fig. 9, B and C). We next examined whether removal of all glycosylation sites resulted in any alteration in transporter expression and function. As shown in Fig. 9B, the triple glycosylation mutant (TGM) migrated as a protein of ~70 kDa, consistent with the contribution of the three glycosylation sites to the size of the transporter. Uptake assays revealed an approximate 50% decrease in Vmax with no alterations in Kmic (1.7 μM in cells expressing the wild-type DAT versus 1.9 μM in cells expressing the TGM transporter). Subcellular localization of the non-glycosylated transporter using immunofluorescence microscopy and cell surface biotinylation showed that the reduction in transport function is associated with an increase in the intracellular retention of the mutated transporters (Fig. 9, A and C). Thus, removal of all three glycosylation sites partially impairs the trafficking of DAT to the plasma membrane. However, the non-glycosylated transporter is still capable of forming functional transporter proteins at the plasma membrane.

Transporter Fragments Containing TM2 Inhibit Wild-type Transporter Activity—Although the results obtained with the TM24LA mutant suggest that TM2 is an interacting domain within DAT, they do not exclude the possibility that the lack of interaction observed might be due to the improper folding of the mutant protein. To provide direct evidence for the contribution of TM2 in transporter assembly, we generated two transporter fragments containing the leucine repeat, TM1–2, and TM1–5 (Fig. 10A). TM1–2 contains a stop codon at the end of the first intracellular loop at amino acid position 141, whereas TM1–5 contains a stop codon in the extracellular loop of DAT between TMs 5 and 6 at amino acid position 293. As
predicted, these transporter fragments are not functional when expressed in HEK-293 cells (Fig. 10B). However, when co-expressed with the wild-type transporter, each of these fragments showed dominant-negative effect (Fig. 10B). Confocal microscopy images of cells transfected with the full-length DAT and either TM1–2 or TM1–5 mutant proteins revealed an increase in the intracellular distribution of the full-length DAT (data not shown). To further support the involvement of the leucine-repeat motif from TM2 in DAT assembly, we generated two additional fragments. TM1-EL1 contains a stop codon at the beginning of TM2 at amino acid position 94, whereas TM1–TM2L4A as revealed with the anti-DAT antibody. Arrows represent the glycosylated and non-glycosylated forms of the transporter. C, [3H]CFT binding experiments performed in cells expressing wild-type DAT or TM2LA. The total binding of the TM2LA mutant was ~25% of the wild-type transporter. D, the TM2 leucine-repeat mutant does not exhibit dominant-negative effect when co-expressed with the wild-type transporter. E, confocal microscopy images shows lack of co-localization between the GFP-tagged full-length DAT and the HA-tagged TM2LA leucine-repeat mutant.

Table I

| Construct(s) | Uptake activity % DAT |
|--------------|----------------------|
| wt-DAT       | 100                  |
| wt-DAT/Y335A | 38                   |
| wt-DAT/D79G  | 43                   |
| wt-DAT/S582  | 54                   |
| wt-DAT/Δ60   | 46                   |
| wt-DAT/TM2LA | 97                   |
| wt-DAT/Y335A-TM2LA | 96               |
| wt-DAT/D79G-TM2LA | 94               |
| wt-DAT/S582-TM2LA | 96               |
| wt-DAT/Δ60-TM2LA | 92               |

Fig. 8. **Integrity of a leucine repeat in TM2 is important for DAT assembly and trafficking.** A, transport activity of wild-type DAT, TM2 leucine-repeat mutant (TM24LA), or TM9 leucine-repeat mutant (TM94LA) in the absence or the presence of 10 μM cocaine (TM4LA + C). Results are representative of three independent experiments. B, Western blot analysis of whole cell transporter proteins from HEK-293 cells transfected with wild-type DAT or TM2LA as revealed with the anti-DAT antibody. Arrows represent the glycosylated and non-glycosylated forms of the transporter. C, [3H]CFT binding experiments performed in cells expressing wild-type DAT or TM2LA. The total binding of the TM2LA mutant was ~25% of the wild-type transporter. D, the TM2 leucine-repeat mutant does not exhibit dominant-negative effect when co-expressed with the wild-type transporter. E, confocal microscopy images shows lack of co-localization between the GFP-tagged full-length DAT and the HA-tagged TM2LA leucine-repeat mutant.

Fig. 9. **Role of N-linked glycosylation in the functional expression of DAT.** A, transport activity of wild-type DAT, single N-linked glycosylation mutants N181Q, N188Q, and N205Q, and the triple glycosylation mutant (TGM). Results are representative of three independent experiments. B, biotinylation of cell surface wild-type DAT and N-linked glycosylation mutants revealed with the anti-DAT antibody. Arrows represent the sizes of the fully, partially, and non-glycosylated proteins. C, confocal microscopy images from HEK-293 cells transfected with wild-type DAT or N-linked glycosylation mutants. Immunostaining was performed with the anti-DAT antibody.
DISCUSSION

In this study, we have examined several aspects of the cell biology of the human dopamine transporter expressed in HEK-293 cells. The main conclusions from the experiments presented here are that DAT exists as an oligomeric complex in intact cells and that the assembly process is required for the proper trafficking of the transporter complex to the plasma membrane. Several independent lines of evidence support these conclusions. First, we have provided biochemical evidence for the physical association between differentially epitope-tagged transporter proteins, indicating that the transporter exist as an oligomeric complex in intact cells. Second, we have used non-functional mutants of DAT that are expressed at the plasma membrane and exhibit dominant-negative effect when co-expressed with the wild-type transporter. This effect is not the result of reduced levels of the wild-type transporter at the plasma membrane but is due to specific protein-protein interactions between wild-type and mutant transporter. Third, trafficking-defective amino and carboxyl-truncated transporter mutants that do not reach the plasma membrane reduce the function of the wild-type transporter by a mechanism involving a decrease in the levels of cell surface DAT. Immunofluorescence analysis demonstrated that the truncated transporters accumulate in the cytoplasm where they retain the wild-type transporter and greatly reduce its ability to reach the cell surface. We conclude that dominant-negative mutants reduce the activity of the transporter by forming oligomeric complexes at the cell membrane (Y335A and D79G) or by interfering with the normal processing and trafficking of the wild-type DAT (A60 and S582C).

The present findings also highlight the importance of the leucine-repeat motif from TM2 as an important domain potentially contributing to transporter assembly. Substitutions of this repeat by mutagenesis results in a mutant transporter devoid of uptake activity due to the inability of this protein to be delivered to the plasma membrane. This mutant protein does not exhibit dominant-negative effect upon co-expression with the wild-type transporter suggesting that the leucine repeat participate in monomer-monomer interaction. However, an alternative explanation of our results is that the leucine-repeat mutant presents a defect in folding steps necessary for assembly and trafficking. The latter alternative does not appear to be consistent with our additional findings. A small fragment of DAT containing only the first two TMs is also capable of inhibiting wild-type DAT function, an effect that is lost upon mutation of the leucine residues in the motif. Thus, it appears that the leucine motif is important for the initial steps in the assembly of DAT. Leucine-repeat motifs have been implicated in the assembly process of several membrane proteins. In several cases, as in the human immunodeficiency virus type 1 transmembrane glycoprotein (24, 25), the murine coronavirus spike protein (26), and the Arabidopsis thaliana somatic embryogenesis receptor kinase 1 protein (27), these motifs are not part of the transmembrane domains. However, there are examples such as the cardiac ion channel phospholamban (28, 29) and the erythropoietin receptor (30), in which leucine repeats are part of transmembrane domains that mediate oligomerization. Interestingly, a similar leucine repeat located in TM9 does not appear to be required for transporter oligomerization. Substitutions of the leucine repeat in TM9 did not impair the trafficking or the function of the transporter. These results suggest that specificity in the location of the leucine repeat is important in determining assembly.

While our results provide evidence for some involvement of TM2 in the assembly of DAT, these findings do not rule out the possibility that other domains might also participate in the oligomerization of this transporter complex. Indeed, during the course of our study, Hastrup et al. (16) demonstrated that DAT could be cross-linked as a homodimer at the plasma membrane of HEK-293 cells (16). These complexes were revealed via the selective chemical cross-linking of a cysteine residue on the extracellular side of TM6. The TM6 in this family of transporters contains a glycophorin A motif that has been shown to mediate dimerization of membrane proteins (31). Interestingly, mutations of the conserved glycine residues of this motif in DAT led to loss of function and lack of cell surface targeting (16), similar to the DAT TM2 leucine-repeat motif mutant reported here. Hastrup’s findings and those reported here are not mutually exclusive as many membrane-bound proteins are known to oligomerize through interactions mediated by multiple domains. The best characterized example is the muscle nicotinic acetylcholine receptor (nAChR), which contains four different subunits that combine to form pentameric hetero-oligomers. nAChR complexes are assembled using a stepwise pathway (32–34). Critical motifs involved in initial subunit assembly events reside in several domains of each channel.
subunit. It remains to be determined whether a multi-step process involving different interacting domains also occurs in the assembly of DAT. Alternatively, it is possible that TM2 might be involved in early steps during the assembly process of DAT, whereas residues in TM6 might be important in maintaining the oligomeric structure of DAT at the plasma membrane as demonstrated by Hastrup et al. (16).

Interestingly, the mutant lacking the leucine repeat from TM2 was not glycosylated. A conceivable explanation is that assembly precedes glycosylation during the trafficking of the transporter and thus, lack of assembly in the leucine-repeat mutant results in lack of glycosylation. Alternatively, it is also plausible that the impaired delivery of this mutant to the plasma membrane results as a consequence of lack of glycosylation. Our results using glycosylation mutants demonstrate that glycosylation is not essential, under our experimental conditions, for the functional expression of the transporter. In the case of SERT, Tate and Blakely (35) demonstrated that lack of glycosylation did not affect the affinity of serotonin for the transporter. The carbohydrate moieties of glycoproteins in general are believed to be important for a variety of functions including normal protein folding, protection from proteolytic degradation, intracellular trafficking, and/or cell surface targeting (36). For instance, the loss of N-linked glycosylation impaired the trafficking of the AT1A receptor to the plasma membrane (37). On the other hand, Bisello et al. (38) showed that N-linked glycosylation is not essential for the expression of the parathyroid hormone-related protein receptor on the plasma membrane (38). Thus, the loss of N-linked glycosylation appears to have a spectrum of effects on membrane-bound proteins, and no general predictions can be made based exclusively on the occurrence of glycosylation for any given protein. Our results demonstrate that glycosylation is not essential for DAT functional expression and suggest that oligomerization takes place during the early stages of transporter processing and appears to be required for the integration of the complex into intracellular membranes for subsequent glycosylation and trafficking.

Interestingly, our data indicate that the carboxyl terminus of DAT plays a critical role in the targeting of the protein to the plasma membrane. Progressive truncation of the transporter causes progressive reduction of function as a result of the impairment of these mutants to reach the plasma membrane. It is tempting to speculate that the tail of DAT contains sequences that mediate interactions with intracellular proteins and that those interactions are important for the proper trafficking of the transporter to the plasma membrane. In fact, DAT-interacting proteins that regulate transporter trafficking appear to exhibit dominant-negative effect may have important physiological implications.

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