Cell-specific Sorting of Biogenic Amine Transporters Expressed in Epithelial Cells*

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We have utilized polarized epithelial cells stably expressing neurotransmitter transporters to analyze the sorting behavior of these membrane proteins. The transporters for serotonin (5-HT), dopamine (DA), and norepinephrine (NE) are expected to be present in situ in the most distal extremities of axonal membranes, where they terminate the action of their biogenic amine substrates. Both Madin-Darby canine kidney (MDCK) and LLC-PK1 cells were stably transfected with cDNAs encoding either the rat 5-HT transporter (SERT), the human NE transporter (NET), or the rat or human DA transporter (DAT). These cells were grown on permeable filter supports, and the transporters were localized by three independent techniques. Confocal immunofluorescence microscopy indicated that each of the transporters expressed in LLC-PK1, cells was sorted to the basolateral membrane, co-localizing with the Na+/K+-ATPase. In MDCK cells, however, DAT was located primarily on the apical surface, while SERT and NET were found on the basolateral membranes. Cell surface biotinylation using an impermeant biotinylating reagent confirmed the immunocytochemistry results. Thus, SERT and NET in MDCK cells were labeled more efficiently from the basolateral medium than the apical medium, and DAT in MDCK cells was labeled more efficiently from the apical side than the basolateral side. Transport measurements in transfected MDCK cells agreed with the immunocytochemistry and biotinylation results. These results suggest the existence of cell-specific mechanisms that discriminate between neurotransmitter transporters for surface expression and render unlikely any simple hypothesis that sorting mechanisms in neurons and epithelia are identical.

Neurotransmitter transporters are widely believed to terminate the action of many neurotransmitters. These transporters, localized near sites of neurotransmitter release, remove transmitters from the synaptic cleft and transport them back into the presynaptic neurons. Recently, cDNAs encoding Na+- and Cl−-dependent transporters for neurotransmitters, amino acids, and other substrates have been cloned (1). These include transporters for the biogenic amines norepinephrine (NE),1 dopamine (DA), and serotonin (5-HT) (2). These transporters share extensive sequence similarity and constitute a multigene family (1). Drugs that modulate the activity of the biogenic amine transporters produce profound behavioral effects, leading to their therapeutic use in depression, obsessive-compulsive disorder, and other mental diseases (3–5) and also to their abuse as stimulants (6–8).

The plasma membranes of polarized cells are divided into functionally and morphologically distinct domains with different lipid and protein compositions (9, 10). Neurons and epithelial cells are examples of such polarized cells. The neuronal plasma membrane is traditionally divided into axolemmal and somatodendritic domains, while that of epithelial cells is divided into apical and basolateral domains. Dotti and Simons (11) showed that some viral glycoproteins were sorted preferentially in both virally infected neurons and epithelial cells, and that those proteins that are sorted to the apical or basolateral membranes of infected epithelial cells were found, respectively, in axons or dendrites of infected neurons. Moreover, Thy-1, a GPI-linked protein which is delivered to the apical membranes of epithelial cells, was reported to be exclusively located on the axonal surface of cultured hippocampal neurons (12). Therefore, they proposed that the sorting mechanisms in neurons and epithelial cells share common features and that the same mechanisms responsible for axonal targeting in neurons may result in apical sorting in epithelial cells (11, 12).

Because neurotransmitter transporters are known to be sorted to axons, we have used them to test the proposal that similar mechanisms mediate sorting in neuronal and epithelial cells. We observed that the γ-aminobutyric acid (GABA) transporter GAT-1, which is known to be axonal in neurons (13), was sorted to the apical membranes of MDCK cells (14), while a homologous non-neuronal protein, the betaine transporter expressed in kidney (15), was sorted to the basolateral membranes. Recently, we examined the sorting behaviors of two isoforms of GABA transporters, GAT-2 and GAT-3 (16). GAT-3, like GAT-1, is expressed in brain and retina and is expected to be exclusively neuronal and involved in GABA reuptake, but GAT-2 mRNA is found also in liver and kidney, and might not function in neurotransmitter reuptake (17). Expression of these two transporters in MDCK cells led to apical localization of GAT-3 and basolateral localization of GAT-2 (16). 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data from GABA transporter expression were consistent with a common sorting mechanism in neurons and epithelial cells. Recently, the β-amyloid precursor protein has been shown to be sorted to and secreted from the basolateral membranes of MDCK cells despite its axonal localization in neurons (18). In light of this result with a secreted protein and the relatively limited amount of data on sorting in neuronal versus epithelial cells, we examined the sorting behavior of three biogenic amine transporters, the human norepinephrine transporter (NET), the rat serotonin transporter (SERT), and the human and rat dopamine transporters (DAT), in epithelial cells. Biogenic amine transporters are targeted to the axonal domain of neuronal plasma membranes. Thus, the transporters have been localized by ligand binding (19–22) and immunocytochemistry (23, 24) to brain regions quite distant from the cell bodies where they are synthesized. Immunoreactivity and binding activity are also observed in cell bodies of these monoaminergic neurons, but are likely to represent intracellular biosynthetic precursor forms of the mature axonal transporters. Recent electron microscopic evidence supports the idea that the DA transporter is absent from cell bodies, but also provides evidence for DAT near sites of dendritic DA release (25, 26). Although many studies have supported dendritic release and transport of amines in the nervous system (27, 28), quantitatively, most of the transporter protein is believed to be localized to axonal varicosities and synaptic terminals (23, 24). Based on the proposal of Dotti and Simons (11), these transporters would be expected to be sorted to the apical membranes of epithelial cells as we observed for the GABA transporters GAT-1 and GAT-3. However, the current study shows that NET and SERT are sorted primarily to the basolateral domain of epithelial plasma membranes (in both MDCK and LLC-PK1 cell lines). In contrast, DAT is sorted to the apical membranes of MDCK cells and the basolateral membranes of LLC-PK1 cells.

EXPERIMENTAL PROCEDURES

Materials—LLC-PK1 cells stably expressing dopamine, norepinephrine, or serotonin transporters (LLC-DAT, LLC-NET, and LLC-SERT) were previously generated in this laboratory (29). The cDNA encoding the rat DA transporter was a generous gift from Dr. George Uhl, NIDA Addiction Research Center, Baltimore, MD. Human DAT was kindly supplied by Dr. Aaron J. Ankney, Oregon Health Sciences University, Portland, OR. NET cDNA, encoding the human NE transporter, was generously supplied by Dr. Susan Amara, Vollum Institute, Portland, OR. SERT cDNA, coding for the rat 5-HT transporter, was kindly contributed by Dr. Beth Hoffman, NIMH, Bethesda, MD. Affinity-purified rabbit anti-sera N430, CT2B, and mouse monoclonal antibody 6H, recognizing human NE transporter, rat serotonin transporter, and the Na+/K+-ATPase α-subunit, respectively, were described elsewhere (23, 30–32). Purified antibody against human DAT is described in a separate publication.2 Vector pRC/CMV was from Invitrogen (San Diego, CA). Radiolabeled substrates 3,4,5,7-tetrahydroxyphenylethylamine (DA), 5,6,7,8-tetrahydroxytryptamine (5-HT), and N-methyl-4-[(methyl-1H)phenyl]pyridinium (MPP+) were purchased from DuPont NEN Research Products. PNGase F was purchased from New England Biolabs. All other reagents were purchased from commercial sources.

Cell Culture—The parental LLC-PK1 cells were maintained in α modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C, 5% CO2. The transfected LLC-PK1 cell lines were maintained in the same medium except that G418 (Life Technologies, Inc.) was added at a concentration of 1.8 g/ml. The MDCK cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine at 37 °C, 5% CO2. The MDCK cell lines expressing transporters were maintained in the same medium plus 0.9 g/L G418.

Subcloning and Transfection—cDNAs encoding the transporters were subcloned into vector pRC/CMV which has a bacteriophage T7 promoter sequence and enhancer/promoter sequences from the immediate early gene of human cytomegalovirus (CMV). The pRC/CMV vectors carrying the neomycin-resistant gene for selection and the transport cDNAs were used to generate clonal LLC-PK1 cell lines expressing DAT, NET, or SERT (LLC-DAT, LLC-NET, and LLC-SERT) as described elsewhere (29). Using a similar procedure, we transfected MDCK cells with the same plasmids. After selection in 0.9 g/L G418 for 10 days, single colonies were picked with Dulbecco's medium and tested for transport activities. The MDCK cell lines stably expressing DAT, NET, and SERT are termed MDCK-DAT, MDCK-NET, and MDCK-SERT.

Immunocytochemistry—Parental or transfected LLC-PK1 or MDCK cells were plated at 50% confluence on Transwell tissue culture inserts (Costar Co., Cambridge, MA) and grown for 7 days. Cells were then rinsed with PBS (34) plus 1 mg/mL MgCl2 and 0.1 mg/mL CaCl2, (PBS/Mg/Ca), fixed for 10 min in methanol, and stored in PBS at 4 °C. After rehydration for 5 min in PBS/Mg/Ca, the cells were permeabilized for 15 min in PBS/Mg/Ca plus 0.3% Triton X-100 and 0.1% bovine serum albumin (permeabilization buffer) and blocked for 30 min in GSDB buffer (16 g/L goat serum (Sigma), 0.3% Triton X-100, 20 mg/mL sodium phosphate, pH 7.4, 0.45 mM NaCl). The cells were then incubated for 1 h in GSDB with anti-transporter antibodies (rat anti-DAT, 1:1200 dilution; rabbit anti-NET (N430), 5 μg/mL; or rabbit anti-SERT (CT2B), 5 μg/mL) and anti-Na+/K+-ATPase α-subunit antibodies (6H, mouse), 1:100 dilution. After three 5-min washes with permeabilization buffer, goat anti-rat, anti-rabbit, or anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC), rhodamine (Texas Red), or Cy5 (Amersham, Arlington Heights, IL), was added at 1:100 dilution. Different dyes were used for staining the transporters and the Na+/K+-ATPase α-subunit as described in each figure legend, so that they could be distinguished during microscopy. At the end of the incubation, the cells were again washed three times with permeabilization buffer and once with 5 mM sodium phosphate, pH 7.5, for 5 min. The cells on filters were then fixed in Vectashield (Vector Laboratories, Burlingame, CA). Immunofluorescence was observed and photographed with a Zeiss Axioshot epifluorescence microscope. Confocal microscopy was performed on a Zeiss laser scanning confocal microscope. Confocal images were line averaged eight times. Contrast and brightness settings were chosen to ensure that all pixels were within the linear range. Confocal X-Z cross sections were generated using a 0.2-μm motor step.

Transport Assay—Cells were plated at 50% confluence on 0.4-μm pore size 6.5-mm Transwell cell culture filter inserts and grown for 7 days. A cell monolayer growing on the porous membrane of the cell culture filter insert effectively separates each well in the cell culture plate into two chambers. The apical membranes of epithelial cells face the top side of the filter, while the basolateral membranes face the lower chamber through the filter. After one wash each of the apical (upper chamber) and basolateral (lower chamber) sides of the monolayer with PBS/Ca/Mg, the cells were incubated in PBS/Ca/Mg containing 3H-labeled substrate either in the upper or the lower chamber at 22 °C. At the end of the incubation cells were washed three times from the apical side and once from the basolateral side (when 3H-labeled substrate was present in the upper chamber) or once from the apical side and three times from the basolateral side (when substrate was present in the lower chamber). The apical side of the cells was washed by adding 0.2 ml of ice-cold PBS to the upper chamber and aspirating. The basolateral side of the cells was washed by pipetting ice-cold PBS over the bottoms of the filter inserts. After the washes, the filters with cells attached were excised from the insert cups, submerged in 3 ml of OptiFluor scintillation fluid (Packard Instrument Co., Downers Grove, IL), and counted in a Beckman LS-3801 liquid scintillation counter. Transport assays on 48-well plates were described previously (29).

Surface Biotinylation—Cells were plated at 50% confluence on 0.4-μm pore size 25-mm Transwell cell culture filter inserts for 6-well plates and grown for 7 days. After two washes with 2 ml of ice-cold PBS/Ca/Mg, the surface proteins of either the apical or the basolateral domains of cell plasma membrane were biotinylated (35) by exposing that side of cell monolayer to 1 mM of NHS-sbstion (Pierce) (1.5 mg/ml) in a solution of methanamine adjusted to pH 9.0 with HCl, containing 2 mM CaCl2 and 150 mM NaCl) for 20 min on ice. The biotinylation process was repeated with a fresh addition of NHS-sbstion solution. Cells were then washed with 2 ml of PBS/Ca/Mg plus 100 mM glycine and incubated in the same solution for 20 min on ice to make sure all the biotinylation reagent was quenched. After two more washes with PBS/Ca/Mg, filters were excised and cells were lysed with 1 ml lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5) for one hour on ice. The cell lysates were clarified by centrifugation at 14,000 × g for 10 min. Biotinylated proteins were recovered by adding one half
Table I

| Transporter | Substrate | $K_m$ (μM) | $V_{max}$ (pmol/min/mg protein) |
|-------------|-----------|-----------|-------------------------------|
| MDCK-DAT    | DA        | 3.5 ± 0.4 | 11.3 ± 0.8                    |
|             | MPP$^+$   | 0.47 ± 0.02 | 0.24 ± 0.08                  |
| LLC-DAT     | DA        | 4.3 ± 0.5 | 9.0 ± 0.1                     |
|             | MPP$^+$   | 0.50 ± 0.2 | 0.49 ± 0.02                   |

Kinetic parameters determined for MDCK-DAT and LLC-DAT with DA or MPP$^+$ as substrates

Cells were grown to confluence on 48-well plates. Three transport experiments performed with confluent cells on 48-well plates each gave similar results. Results from a representative experiment are shown. Transport measurements were performed as described under "Experimental Procedures." DA and MPP$^+$ were tested as substrates on the same batch of cells in each experiment. $K_m$ and $V_{max}$ values and their associated uncertainties were obtained by nonlinear regression analysis.

Expression of Biogenic Amine Transporters in Epithelial Cells—We previously reported the stable expression and characterization of DAT, NET, and SERT in the LLC-PK$_1$ cell line which is derived from pig kidney and resembles proximal tubules (29). These cells grow in a polarized fashion, with distinct apical and basolateral plasma membrane domains. MDCK cells, which are derived from canine kidney and resemble distal tubules, were also transfected with transporter cDNAs carried in the vector pRC/CMV using the same procedure as used previously with LLC-PK$_1$ cells (29). The transfected MDCK cells demonstrated cocaine-sensitive biogenic amine transport similar to LLC-PK$_1$ cells transfected with the same cDNA. Table I lists the kinetic parameters for DA and MPP$^+$ uptake by MDCK and LLC-PK$_1$ cells transfected with human DAT cDNA.

Immunocytochemistry—To study the sorting properties of biogenic amine transporters in these epithelial cells, we stained the transfected cells with primary antibodies specific for each transporter and secondary antibodies, conjugated with fluorescent dyes, and examined their localization by laser scanning confocal microscopy. Cells were double labeled with antibodies recognizing the α-subunit of Na$^+/K^+$-ATPase, and examined by enhanced chemiluminescence (Amersham Corp.). The films were then quantitated with a Digital Imaging System model IS-1000 from Alpha-Innotech Corporation.

RESULTS

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Figs. 1–3 show immunofluorescence micrographs of LLC-PK$_1$ and MDCK cells expressing SERT, NET, and DAT. In each case, the same cells were stained with antibodies against both the specific transporter expressed (right panels) and the α-subunit of Na$^+/K^+$-ATPase (left panels). An X-Z cross section representative of each field was generated, and is shown below each en face view. Previous studies with GABA transporters (14, 16) were in agreement with the prediction of Dotti and Simons (11) that axonal proteins should be sorted apically in epithelial cells.

In contrast, however, biogenic amine transporters expressed in LLC-PK$_1$ cells are restricted to the basolateral domain of the plasmalemma. Panels B and D of Figs. 1–3 show a distribution of SERT, NET, and DAT, respectively, that is essentially identical to the distribution of Na$^+/K^+$-ATPase (panels A and C). In MDCK cells, expression of SERT and NET results in a similar distribution, with essentially all of the transporter localized to the basolateral membrane (Figs. 1 and 2, panels F and H) as is the Na$^+/K^+$-ATPase (panels E and G).

To our surprise, the dopamine transporter was sorted differently in the two epithelial cell lines. The same DAT cDNA was transfected into the LLC-PK$_1$ and MDCK cells using the same procedure, but the expressed dopamine transporter was sorted predominately to the apical domain of MDCK cell plasma membranes (Fig. 3, panels F and H), while it was excluded from the apical membrane domain of LLC-PK$_1$ cells (panels B and D). Human DAT was also transfected into MDCK cells, and its...
Localization was identical to that of rat DAT (data not shown). The level of expression of DAT, as judged by immunofluorescence, biotinylation, and transport assays (see below) was not significantly different from that of NET or SERT in MDCK cells.

Cell Surface Biotinylation—To confirm the immunocytochemistry results described above, and to evaluate the approximate molecular sizes of the transporters expressed on the surface of these cells, we used cell surface biotinylation and Western blot analysis to estimate the relative expression levels of biogenic amine transporters on the apical or basolateral membranes of MDCK cells (Fig. 4) as described under “Experimental Procedures.” The right panel of Fig. 4 shows that SERT expressed in MDCK cells was labeled when the biotin reagent was present at the basolateral cell surface and was detected as a strong 75-kDa band (lanes B) similar to the band observed in platelet membranes detected with the same antibody (23). When cells were biotinylated from the apical surface (lanes A), little or none of the serotonin transporters were labeled, and the band is correspondingly weak or absent.

The middle panel of Fig. 4 indicates that, in MDCK-NET cells, the norepinephrine transporter was readily biotinylated from the basolateral side of cell monolayer (lanes B). In contrast to NET and SERT, DAT was found to be more abundant in the apical membrane of MDCK-DAT cells (left panel). These results are consistent with the immunocytochemical data presented in Figs. 1–3. All three transporter proteins appeared on Western blots as broad bands, probably due to heterogeneous glycosylation. The surface biotinylated band observed with NET was similar in mobility to the band previously detected in total extracts from LLC-NET cells (30). The mobility of the biotinylated DAT band also was similar to that previously observed in brain membranes and transfected cells (24).

Sorting of Biogenic Amine Transporters—To investigate the sorting mechanisms of the biogenic amine transporters, we used cell surface biotinylation in MDCK cells expressing human DAT (MDCK-DAT), human NET (MDCK-NET), or rat SERT (MDCK-SERT). The biotinylated cell surface proteins were recovered from solubilized cell extracts with streptavidin-conjugated agarose beads, separated on SDS-polyacrylamide gels, and transferred to nitrocellulose filters. The blots were then probed with antibodies against DAT, NET (N430), or SERT (CT2B) and visualized by enhanced chemiluminescence. The experiments were performed in duplicate. Each lane represents equal amounts of detergent-solubilized cell extract. Molecular mass markers are indicated at the right of each blot.
The apical membranes of LLC-PK1 cells are difficult to biotinylate; membranes of MDCK-DAT cells, and about 80% of plasmalemmal DAT on apical NET and SERT are expressed on the basolateral surfaces of this quantitation suggests that 90% or more of plasmalemmal densities of the protein bands as shown in Fig. 4, determined Western blots to avoid filmsaturation. Fig. 5 showsthe relative densities of the protein bands as shown in Fig. 4, determined using two or more independent Western blots for each cell line. This quantitation suggests that 90% or more of plasmalemmal NET and SERT are expressed on the basolateral surfaces of MDCK cells, and about 80% of plasmalemmal DAT on apical membranes of MDCK-DAT cells.

We have previously determined that proteins expressed in the apical membranes of LLC-PK1 cells are difficult to biotinylate efficiently (37). Thus, in contrast with MDCK cells, quantitative estimates of protein polarity can not be performed by biotinylation in LLC-PK1 cells. However, we tested LLC-SERT, LLC-NET, and LLC-DAT cells using apical and basolateral biotinylation to be certain that it did not indicate a predominantly apical distribution. The results were qualitatively consistent with the basolateral distribution obtained from the same cells by immunofluorescence (data not shown). There is no way to be certain that apical and basolateral biotinylation efficiencies are identical, even in MDCK cells. Nevertheless, the dramatic differences in labeling between DAT and either NET or SERT in MDCK cells (Figs. 4 and 5) are unlikely to result from a difference between apical and basolateral labeling efficiency.

Transport Assays—As a third method for assessing the distribution of biogenic amine transporters in epithelial cells, the transport activities of the transfected cell lines were also examined and compared. By adding the transport substrate separately to each side of the cell monolayer, we independently measured the activity of transporters expressed on apical and basolateral membranes. LLC-PK1 cells do not form sufficiently tight cell monolayers and, therefore, are not suitable for these polarized transport assays to examine localization. We observed substrate leakage through the LLC-PK1 cell monolayer (data not shown). MDCK cells, in contrast, form tightly sealed cell monolayers on filter inserts. We measured and compared transport activities of MDCK cells expressing DAT, NET, or SERT. The results, shown in Fig. 6 are generally consistent with the distribution suggested by immunocytochemistry and cell surface biotinylation experiments. For both MDCK-SERT and MDCK-NET cells, transport was more rapid when the substrate (5-[3H]HT or [3H]DA, respectively) was added to either the apical or the basolateral sides of the cell monolayers at a concentration of approximately 200 nM. In the figure the transport activities from apical and basolateral sides of each transporter are compared and shown as relative percentage with the sum of the transport activities from both sides as 100%. The experiments were performed in triplicate. Standard deviations are shown as error bars. The 100% values for each cell line were as follows: MDCK-SERT, 15.5 ± 1.3 pmol/mg/min; MDCK-NET, 9.4 ± 0.1 pmol/mg/min; MDCK-DAT, 5.4 ± 0.5 pmol/mg/min.

**DISCUSSION**

Polarized cells such as neurons and epithelial cells possess plasma membranes which are divided into distinct domains with different protein and lipid compositions (38). Based on the sorting behaviors of viral glycoproteins in both neurons and
epithelial cells, Dotti and Simons (11) proposed that the sorting mechanisms of neurons and epithelial cells share common features, and that the mechanisms responsible for axonal targeting in neurons may lead to apical sorting of neuronal proteins in epithelial cells. Our previous observation that axonal GABA transporters were localized apically in epithelial cells is consistent with their proposal (14, 16). However, the results presented here demonstrate that this simple model is not sufficient to explain sorting of the biogenic amine transporters SERT, DAT, and NET.

Each of the biogenic amine transporters is believed to be the product of a single gene. No alternative splicing or other isoforms have been detected. (GABA transporters, in contrast, include at least four different isoforms, plus a taurine transporter that also transports GABA (1)). Therefore, the biogenic amine transporter polypeptides expressed in transfected epithelial cells should be the same as the presynaptic biogenic amine transporters in neurons. These transporters have been localized at or near sites of biogenic amine transmitter release using specific affinity probes (19–22) and antibodies (23, 24).

Recent evidence suggests that DAT is localized adjacent to sites of DA release in both axonal and dendritic plasma membranes (25, 26). The transporters are also found in cell bodies, although these preliminary results suggest that most of the cell body labeling is intracellular. Despite the axonal localization of the transporters in vivo, each of the biogenic amine transporters expressed in LLC-PK1 cells and SERT and NET in MDCK cells are excluded from the apical membrane, contrary to the prediction of Dotti and Simons (11).

The results of immunocytochemistry, cell-surface biotinylation, and transport assays agree with each other with respect to the predominant location for each of the transporters. There is, however, a significant quantitative discrepancy between transport and biotinylation measurements of the distribution of NET and DAT in MDCK cells. For both transporters, transport measurements report a less polarized distribution than does biotinylation. For example, although [3H]DA transport into MDCK-NET cells is higher from the basolateral than from the apical side of the monolayer (Fig. 6), the basolateral:apical ratio is only 1.3:1. In the corresponding biotinylation experiment (Fig. 5) the ratio is 49:1. The same discrepancy was observed previously for the GAT-1 transporter in MDCK cells (14). In that case, GAT-1 was predominantly apical by both biotinylation and transport measurements, but the apical/basolateral ratio was much higher by biotinylation.

The explanation for this discrepancy may lie in the different conditions used in the two methods. Transport measurements were performed at 22 °C with a small transport substrate but biotinylation was performed at 0 °C using a larger, less permeant reagent. Permeability of the tight junctions between cells to transport substrates at 22 °C may allow substrate present on one side of the monolayer to be taken up on the opposite side of the cell, and this effect may be more pronounced in LLC-PK1 cells, which grow in a less ordered monolayer than do MDCK cells. Thus, it is possible that such leakage was responsible for our inability to measure polarization of transport in transfected LLC-PK1 cells, although biotinylation and immunofluorescence demonstrated a polarized distribution of the transporters. Another possible explanation is that a much larger portion of the NE transporter that reaches the basolateral surface of MDCK cells is inactive for transport, relative to transporters that reach the apical membrane. We consider this explanation to be less likely since the selective inactivation would need to occur on the opposite side of MDCK cells expressing the DA and GABA transporters to account for the differences in distribution for DAT and GAT-1 as measured by transport and biotinylation.

The most direct conclusion from the results presented here is that the mechanisms of protein sorting in neurons and epithelial cells are not identical. This contention is also supported by studies in which Na+/K+-ATPase isoforms were localized in neurons and epithelia. Both the α2 and α3 polypeptides were present in axons and dendrites of hippocampal neurons in culture, but both proteins were restricted to the basolateral membrane when expressed in LLC-PK1 cells (32). More recently, the β-amyloid precursor protein has been shown to be sorted to and secreted from the basolateral membranes of MDCK cells despite its axonal localization in neurons (18).

Another possibility, however, is that the ability of these proteins to be recognized by the sorting machinery may be different in the two cell types. Post-translational modification of the transporters could be different, or additional proteins, specific to each cell type, might associate with the transporters. There is evidence, at least for the dopamine transporter, that expression in different cell types alters the catalytic function of the protein (39), and a C-terminal truncation of DAT has been shown to have altered transport properties (lower K_m) (40). Moreover, the Na^+-dependent glucose transporter is known to associate with a protein that alters its function (41). Subunit-specific interactions are believed to be responsible for differential sorting of GABA-A receptor isoforms in brain (42). The finding that DAT is associated with release sites in both axons and dendrites (25, 26) suggests that other proteins present at release sites may serve as determinants for transporter localization. Other possibilities, for example that differences in lipid composition between cell types may selectively affect sorting, are also possible.

An unexpected finding was that DAT expression exhibits two different distributions in the two epithelial cell lines. Basolateral expression of DAT in LLC-PK1 cells is similar to that observed with NET and SERT. However, apical DAT expression in MDCK cells is in marked contrast not only with the basolateral expression of NET and SERT in MDCK, but also with the basolateral expression of DAT in LLC-PK1 cells. The apical distribution of DAT in MDCK is not due to overexpression and saturation of the sorting machinery, since by all of the criteria applied in our studies, DAT is expressed at the same (or lower) levels as NET or SERT in MDCK. We have previously shown that proteins bearing tyrosine-based basolateral targeting signals accumulate at the basolateral surface of MDCK cells and at the apical surface of LLC-PK1 cells. The results presented here for DAT are the first example of a protein which sorts apically in MDCK and basolaterally in LLC-PK1. It will be interesting in future studies to use these differentially sorted proteins to further evaluate the distinct sorting mechanisms which operate in these two homologous renal epithelial cell lines.

We considered the possibility that significant modification of DAT occurs in LLC-PK1 or MDCK cells, leading to the observed difference in sorting patterns in these two cell types. Such modifications might also affect the electrophoretic mobility of the protein or its kinetic properties. However, Western blot analysis revealed no difference in the size of the DAT protein before or after treatment with PNGase F (data not shown). Measurements of K_m and V_max values for transport of DA and MPP^+ into LLC-DAT and MDCK-DAT cells were mostly similar (Table I). The only difference observed was that MPP^+ appeared to have a larger V_max relative to DA in LLC-
DAT cells when compared with MDCK cells. Even this difference, however, was rather small. Although we detected no obvious differences in the properties of DAT expressed in LLC-PK₁ and MDCK cells, we cannot rule out the possibility that a difference in post-translational modification of DAT between the two cell types is responsible for the different sorting behavior.

Although DAT expression has been observed only in neuronal preparations, NET and SERT are expressed also in peripheral cells such as platelets, mast cells, and placenta. In placenta, NET and SERT are localized in the syncytiotrophoblast, which is derived from polarized epithelial cells. NET and SERT transport activity is enriched in membrane vesicles derived from the apical surface of the syncytiotrophoblast from term human placenta (44, 45) suggesting that they are specifically sorted to the apical membrane. This pattern is opposite to the one reported here for LLC-PK₁ and MDCK cells, reinforcing the evidence presented here for cell-specific mechanisms involved in the sorting of these proteins. Among the neurotransmitter transporters studied in MDCK cells, a common finding is that transporters whose endogenous expression is limited to a single tissue (e.g., NET in placenta) are localized in the syncytiotrophoblast or the syncytial plasma membrane. Thus, these transporters are likely to include the relatively restricted regions where their sequences differ. The availability of functional chimeric transporters derived from NET and DAT sequences (33, 43) provides an opportunity to localize these sorting signals. These experiments are currently underway in our laboratory.

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