Simultaneous Expression of hCNT1-CFP and hENT1-YFP in Madin-Darby Canine Kidney Cells

LOCALIZATION AND VECTORIAL TRANSPORT STUDIES*

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To test the hypothesis that human concentrative and equilibrative nucleoside transporters (hCNT1 and hENT1) are present on the apical and basolateral membrane, respectively, we constructed a Madin-Darby canine kidney (MDCK) cell line that simultaneously and stably expresses recombinant hCNT1 and hENT1 gene products tagged with CFP and YFP fluorescent proteins, respectively. Using a confocal microscope, both hCNT1-CFP and hENT1-YFP were found to be distributed uniformly on the plasma membrane of undifferentiated MDCK cells. Upon differentiation of the MDCK cells on Transwell filter inserts, hCNT1-CFP was visualized exclusively on the apical membrane, whereas hENT1-YFP appeared predominantly on the basolateral membrane. As differentiation proceeded, there was an increase in alkaline phosphatase activity, and activity of hENT1 in the apical compartment decreased while hCNT1 activity remained constant. These results suggest that, on differentiation, hENT1 is sorted to the basolateral membrane. This was confirmed when the hCNT1-mediated uptake of [3H]uridine from the apical compartment of the differentiated cells was found to be 20-fold higher and that for hENT1 was 4-fold lower than the corresponding uptake from the basal compartment. As observed in vivo, the net transport of [3H]adenosine was from the apical to the basal compartment, whereas that for [14C]-deoxyadenosine was from the basal to the apical compartment. In summary, we have shown for the first time that hCNT1 and hENT1 are present in polarized MDCK cells on the apical and basolateral membrane, respectively, allowing vectorial transport in both directions depending on the relative activity (ratio of maximal transporter activity to affinity) of each transporter for their substrates.

Nucleoside transporters (NTs) are important in mediating the transport of nucleosides and nucleoside drugs (e.g. antiviral and anticancer drugs) across cell membranes (1). Physiologically, the sodium-dependent concentrative nucleoside transporters mediate the influx of nucleosides. Human concentrative nucleoside transporter 1 (hCNT1) is pyrimidine-specific, whereas hCNT2 is purine-specific. Both hCNT1 and hCNT2 transport uridine and adenosine (2) and are insensitive to inhibition by nitrobenzylthioinosine (NBMPR). hCNT1 and hCNT2 are expressed on specialized cells such as intestine and kidney epithelia (3, 4). The equilibrative transporters mediate both the influx and efflux of nucleosides and exhibit broad substrate specificity, accepting both purine and pyrimidine nucleosides as permeants. Human equilibrative nucleoside transporter 1 (hENT1 or es) is inhibited by NBMPR concentrations as low as 0.1 nM (IC50 = 0.4 nM), whereas hENT2 (ez) transporter is insensitive to inhibition as high as 1 μM (IC50 = 2.8 μM) (1, 4, 5). One or both of the equilibrative transporters are expressed in most, if not all, cell types.

Although functional measurement of transporter activity has helped elucidate the tissue expression of the concentrative and equilibrative nucleoside transporters, because of the lack of antibodies little information is available on the cellular localization of these transporters. Functional studies using membrane vesicles have shown that the concentrative transporters are found only on the apical membrane of the polarized epithelial cells (3, 6, 7). In contrast, such studies have resulted in controversial findings regarding the membrane localization of the equilibrative transporters. For example, we have shown that the equilibrative nucleoside transporters, hENT1 and hENT2, are absent from the apical membrane of the human enterocytes (3) and we have hypothesized that they are present on the basolateral membranes (8). In contrast, others have found ENT1 (but not ENT2) to be present on the apical membrane (9) or on both membranes (10) of the polarized epithelial cells. Because of the lack of availability of antibodies for immunolocalization of the equilibrative transporters, it has been difficult to resolve this controversy. To test the hypothesis that the concentrative and equilibrative nucleoside transporters are present on different faces of the polarized epithelial cells, we have used the model epithelial cell line derived from the kidney, namely the MDCK cells. We constructed a stable MDCK cell line that simultaneously expresses recombinant hCNT1 and hENT1 gene products tagged with CFP and YFP fluorescent proteins, respectively. These cells were then used to test the localization of these two transporters in both the nonpolarized and polarized MDCK cells. In addition, we tested the hypothesis that the directionality of vectorial transport of nucleosides across polarized cells will depend on the localization and activity (Vmax/Km) of these nucleoside transporters for their substrates. Specifically, we asked whether differential localization of hCNT1 and hENT1 could explain the paradoxical observation in vivo that adenosine is actively reabsorbed by the human kidney, whereas deoxyadenosine is secreted.

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‡ The abbreviations used are: NT, nucleoside transporter; hCNT, human concentrative nucleoside transporter; hENT, human equilibrative nucleoside transporter; NBMPR, nitrobenzylthioinosine; MDCK cells, Madin-Darby canine kidney cells; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.
FIG. 1. Coexpression of hENT1-YFP and hCNT1-CFP in MDCK cells. The coexpressing cells were seeded on a Lab-Tek chambered coverglass and visualized with an epi-fluorescence microscope. hCNT1-CFP fluorescence (A) was detected by excitation at 458 nm and collecting the emission at 460–470 nm, whereas hENT1-YFP fluorescence (B) was detected by excitation at 488 nm and collecting the emission at 500–630 nm. Both hCNT1-CFP and hENT1-YFP are localized on the plasma membrane. C, merging of images A and B shows that more than 90% of the cells coexpress the two gene products. D, in contrast, MDCK cells transfected with pEFP empty vector (mock cells) showed diffuse expression throughout the cells.

EXPERIMENTAL PROCEDURES

Gene Construction—A 1.4-kb human intestinal ENT1 (hENT1) and a 2.0-kb CNT1 (hCNT1) fragment was amplified from the plasmid constructed previously (11) using primer pairs overlapping the start or end codons. BglII and KpnI sites were added as follows: hENT1, sense (TGATGAATGATCTATGCAACCCGATCCGAGC) and antisense (TAGTAGGTAACCTCACACAATTGCCCGGAACA); hCNT1, sense (TGATGAATGATCTATGCAACCCGATCCGAGC) and antisense (TAGTAGGTAACCTCACACAATTGCCCGGAACA); hENT1, sense (TGATGAATGATCTATGCAACCCGATCCGAGC) and antisense (TAGTAGGTAACCTCACACAATTGCCCGGAACA); hENT1, sense (TGATGAATGATCTATGCAACCCGATCCGAGC) and antisense (TAGTAGGTAACCTCACACAATTGCCCGGAACA).

All constructs were confirmed by automated sequence analysis using BigDye terminator cycle sequencing ready reaction kits (PE Applied Biosystems, Foster City, CA). The resulting constructs, hENT1-YFP and hCNT1-CFP, were used to produce a stable MDCK cell line expressing hENT1 and hCNT1. All constructs were confirmed by automated sequence analysis using BigDye terminator cycle sequencing ready reaction kits (PE Applied Biosystems, Foster City, CA). To construct expression plasmids without tagged fluorescence protein, hENT1-YFP and hCNT1-CFP constructs were double-digested with AgeI and BglII. A 5.4- (hENT1) or 6.0-kb (hCNT1) fragment was isolated and ligated after blunting the cutting end to yield hENT1-pE and hCNT1-pE expression cassettes.

Selection of MDCK Cells Stably Coexpressing hCNT1 and hENT1—MDCK cells were cultured in minimum Eagle’s medium with Earle’s salts and l-glutamine containing 10% fetal bovine serum, 100 units of penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in 5% CO2 with 95% humidity. To generate stable hCNT1 and hENT1 transfectants, ~7 × 10^6 cells/well were seeded in six-well plates in minimum Eagle’s medium 1 day before transfection. The expression cassettes were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, 2.5 μg of the hENT1-YFP and 2.5 μg of the hCNT1-CFP constructs were diluted in 0.25 ml of Dulbecco’s modified Eagle’s medium without serum and mixed with 15 μl of LipofectAMINE 2000 reagent diluted in 0.25 ml of Dulbecco’s modified Eagle’s medium without serum. Then the mixture was incubated for 30 min at room temperature. The mixture was applied to MDCK cells maintained in a six-well plate at ~90% confluence. Forty-eight hours later, the cells were transferred to a 100-mm dish and cultured in growth medium containing G418 (Invitrogen). The medium was changed every 3 days, and the G418 concentration was varied from 200 to 1000 μg/ml depending on the status of the cells. After selection for 2 to 3 weeks, cell colonies were isolated using cloning cylinders by checking their fluorescence intensity using a fluorescence microscope (Zeiss, Thornwood, NY). Cells highly expressing both CFP and YFP were subsequently cloned on 96-well plates using limited cell dilution.

Uridine Uptake Experiments—All uptake experiments were carried out in triplicate in sodium-containing transport buffer (Tris-HCl 20 mM, K2HPO4 3 mM, MgCl2·6 H2O 1 mM, CaCl2 2 mM, glucose 5 mM, NaCl 130 mM, pH 7.4) or sodium-free transport buffer in which NaCl was replaced by N-methyl-t-glucamine 130 mM (pH 7.4). The uptake experiments were conducted 3 days after seeding in 24-well plates. To measure sodium-dependent uptake, the cells were washed three times with sodium-free buffer and preincubated with sodium-free buffer with 10 μM NBMPR (in 0.1% MeSO) for 15 min at 37 °C. Then 0.5 ml of sodium transport buffer or sodium-free buffer containing 1 μM [3H]uridine (1 μCi/ml, 17.7 Ci/mmol, Moravek Biochemicals, Brea, CA) and 10 μM NBMPR were added to each well. To measure Na+-independent facilitated uptake, the cells were washed three times with sodium-free buffer and preincubated with sodium-free buffer with or without 10 μM NBMPR for 15 min. Then 0.5 ml of sodium-free buffer containing 1 μM [3H]uridine with or without 10 μM NBMPR was added to the wells. To control for any solvent effect, an equal amount of MeSO was included in all experiments. After incubating at 37 °C for 5, 10, or 15 min, the wells were washed rapidly three times with ice-cold Na+ -free buffer containing 10 μM NBMPR. The cells were solubilized with 0.5 ml of 1 N NaOH and then neutralized with 0.5 ml of 1 N HCl. Then 0.8 ml of the cell lysate was counted on the scintillation counter. Protein in the cell homogenate was quantified using the BCA protein assay kit (Pierce). Bovine serum albumin was used to generate a standard curve to determine the protein concentration. Nucleoside transporter-mediated uridine uptake was calculated as the difference between the uptake by the MDCK cell transfected with hCNT1 and hENT1 cDNAs and the uptake by mock-transfected cells.

Transwell Transport Experiments—1 × 10^5 stable coexpressing cells were seeded on six-well polycarbonate Transwell filter inserts (Corning Costar Quality Biological, Gaithersburg, MD) and cultured with regular changes of medium for 10–12 days after reaching confluence. To ensure that the cells had polarized and formed tight junctions, transport experiments were conducted when the transepithelial electrical resistance (TEER) values (measured by milliQ-ERS; Millipore, Bedford, MA) reached 300–500 ohms/cm2 in representative wells. The Transwell filter inserts were washed three times with Na+-free buffer, and then 1 μM [3H]uridine was added to either the apical or the basal side. At various times, 50 μl of buffer was collected from the opposite compartment, either apical or basal. The transport experiments were termi-
nated by aspirating the buffer, and filters were washed with chilled Na"-free buffer containing 10 µM NBMPR. The whole filter was wiped with tissue to remove any excess buffer, the filter was removed from the plastic support, and the filter was counted on a scintillation counter.

**Visualization of hCNT1 and hENT1 Tagged with Fluorescence Proteins**—1 × 10^6 stable coexpressing cells were grown in either two-well Lab-Tek borosilicated coverglass chambers (Nalge Nunc International Corp., Naperville, IL) or six-well Corning Costar polycarbonate Transwell filter inserts for 10–12 days after reaching confluence. The filters were washed with phosphate-buffered saline, excised, then loaded on a glass slide and covered with a coverslip. Between the slide and the coverslip, an ~1-mm gap was filled with medium to keep the cells alive. Images were obtained using a Leica TCS NT laser-scanning confocal microscope equipped with a krypton/argon laser as the light source. Images were captured by excitation at 458 nm and emission at 470–480 nm (CFP) or excitation at 488 nm and emission at 610–640 nm (YFP).

**Measurement of Alkaline Phosphatase Activity, a Marker of Cell Differentiation**—Alkaline phosphatase activity was measured at 410 nM in 75 mM alkaline buffer solution (pH 10.3, Sigma). The confluent monolayers in 24-well plates were washed twice with buffer solution. Then a buffer containing 3 mM 4-nitrophenylphosphate was added to the confluent monolayers. The buffer was then loaded on a glass slide and covered with a coverslip. Between the slide and the coverslip, an ~1-mm gap was filled with medium to keep the cells alive. Images were captured at excitation at 458 nm and emission at 470–480 nm (CFP) or excitation at 488 nm and emission at 610–640 nm (YFP).

**Vectorial Transport of [3H]Adenosine and [14C]Deoxyadenosine by MDCK Monolayer Cells Grown on Transwell Filters**—hENT1-YFP and hCNT1-CFP coexpressing and mock cells were seeded on six-well polycarbonate Transwell filter inserts and cultured for 10–12 days with a regular change of medium. Transport experiments were conducted when the transepithelial electrical resistance reached 300–500 ohms/cm^2 in a representative well. The filter inserts were washed three times and preincubated with 500 µM adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride for 30 min. Then 8 µM [3H]adenosine (32.1 Ci/mmol, Moravek Biochemicals) and 8 µM [14C]deoxyadenosine (56 mCi/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO) were loaded in the basal or apical compartment of the Transwell filter inserts. Transport experiments were conducted with buffer (1.0 ml in the apical compartment and 1.5 ml in the basal compartment) containing sodium but not NBMPR on both sides of the Transwell filters. Fifty µl of buffer from the compartment opposite the loading side were sampled at various times (up to 60 min), and the buffer was replenished immediately. At the end of the experiments, the filters were washed and removed from plastic supports to measure the cell contents of adenosine and deoxyadenosine. The cells on the filters were solubilized by 0.3 ml of 1N NaOH and then neutralized by 0.3 ml of 1N HCl. Radioactivity was counted using a dual channel scintillation counter.

**HPLC Analysis**—To determine the maximum possible metabolism of [3H]adenosine and [14C]deoxyadenosine under the above described transport conditions, the above experiment was repeated for 60 min on a separate batch of cells. Buffer samples from the loading and the contralateral compartments were collected, and cells on the filters were sonicated in the presence of 1 ml of methanol and then centrifuged at 700 × g for 15 min. The cell lysates were diluted twice with water. Fifty-µl samples were injected onto a C18 HPLC column (Econosil, 250 × 4.6 mm, 5 µ, Alltech Associates, Inc., Deerfield, IL). The column was eluted with a mobile phase consisting of 20% methanol and 80% water (0.8 ml/min). The HPLC effluent was collected every 30–60 s for 30 min and counted using a dual channel scintillation counter. [Fig. 2.]
times of adenosine and deoxyadenosine were confirmed by injection of cold standards and detection at 254 nm.

**Data Analysis**—Data are expressed as the mean ± S.D. of uptake values obtained in three wells or filter inserts. Data are representative of a minimum of two experiments carried out on different days on different batches of cell.

**RESULTS AND DISCUSSION**

We chose to use MDCK cells for expression of human nucleoside transporters for several reasons. First, they readily express heterologous proteins. Second, the cells can be readily differentiated into polarized cells, allowing the study of localization of nucleoside transporters in the undifferentiated and differentiated cells. Third, these cells have been studied extensively in the past to determine localization and mechanisms of sorting of numerous other membrane proteins.

To conveniently and simultaneously detect the localization of both hCNT1 and hENT1 in polarized MDCK cells, we constructed cyan and yellow fluorescence fusion proteins of these gene products. Cells expressing individual proteins were used to confirm that there was no overlap in the emission signal between YFP and CFP (data not shown). Localization of hCNT1 and hENT1 in undifferentiated MDCK cells was visualized by confocal microscopy. CFP-tagged hCNT1 and YFP-tagged hENT1 exhibited an even distribution on the plasma membrane of the cell (Fig. 1, A and B). In cells expressing both proteins, their areas of localization overlapped (Fig 1C). In contrast, expression of the empty YFP vector showed diffuse localization in the entire cell (Fig 1D).

Because uridine is a substrate for both hCNT1 and hENT1, we used it in our studies to quantify the functional activity of hCNT1 and hENT1 coexpressed in MDCK cells. When the cells are incubated in Na+-free medium and [3H]-labeled uridine, only the equilibrative nucleoside transporter activity can be observed (Fig 2A). At 15 min, hENT1 shows an almost 30-fold greater uptake of [3H]uridine in the coexpressing cells than in the mock (vector only) cells. In the presence of NBMPR (10 μM), which specifically inhibits hENT1 (but not hCNT1), almost all of the hENT1-mediated uptake of [3H]uridine is inhibited, thus showing very clearly the functional activity of hENT1 in these coexpressing cells. Similarly, when the cells are incubated with Na+-containing buffer and 10 μM NBMPR, only hCNT1-mediated uptake of [3H]uridine is observed (Fig. 2B). At 15 min, hCNT1-mediated uptake of [3H]uridine by the coexpressing cells is ~60-fold greater than that by mock cells. Mock cells demonstrated low hENT-type activity, whereas hCNT1 activity was absent. As previously shown by Mangravite et al. (12), we found that [3H]uridine uptake by hCNT1 was comparable with that by hCNT1-CFP. Likewise, [3H]uridine uptake by hENT1 was similar to that by hENT1-YFP (data not shown). These results demonstrate that both hENT1-YFP and hCNT1-CFP are functional when simultaneously expressed in undifferentiated MDCK cells.

We used the doubly transfected MDCK cells to test the hypothesis that hCNT1 and hENT1 are expressed on the apical and basolateral membrane, respectively, of polarized epithelial cells, allowing vectorial transport of nucleosides and nucleoside drugs from the apical compartment to the basal compartment or vice versa. As described below, our data show that this hypothesis is correct. Unlike in undifferentiated cells, in the differentiated polarized MDCK cells the localization of hCNT1 and hENT1 on the plasma membrane did not overlap. In the x-y plane, hCNT1 is localized predominately on the apical layer and hENT1 is localized mostly on the layer adjacent to the filter (Fig. 3A, B and E). Indeed, vertical cross-sections of the images (z plane) showed that hCNT1-CFP was found exclusively on the apical membrane (Fig. 3D), whereas hENT1-YFP was distributed predominantly around the basolateral membrane (Fig. 3E). The merged images of CFP and YFP confirmed this localization (Fig. 3F). As a control, MDCK cells transfected with the YFP empty vector showed diffuse localization in the cell in both the x-y and the z planes (Fig. 3C and G).

To confirm this differential localization functionally, we measured both hCNT1 and hENT1 activity in the apical and basal compartment of differentiated cells grown on Transwell filters. [3H]Uridine (1 μCi/ml, 0.2 μM) was loaded on either the apical or the basal compartment. As measured by cellular...
accumulation of uridine radioactivity, hENT1 activity in the apical compartment was ~4-fold lower than in the basal compartment, whereas hCNT1 activity in the apical compartment was ~20-fold greater than in the basal compartment (Fig. 4, A and B). In contrast, in the mock cells, no difference was observed in cellular accumulation of radioactivity whether uridine was loaded on the apical or the basal compartment.

As the localization of hENT1 and hCNT1 in differentiated and undifferentiated MDCK cells was different, we tested the hypothesis that differentiation of MDCK cells results in sorting of hENT1 to the basolateral compartment. To test this hypothesis, we measured the time course of uptake of [3H]uridine by hENT1 and hCNT1 when loaded in the apical compartment of MDCK cells grown on 24-well plates. To measure the time course of differentiation of these cells, alkaline phosphatase activity in the apical compartment, a marker of differentiation, was also measured. The cells began to differentiate at about 6–8 days from confluence and reached complete differentiation at about 13 days from confluence. As cells began to differentiate, the activity of hENT1 in the apical compartment began to decrease and reached a minimum at about 13 days after confluence (Fig. 5). Meanwhile the apical uptake by hCNT1 remained relatively constant throughout the entire experimental period. These results suggest that upon differentiation, hENT1 is sorted to the basolateral membrane of MDCK cells. Somewhat different results have been obtained with the OK cells (epithelium cells from the opossum kidney) (10). At day 10 from confluence, both Na+-dependent and Na+-independent endogenous transporter activity are observed in the apical compartment, whereas only Na+-independent activity is observed in the basolateral compartment of these polarized cells. In contrast, at day 1 from confluence (presumably nonpolarized cells), the majority of transporter activity is Na+-independent (10). These data suggest that in OK cells, on differentiation the concentrative transporter(s) sort to the apical membrane, whereas the equilibrative transporter(s) are present on both the apical and basolateral membrane.

We hypothesized that the differential localization of hCNT1 and hENT1 transporters and their activities in the polarized cells should affect the directionality of vectorial transport of various nucleoside and nucleoside drugs across these cells. At subsaturating concentrations, this directionality of transport will be determined by the relative ratio of the maximal transport activity ($V_{max}$) and the affinity ($K_m$) of the transporters for the nucleosides, provided the nucleoside is not completely metabolized within the cells. Therefore, we tested the hypothesis that this differential localization of nucleoside transporters explains

**FIG. 4.** Functional localization of hENT1-YFP and hCNT1-CFP in MDCK cells. 1 x 10^6 cells were seeded on six-well Corning Costar Transwell inserts. At 10–12 days after confluence, the uptake (at 37 °C) from the apical or the basal compartment of [3H]uridine (1 μCi/ml, 0.2 μM) mediated by hENT1 (A) or hCNT1 (B) was determined at 10 min. hENT1-mediated [3H]uridine influx was measured in the presence and absence of sodium (130 mM) but in the presence of 10 μM NBMPR. hCNT1-mediated [3H]uridine influx was measured in the absence of sodium and with or without 10 μM NBMPR. The data shown are the means of triplicate determinations.

**FIG. 5.** Time-course of [3H]-labeled uridine uptake (left scale) and alkaline phosphatase activity (right scale) in stable hENT1-YFP- and hCNT1-CFP-expressing MDCK cells. 1 x 10^6 MDCK cells were plated on 24-well cell culture plates. [3H]Uridine uptake from the apical compartment was measured at 37 °C at 15 min. hCNT1 transporter activity was measured in the presence and absence of sodium (130 mM) but in the presence of 10 μM NBMPR. hENT1 transport activity was measured in the absence of sodium and with or without 10 μM NBMPR. MDCK cells stably transfected with pEYFP empty vector were used as a control. Data are the mean ± S.D. of triplicates.
the paradoxical observation that adenosine and deoxyadenosine are handled quite differently in vivo by the kidney. In the presence of adenosine deaminase deficiency or an adenosine deaminase inhibitor, adenosine is actively absorbed, whereas deoxyadenosine is secreted (13–16). Indeed, as observed in vivo, in the presence of an adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (500 μM), over a time span of 60 min. A, time course of transport of adenosine and deoxyadenosine across MDCK monolayer. B, at 60 min, the net transport of adenosine is from the apical to the basal compartment, whereas that of deoxyadenosine is from the basal to the apical compartment. C, intracellular content of [3H]adenosine- or [14C]deoxyadenosine-associated radioactivity when loaded on the apical or the basal compartment. D, ratio of adenosine or deoxyadenosine efflux (elimination ratio) as a percent of total radioactivity (intracellular + effluxed). E, schematic representation of the activity of the transporters. hCNT1 is located on the apical membrane and reabsorbs adenosine; hENT1 is located on the basolateral membrane and mediates both adenosine and deoxyadenosine influx and efflux.

Fig. 6. [3H]Adenosine and [14C]deoxyadenosine transport in coexpressing MDCK cells. hENT1 and hCNT1 coexpressing MDCK cells were grown on six-well Transwell filter inserts for 10–12 days to obtain cell differentiation. 1 μCi (8 μM) of deoxyadenosine and adenosine were added to the apical or basal compartment in sodium-containing (130 mM) transporter buffer in the absence of NBMPR. The apical-to-basolateral (A → B) or basolateral-to-apical (B → A) compartment of deoxyadenosine and adenosine was measured in the presence of an adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (500 μM), over a time span of 60 min. A, time course of transport of adenosine and deoxyadenosine across MDCK monolayer. B, at 60 min, the net transport of adenosine is from the apical to the basal compartment, whereas that of deoxyadenosine is from the basal to the apical compartment. C, intracellular content of [3H]adenosine- or [14C]deoxyadenosine-associated radioactivity when loaded on the apical or the basal compartment. D, ratio of adenosine or deoxyadenosine efflux (elimination ratio) as a percent of total radioactivity (intracellular + effluxed). E, schematic representation of the activity of the transporters. hCNT1 is located on the apical membrane and reabsorbs adenosine; hENT1 is located on the basolateral membrane and mediates both adenosine and deoxyadenosine influx and efflux.

The paradoxical observation that adenosine and deoxyadenosine are handled quite differently in vivo by the kidney. In the absence of adenosine deaminase deficiency or an adenosine deaminase inhibitor, adenosine is actively absorbed, whereas deoxyadenosine is secreted (15–16). Indeed, as observed in vivo, in the absence of an adenosine deaminase inhibitor, adenosine is preferentially transported from the apical to the basolateral compartment by the coexpressing polarized MDCK cells (Fig. 6, A and B), whereas deoxyadenosine is preferentially transported from the basolateral to the apical compartment (Fig. 6, A and B). At 60 min, there is a net A → B transport of adenosine and a net B → A transport of deoxyadenosine (Fig. 6D). This preferential transport of adenosine is confirmed by its greater uptake from the apical compartment when compared with deoxyadenosine (Fig. 6C). hCNT1 has a high affinity (Km, ~50 and 46 μM, respectively) for adenosine and deoxyadenosine (17). However, transport of deoxyadenosine by hCNT1 is slower than that of adenosine. The hCNT1-mediated adenosine-to-deoxyadenosine flux ratio is 4:1 (17, 18). In contrast, adenosine and deoxyadenosine are transported by hENT1 with about equal affinity (Km, 60 and 71 μM, respectively) (1). Thus, in coexpressing MDCK cells, when adenosine and deoxyadenosine are placed in the apical compartment, hCNT1 transports adenosine into the cells more efficiently than deoxyadenosine. At 60 min the majority of adenosine is trapped in the cells by metabolism (~95%; this and subsequent values of percent metabolism were determined by HPLC), but a small percentage escapes into the basolateral compartment (Fig. 6D). Of that which escape, ~65% is unchanged adenosine presumably transported by hENT1 (Fig. 6D) and 35% is metabolites (perhaps transported by nucleotide efflux pumps). In contrast, at 60 min very little of the deoxyadenosine radioactivity in the cells is metabolized (~10%) (Fig. 6C), and the radioactivity of that which
Simultaneous Expression of hCNT1 and hENT1

37717

escapes to the basal compartment (Fig. 6D) is predominantly unchanged deoxyadenosine (~90%). When these two nucleosides are introduced into the basal compartment, both adenosine and deoxyadenosine are transported into the MDCK cells by hENT1 (Fig. 6C). Again, at 60 min, the greater part of the adenosine is trapped intracellularly by metabolism (~95%) (Fig. 6C), and a smaller portion, which escapes into the apical compartment by diffusion or via the low level of hENT1 transporter present there, is reabsorbed by hCNT1 present on the apical membrane (Fig. 6D). In contrast, at 60 min only a small amount of the deoxyadenosine transported into the cells is trapped intracellularly by metabolism (~15%) (Fig. 6C), whereas most of it is eliminated (~85% deoxyadenosine) into the apical compartment either by the low level of hENT1 transporter activity present there or by diffusion (Fig. 6D). This differential localization of hCNT1 and hENT1 (Fig. 6E), with differing capacities to transport adenosine and deoxyadenosine, appears to explain the paradoxical observation that adenosine is reabsorbed, whereas deoxyadenosine is secreted by the kidney in vivo. Although the level of expression of hCNT1 and hENT1 in the human kidney may differ from that in our doubly transfected MDCK cells, such a difference will not change our general conclusions. This is because our results are dependent primarily on the expression of both transporters in series and on the relative transport (Vₘₐₓ/Kₘ) of adenosine and deoxyadenosine by each of the transporters, hCNT1 and hENT1. Only if one or both of the transporters are saturated (or if one of the transporters is absent) would our observed results change and differ from those obtained in vivo. Indeed, the transporter on the apical membrane of the kidney (presumably CNT1) can be saturated in the perfused rat kidney by high concentrations of adenosine. In this event, adenosine is secreted by the kidney and not actively reabsorbed (9). The concentrations of adenosine and deoxyadenosine used in our experiments (8 μM), are well below the Kₘ of adenosine and deoxyadenosine for hCNT1 (~50 and ~46 μM, respectively) or hENT1 (60 and 71 μM, respectively). At the physiological concentrations of adenosine (<1 μM) (15), both hENT1 and hCNT1 are unlikely to be saturated. The reabsorption of adenosine by the kidney is an important mechanism for regulation of the pharmacological activity of adenosine in the kidney. As reviewed by Jackson and Dubey (19), adenosine regulates preglomerular and postglomerular vascular resistances, glomerular filtration rate, rennin release, epithelial transport, and intrarenal inflammation. If other nucleoside transporters are present in the kidney epithelial cells, they too may influence the renal disposition of this and other nucleosides. Transport experiments with kidney apical and basolateral membrane vesicles or with kidney cell lines have demonstrated only an ENT1 type of activity on the apical (9), basolateral (20), or both faces (10) of these epithelial cells. However, there is no evidence that an ENT2-type transporter is present in the kidney epithelial cells. Although hCNT2 was originally cloned from the human kidney, functional studies with brush border membrane vesicles from the human kidney have not detected hCNT2 activity, but these studies did find hCNT1-type activity that is inhibited by guanosine (6). Immunolocalization studies to determine the localization of hCNTs in the kidney epithelium have not been performed because of a lack of antibodies for such studies. However, even if hCNT2 is present on the brush border membrane of the kidney epithelium, the above findings will likely stand and possibly exaggerate the reabsorption and secretion of adenosine and deoxyadenosine, respectively. This is because adenosine is efficiently transported by hCNT2, whereas deoxyadenosine appears to be a poor permeant of hCNT2 (21).

In summary, we have shown for the first time, using fluorescent-labeled proteins, that the concentrative and equilibrative transporters are localized on the different faces of the polarized kidney epithelium cell. This differential localization appears to be triggered by differentiation and may explain the vectorial transport of nucleosides and nucleoside drugs across the cell membrane. The directionality of the vectorial transport will depend on the relative affinity and maximal transport activity of the two transporters for the nucleosides and nucleoside drugs and likely explains why some nucleosides and nucleoside analogs are absorbed, whereas others are secreted.

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