Localization of human equilibrative nucleoside transporters, hENT1 and hENT2, in renal epithelial cells

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Two human equilibrative nucleoside transporters have been cloned and characterized, hENT1 and hENT2. The primary goal of this study was to localize these transporters in polarized renal epithelia. hENT1 and hENT2 were tagged with green fluorescence protein, stably expressed in renal epithelial cells, and localized by immunofluorescence and functional analysis. Our data demonstrated that both transporters are expressed on the basolateral membrane. hENT1 is also present on the apical membrane. Additionally, we examined the importance to basolateral targeting of two COOH-terminal targeting motifs: a RXXV motif for hENT1 and a dileucine repeat for hENT2. Neither motif appeared to affect targeting, but the dileucine repeat was implicated in surface expression of hENT2. In addition, a splice variant of hENT2 was identified that is predicted to result in a 156-residue COOH-terminal truncation. This variant had a tissue distribution similar to wild-type hENT2 but was retained intracellularly. These data suggest that hENT1 and hENT2 on the basolateral membrane function with concentrative nucleoside transporters on the apical membrane to mediate active reabsorption of nucleosides within the kidney.

Equilibrative nucleoside transporter; Madin-Darby canine kidney; dileucine; splice variant

Nucleoside transporters are polytopic membrane proteins that mediate both the uptake and release of hydrophilic nucleosides across lipophilic membranes. Nucleoside transporters are essential for cellular uptake of many clinically relevant nucleoside analogs used in the treatment of cancers and viral infections. Additionally, they are highly abundant in the kidney where they are hypothesized to play a major role in the salvage of endogenous nucleosides used for nucleotide synthesis.

Two major classes of nucleoside transporters, equilibrative nucleoside transporters (ENT, SLC29) and concentrative nucleoside transporters (CNT, SLC28), have been characterized from a variety of species, including humans and rats (12, 13, 33, 34, 40, 47, 48). The CNT family are secondary active transporters that couple cellular transport of nucleosides to an internally directed sodium or proton gradient (10, 33, 47). In contrast, the ENT family mediates passive transport of nucleosides. Classically, the ENT family can be further subdivided into two types of transporters (es and ei) based on their sensitivity to inhibition by nitrobethylthioinosine (NBMPR). Es-type transport is sensitive to NBMPR, whereas ei-type transport is not (2, 48). Two members of the ENT family have been cloned and functionally characterized: ENT1, which mediates es-type transport, and ENT2, which mediates ei-type transport (15, 19, 48).

Members of both the CNT and ENT family are present in renal epithelium that forms the barrier between the tubule lumen and the circulatory system (11, 24, 25, 45, 46). These transporters are hypothesized to act in series to mediate the vectorial flux of nucleosides through this epithelium in a reabsorptive direction, providing a means to salvage nucleosides from the filtrate. The ability of the epithelial cells to perform this function depends on the asymmetric cellular distribution of nucleoside transporters to the apical and basolateral membrane. Early studies using apical and basolateral membrane vesicles from renal epithelium in animal models indicate that transport at the apical membrane is predominantly concentrative while basolateral transport is predominantly equilibrative (3, 23, 25, 31, 37, 39, 46). Some studies additionally report equilibrative nucleoside transport activity on the apical membrane (6, 9). Molecular localization studies in our laboratory provided the first direct evidence that CNT1 and CNT2 are localized to the apical membrane in cultured renal cells (27). This result is supported by recent immunohistochemical studies demonstrating apical expression of CNT1 in epithelia using rat kidney tissue (14). Recent work by Lai et al. (22) localized ENT1 predominantly to the basolateral membrane of differentiated renal epithelial cells. Immunofluorescence studies visualized ENT1 entirely on the basolateral membrane, but functional assays indicated low levels of ENT1-mediated trans-
port on the apical membrane as well (22). To date, there is no information regarding intracellular localization of ENT2 in renal epithelial cells. Knowledge of the localization of these transporters will enhance our understanding of how ENT1 and ENT2 work in concert with the CNT family to mediate transepithelial flux of nucleosides and nucleoside analogs within the kidney. Furthermore, this information will contribute to understanding the differential functions of these two transporters.

In polarized cells such as renal epithelium, plasma membrane proteins are sorted in the trans-Golgi network and specifically sent to either the apical or basolateral membrane (1). Basolateral targeting appears to be triggered by distinct amino acid sequences (targeting motifs) within the protein itself, which interact with the cellular sorting machinery (50). Some of these targeting motifs [such as the tyrosine motif (NPXY) or dileucine repeat] are related to signals for clathrin-coated pit localization. These signals overlap with those used for endosomal recycling and endocytosis (1). Some proteins contain basolateral targeting motifs unrelated to clathrin-coated pits such as the R/HXXV motif seen in the cation-dependent mannose 6-phosphate receptor (CD-MPR; see Ref. 8). Although the exact mechanisms of action of these unrelated motifs are unknown, their structural orientation appears to allow for interaction with the basolateral sorting machinery. Apical targeting is less well understood but appears to be based on segregation of apical proteins into vesicles or rafts enriched with lipids preferentially delivered to the apical membrane. For some proteins, it appears that incorporation into these rafts is based on glycosylation motifs or glycosylphosphatidylinositol anchors (1).

The goal of this study was to determine the localization of both human (h) ENT1 and hENT2 within renal epithelial cells. We used Madin-Darby canine kidney (MDCK) cells, which have been successfully used to study in vivo intracellular localization of a variety of renal transporters (4, 28, 29, 38). Additionally, we sought to examine the importance of two targeting motifs in basolateral targeting and distribution of hENT1 and hENT2.

MATERIALS AND METHODS

Materials. Cell culture media and supplements were purchased from the University of California, San Francisco Cell Molecular Resource Center at the University of California, San Francisco. The EMBL MDCK II strain was a gift from Dr. Andrew T. Gray (University of California, San Francisco). Plasmid construction. hENT1, hENT2, and the splice variant hENT2A were cloned by PCR using primers flanking the open reading frame (ORF) of hENT1 and hENT2. The primers were designed based on published hENT1 and hENT2 cDNA sequences (7, 12). For hENT1, the sense primer was 5'-gggaaaacggacaaacctca-3'; the antisense primer was 5'-agctgtcactcttcgtaac-3'. For hENT2, the sense primer was 5'-gggacagcggccgacgtg-3', and the antisense primer was 5'-gacctggagggcactcag-3'. hENT1 was then subcloned in frame into pEGFP-C1 vector by adding a Sal I site to the 5' end and a Sac II site to the 3' end. Both hENT2 and hENT2A were subcloned in frame into pEGFP-C1 vector by adding a Sal I site to the 5' end and an Apa I site to the 3' end. All plasmid constructions and DNA sequences were confirmed by enzyme digestion analyses and by automated sequencing at the Biomolecular Resource Center at the University of California, San Francisco.

Site-directed mutagenesis. Mutations of hENT1 (R453A and ΔRAIV) and of hENT2 (L455R and ΔLL) were constructed with the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) using wild-type hENT1 cDNA and hENT2 cDNA as the templates. The sequences of these mutants were confirmed by DNA sequencing at the Biomolecular Resource Center at the University of California, San Francisco.

Stable transfection of MDCK. MDCK cells were grown in MEM with Earle's BSS supplement, 5% heat-inactivated FBS, 100 U/ml penicillin, and 100 U/ml streptomycin in a humidified atmosphere of 5% CO2-95% air at 37°C. Cells were transfected with 1 μg DNA and 16 μg Effectene (Qiagen, Valencia, CA). Cells were grown for 48 h and then diluted in media supplemented with 700 μg/ml G418. Clones were picked after 2 wk of growth in selection media, and positive clones were chosen by Western blot, confocal microscopy, and functional uptake of [3H]-labeled nucleoside.

Confocal microscopy. Samples were prepared for confocal microscopy as described previously (27). Samples were grown for 4–7 days on permeable support and then fixed with 4–8% paraformaldehyde, permeabilized with 0.25% (wt/vol) saponin in PBS, stained with Texas red-conjugated phalloidin for visualization of actin, and mounted on slides in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Samples were analyzed using a Bio-Rad MRC-1024 laser scanning confocal microscope.

Functional uptake in MDCK. Stably transfected MDCK cells were grown for 5–7 days on permeable support and then assayed for membrane specific functionality, as described previously (27). Briefly, cells were treated with 0.1 μM [3H]-inosine in choline buffer (in mM: 128 choline, 4.73 KCl, 1.25 CaCl2, 1.25 MgSO4, and 5 HEPES, pH 7.4) in the absence or presence of 1 mM inosine. All buffers in hENT2 experiments also contained 10 μM NBMPR to reduce background levels of endogenous es-type function. Reaction mix was applied to either the apical or basolateral membrane for 2 min and removed, and cells were washed three times in ice-cold choline buffer to terminate the reaction. Cellular uptake of [3H]-inosine was measured by lysing cells and counting in a Beckman Scintillation Counter. All experiments were repeated in duplicate on three separate occasions.

Expression and transport assay in Xenopus laevis oocytes. To study the function of wild-type and mutant hENTs and green fluorescence protein (GFP)-tagged hENTs, DNA of these transporters was subcloned in pOXX vector by adding a Sal I site to the 5' end and an Xbal I site to the 3' end. DNA sequences were confirmed by enzyme digestion analyses and by automated sequencing at the Biomolecular Resource Center at the University of California, San Francisco. Plasmid construction. hENT1, hENT2, and the splice variant hENT2A were cloned by PCR using primers flanking the open reading frame (ORF) of hENT1 and hENT2. The primers were designed based on published hENT1 and hENT2 cDNA sequences (7, 12). For hENT1, the sense primer was 5'-gggaaaacggacaaacctca-3'; the antisense primer was 5'-agctgtcactcttcgtaac-3'. For hENT2, the sense primer was 5'-gggacagcggccgacgtg-3', and the antisense primer was 5'-gacctggagggcactcag-3'. hENT1 was then subcloned in frame into pEGFP-C1 vector by adding a Sal I site to the 5' end and a Sac II site to the 3' end. hENT2 and hENT2A were subcloned in frame into pEGFP-C1 vector by adding a Sal I site to the 5' end and an Apa I site to the 3' end. All plasmid constructions and DNA sequences were confirmed by enzyme digestion analyses and by automated sequencing at the Biomolecular Resource Center at the University of California, San Francisco.

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Fifty nanoliters of cRNA (~0.4 ng/nl) or water was injected individually in defolliculated oocytes. Oocytes were incubated at 18°C for 30–40 h, and then uptake assays were performed for 40 min at 25°C in 100 μl of transport buffer (2 mM KCl, 1 mM CaCl2, and 10 mM HEPES) containing various concentrations of 3H-labeled nucleosides (Moravek Biochemicals). The reaction was terminated by washing oocytes five times in 3 ml ice-cold choline buffer. Oocytes were lysed individually in 10% SDS, and the amount of radiolabeled nucleoside transported in each oocyte was determined by liquid scintillation counting.

Statistics and data analysis. Groups of 8–10 cRNA-injected or water-injected oocytes were used for each experiment. Uptake values are expressed as means ± SE. For kinetic studies, uptake rates (V) determined at different substrate concentrations (S) were fit to the Michaelis-Menten

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<th>Km, μM</th>
<th>Vmax, pmol·oocyte⁻¹·40 min⁻¹</th>
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<tr>
<td>hENT1</td>
<td>62.0 ± 18.4</td>
<td>181 ± 32.8</td>
</tr>
<tr>
<td>GFP-hENT1</td>
<td>53.5 ± 18.8</td>
<td>115 ± 23.4</td>
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<tr>
<td>hENT2</td>
<td>75.9 ± 21.3</td>
<td>180 ± 34.2</td>
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<tr>
<td>GFP-hENT2</td>
<td>78.0 ± 22.2</td>
<td>164 ± 30.2</td>
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Uptake values are expressed as mean ± SE. These experiments were repeated several times and apparent Km and Vmax from one representative experiment are given. hENT, human equilibrative nucleoside transporter; GFP, green fluorescence protein. hENT1-, GFP-hENT1-, hENT2-, and GFP-hENT2-mediated uptake of adenosine at concentrations ranging from 1 μM to 2 mM were measured as described in MATERIALS AND METHODS.
Localization of hENT1 and hENT2 in polarized renal epithelial cells. To visualize hENT1 and hENT2 in the absence of protein-specific antibodies, we tagged the NH₂ terminus of hENT1 and hENT2 with GFP. Kinetic studies in Xenopus laevis oocytes indicated that there were no significant differences in the uptake of adenosine or thymidine between tagged and untagged transporters (Table 1), suggesting that the GFP tag does not kinetically alter the function of these transporters (Fig. 1).

Results

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Immunofluorescent analysis of tagged hENT1 and hENT2 stably transfected in MDCK indicated that both transporters localized predominantly to the basolateral membrane (Fig. 1). Vertical optical sections indicated the additional presence of a small portion of hENT1 on the apical membrane (Fig. 1A, i–ii). Apical localization was evident upon closer examination of the apical surface using xy-slices (Fig. 1A, iii–v). The apical presence of hENT2 was not observed (Fig. 1B). hENT1-mediated transport of inosine was observed at both the apical and basolateral membranes, whereas hENT2-mediated transport was isolated to the basolateral membrane (Fig. 2). Results were replicated with multiple positive stable clones for each transporter.

Basolateral targeting is independent of the COOH-terminal tail of both hENT1 and hENT2. We were interested in investigating the molecular determinants responsible for polarized localization of hENT1 and hENT2. Based on hydropathy plot analysis and topology studies, hENT1 and hENT2 are each predicted to have eleven transmembrane domains with a four-amino acid COOH-terminal tail (Fig. 3A; see Refs. 12–14). The COOH terminus of both transporters contains a motif implicated in basolateral targeting: an R/HXXV motif in hENT1 (RAIV) and a dileucine repeat in hENT2 (LL). No other targeting motifs were obvious in either sequence at either terminus. We investigated the significance of these two sequences on polarized trafficking of the proteins via mutagenesis studies.

The R/HXXV sequence in hENT1 was mutated in the following two ways: 1) a single mutation was made at position 453, removing the arginine (R453A), or 2) the entire COOH-terminal tail was truncated (ΔRAIV). Both mutants were stably transfected in MDCK and produced full-length protein, as demonstrated by Western blot (data not shown). Neither mutation had a visible effect on localization of hENT1 (Fig. 3B), nor was there an effect on hENT1-mediated transport of inosine at the basolateral membrane (data not shown).

The dileucine repeat in hENT2 was also mutated by both point mutation (L455R) and truncation (ΔLL; Fig. 3A) and stably transfected in MDCK. Transfection efficiencies were extremely low (<10% of cells were transfected). L455R protein levels were too low to be detected by Western blot. ΔLL protein levels were detectable and yielded a single band smaller in size than wild-type hENT2 but equivalent to wild-type hENT1 (data not shown). Wild-type ENT2 clones produced a protein of abnormally large mass (~140 kDa) compared with ENT1 (80 kDa). Evidence for large ENT2 has been seen in other studies as well (18, 42). Both hENT2 L455R and ΔLL trafficked exclusively to the basolateral membrane with no apparent apical
localization. However, surface expression was reduced drastically (Fig. 3B). The L455R mutant displayed some vesicular staining in MDCK (Fig. 3C). The H9004LL mutant displayed significant vesicular staining (Fig. 3D), indicating that the dileucine motif is important for surface expression. This was also true when these proteins were transfected in LLC-PK1 cells, a renal epithelial cell line originating from the proximal tubule (data not shown). Vesicular retention may occur by alteration of protein stability, surface delivery, or surface retention.

Functional localization studies could not be performed on the mutated hENT2 stable MDCK clones because of low transfection efficiencies. Therefore, further studies analyzing the effect of these mutations on hENT2 function were carried out using heterologous expression in oocytes (Fig. 4). Neither truncation of hENT1 (ΔRAIV) nor single mutation of hENT2 (L455R) altered the functional activity of these proteins. In contrast, the hENT2 truncated mutant (ΔLL) showed significant reduction in function, suggesting that the dileucine motif is essential for surface expression of hENT2 in oocytes as well.

Identification of an hENT2 variant. In the process of cloning hENT2, using primers flanking the ORF of the published hENT2 cDNA sequence (7), we found a variant termed hENT2A (GenBank accession no. AF401235). We determined this to be a splice variant based on the genomic sequence of hENT2 (GenBank accession no. AF034102), which has 12 exons and 11 introns. hENT2A uses a different splicing site on the 5′ end of exon 9, causing a 40-bp deletion (positions 1103–
1142) in hENT2A mRNA (Fig. 5). This out-of-frame deletion introduces a premature stop codon in the ORF, encoding a truncated variant that is 156 amino acids shorter than wild-type hENT2 and has an alternative COOH-terminal sequence (Fig. 6). RT-PCR analysis of several tissues found that both wild-type and variant hENT2 are expressed in skeletal muscle, liver, lung, brain, kidney, heart, pancreas, and placenta (data not shown).

hENT2A was heterologously expressed in oocytes and functionally studied. The variant did not take up adenosine or thymidine under our experimental conditions (Fig. 7A). This lack of activity may suggest that the variant does not retain the domains necessary for nucleoside transport. Alternatively, it is possible that the variant is not properly trafficking to the membrane. To further explore this, hENT2A was tagged with GFP and stably expressed in MDCK in the same manner described earlier. hENT2A did not sort to the plasma membrane (Fig. 7, B and C).

Several splice variants of other membrane transporters have been found to have dominant negative effects on the function of wild-type transporters (20, 44). We tested the effect of expression of hENT2A on the function of wild-type hENT2 by coinjecting equal amounts of cRNA for both transporters in oocytes and measuring nucleoside uptake. Uptake in oocytes expressing hENT2 and hENT2A did not differ from that observed in oocytes expressing wild-type hENT2 alone (Fig. 7A).

Fig. 4. Effect of mutations on hENT1- and hENT2-mediated uptake of adenosine and thymidine in oocytes. Uptake of [3H]adenosine (10 μM; A) and [3H]thymidine (10 μM; B) was measured at 25°C for 40 min in oocytes injected with H2O or cRNA for GFP-tagged hENT1 (wild-type [wt]), hENT1 ΔRAIV (Δ), hENT2 (wt), hENT2 L455R (L > R), or hENT2 ΔLL (Δ). Data are expressed as means ± SE of 6–8 oocytes. *Results that are statistically different from GFP-tagged wild-type control (P < 0.05).

Fig. 5. Sequence analysis of hENT2 and hENT2A. A: schematic representation of the hENT2 and hENT2A mRNA and gene organization. Lines and boxes in the hENT2 gene represent introns and exons, respectively. Exons are numbered. Gray boxes represent exons 9. Black boxes represent the 40-bp region in exon 9 that is deleted in hENT2A mRNA. B: 5' and 3' exon-intronic splice sites of exon 9 in hENT2 and hENT2A. Exon region is in bold and italic, and the 40-bp region that is deleted in hENT2A mRNA is underlined.

Fig. 6. Protein sequences of hENT2 and hENT2A. Wild-type hENT2 has 456 amino acid residues. Because of the premature stop codon induced by the out-of-frame 40-bp deletion in the open reading frame of hENT2, hENT2A has only 301 amino acid residues, and its COOH-terminal sequence is changed. Predicted transmembrane domains are overscored. Amino acid residues in hENT2A that differ from hENT2 are in bold type.
DISCUSSION

Past studies have attempted to localize equilibrative nucleoside transport within epithelia with conflicting results. Both the absence and presence of es-type transport activity (presumably ENT1) in brush-border membrane vesicles has been reported (5, 6, 9, 26). In contrast, et-type activity (presumably ENT2) was reported to reside only in basolateral membrane vesicles (5). Because functional studies in isolated plasma membrane vesicles may be confounded by the presence of multiple transport activities or contamination with other membranes, data localizing transporters using functional activity are difficult to interpret.

In this study, we directly examined the localization of GFP-tagged hENT1 and hENT2 in renal epithelial cells. Our data demonstrate that hENT1 and hENT2 are present and functional on the basolateral membrane (Figs. 1 and 2). Interestingly, hENT1 appears in small amounts on the apical membrane where it is also functional. The function of hENT1 on the apical membrane in transfected MDCK was also demonstrated recently by Lai et al. (22). Previous data from this laboratory demonstrated that the concentrative nucleoside transporters, CNT1 and CNT2, are predominantly localized to the apical membrane in renal epithelial cells (27). Together, these data provide a picture of asymmetrically localized CNTs and ENTs working in concert to salvage nucleosides and nucleoside analogs from the tubular filtrate. In vivo studies showing that adenosine is reabsorbed in the kidney support this model (9).
We were additionally interested in examining the molecular determinants responsible for basolateral targeting of these two transporters. The COOH-terminal tail of hENT1 contained an R/HXXV motif (RAIV) that has been implicated in basolateral sorting of CD-MPR (8, 30, 51). Neither mutation nor truncation of this sequence affected hENT1 levels on the basolateral membrane (Fig. 3). In contrast, the COOH-terminal tail of hENT2 contained a dileucine repeat. This motif is implicated as a signal in both basolateral sorting and endosomal recycling of a large number of proteins (16). Both mutation and truncation of the dileucine affected surface expression of hENT2, but, in both cases, all protein that reached the plasma membrane remained confined to the basolateral membrane. This indicates that this motif is important for maintaining steady-state expression of hENT2 on the plasma membrane. Although this does not implicate the dileucine as a targeting motif, the repeat may be important in endosomal recycling or surface retention of hENT2.

Differential localization of hENT1 and hENT2 further substantiates the idea that these two transporters are maintained and regulated by distinct mechanisms within the cell. hENT1 is found ubiquitously throughout the body and is thought to be the major transporter involved in uptake of nucleosides for DNA synthesis. hENT1 is also implicated in terminating adenosine signals in the vicinity of adenosine receptors (32). Within the renal epithelium, the A1 adenosine receptor, which also localizes to both membranes in MDCK, is thought to be the major receptor involved in adenosine signaling (36). Conditions of chronic hypoxia selectively downregulate ENT1 function as a means to increase extracellular adenosine levels at the site of its receptor (21). Symbiosis between hENT1 and the A1 adenosine receptor may explain the levels at the site of its receptor (21). Symbiosis between hENT1 and the A1 adenosine receptor may explain the levels at the site of its receptor (21). Symbiosis between hENT1 and the A1 adenosine receptor may explain the levels at the site of its receptor (21). Symbiosis between hENT1 and the A1 adenosine receptor may explain the levels at the site of its receptor (21).

In contrast, hENT2 is expressed in far lower amounts in all tissues except skeletal muscle. It has a lower affinity for most physiological nucleosides, with the exception of inosine, an adenosine metabolite (32, 43). Recent data indicate that it also interacts with nucleoside bases, preferring the purinergic base hypoxanthine (49). For this reason, it has been proposed that hENT2 is involved in mechanisms requiring heavy adenosine metabolism, such as ATP depletion in skeletal muscle caused by strenuous exercise (7). Within the kidney, its purely basolateral localization suggests that its major role is to function in concert with CNTs in the salvage of nucleosides from the filtrate.

Confocal microscopy of MDCK expressing the GFP-tagged splice variant hENT2A indicates that the variant is not expressed on the surface of these cells (Fig. 7). Furthermore, our data demonstrated that the variant did not function when expressed in oocytes, suggesting that it is not functional or lacks expression on the plasma membrane. In addition, expression of hENT2A did not affect the function of wild-type hENT2, a phenomenon that has been demonstrated for spliced isoforms of other membrane proteins (20, 35, 41, 44). The role of hENT2A is unknown.

In summary, we report that cellular hENT2 is localized exclusively to the basolateral membrane and that hENT1 is localized primarily to the basolateral membrane in renal epithelial cells. The COOH-terminal dileucine motif in hENT2 is implicated in surface expression of this protein; however, neither the dileucine motif nor the RXXV motif in hENT1 appears to be important for basolateral targeting.

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