Conserved residues F316 and G476 in the concentrative nucleoside transporter 1 (hCNT1) affect guanosine sensitivity and membrane expression, respectively

Yurong Lai, Eun-Woo Lee, Carl C. Ton, Shashi Vijay, Huixia Zhang, and Jashvant D. Unadkat

Departments of Pharmaceutics, University of Washington, Seattle, Washington

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Lai, Yurong, Eun-Woo Lee, Carl C. Ton, Shashi Vijay, Huixia Zhang, and Jashvant D. Unadkat. Conserved residues F316 and G476 in the concentrative nucleoside transporter 1 (hCNT1) affect guanosine sensitivity and membrane expression, respectively. Am J Physiol Cell Physiol 288: C39–C45, 2005. First published September 29, 2004; doi:10.1152/ajpcell.00192.2004.—The functional significance of two highly conserved amino acid residues, F316 [putative transmembrane domain (TM)7] and G476 (putative TM11), in the concentrative nucleoside transporter hCNT1 (SLC28A1) was examined by performing site-directed mutagenesis. Conservative mutations at these positions (F316A, F316Y, G476A, and G476L) were generated and expressed in Madin-Darby canine kidney (MDCK) cells as fusion polypeptides with green fluorescent protein (GFP). Unlike wild-type hCNT1, G476A-GFP and G476L-GFP were not expressed in the plasma membrane of undifferentiated or differentiated MDCK cells and had no functional activity. Like wild-type hCNT1, F316A-GFP and F316Y-GFP were expressed in the plasma membrane of undifferentiated MDCK cells and in the apical membrane of differentiated MDCK cells. Remarkably, transport of [3H]uridine by F316Y-GFP or F316A-GFP was highly sensitive to inhibition by guanosine. Furthermore, genotyping of exon 11 of hCNT1 (TM7) in a panel of 260 anonymous human DNA samples revealed a novel F316H variant (TT>CA; 1/260). When expressed in MDCK cells, [3H]uridine transport by F316H was also found to be sensitive to inhibition by guanosine (IC₅₀ = 148 μM). The effect of the F316H mutation resembles the N4 type nucleoside transporter phenotype previously reported to be present in human kidneys. We suggest that the N4 transport system is a naturally occurring variant of hCNT1, perhaps at the F316 position. Collectively, our data show that G476 is important for correct membrane targeting, folding, and/or intracellular processing of hCNT1. In addition, we have discovered that hCNT1 displays natural variation at position F316 and that the variant F316H confers on the transporter an unusual sensitivity to inhibition by guanosine.

localization; inhibition; polymorphism

NUCLEOSIDE TRANSPORTERS PLAY a critical role in modulating the physiological activity of adenosine (19) and in the cellular transport of many therapeutic nucleoside drugs used in the treatment of cancer (e.g., 5-fluorouridine; Ref. 10) and viral diseases (e.g., ribavirin; Ref. 9). In mammalian cells, nucleoside transport is mediated by members of two families of transporters: Na⁺-dependent concentrative transporters (CNT) and Na⁺-independent equilibrative (or facilitative) transporters (ENT) (3). To date, five functionally distinct concentrative Na⁺-dependent nucleoside transporter activities have been described (N1–N5). However, cDNA of only three of these human transporters has been identified: hCNT1 (N2), hCNT2 (N1), and hCNT3 (N3) (12–14). hCNT1 transports pyrimidine nucleosides and hCNT2 transports purine nucleosides, while hCNT3 transports both pyrimidines and purine nucleosides. Uridine and adenosine are substrates of all three transporters. Both hCNT1 and hCNT2 are expressed in specialized cells such as the intestinal epithelia (10), while hCNT3 appears to be present predominately in the human pancreas (11). The N4 transporter activity observed in the human kidney resembles that of the N2 transporter, except that it is sensitive to inhibition by guanosine (4, 5). The N5 transporter activity differs from the other concentrative transporters in that it is sensitive to nitrobenzylthioinosine (NBMPR) and transports guanosine (8). However, there is no clear evidence that the latter two activities are encoded by additional, distinct nucleoside transporter genes. In fact, with the completion of the Human Genome Project, the indication is that all orthologs of the human concentrative nucleoside transporters, as a gene family (SLC28) (2), have been accounted for. The results presented in this article may provide a partial explanation for this apparent paradox.

While investigators at our laboratory (15, 20) and others (17, 18) have made considerable progress in elucidating the structural basis of hENT1 function, progress in dissecting the structural and functional relationships of the CNT has been relatively limited. Nevertheless, chimera studies of hCNT1 followed by site-directed mutagenesis have shown that converting Ser319/Gln320 of transmembrane domain (TM7) and Ser355/Leu354 of TM8 to the corresponding residues in hCNT2 (Gly319/Met314 and Thr347/Val348) changes the substrate selectivity of hCNT1 to resemble that of an hCNT2-like transporter (7). On the other hand, mutation of only the two TM7 residues of hCNT1 produced a protein with an intermediate, CNT3-like activity. In addition, TM7 and TM8 have been identified as potential determinants of substrate selectivity in rat rCNT1 and rCNT2 (21), and mutation of rCNT1 Ser318 (corresponding to hCNT1 Ser319) yields a broad, CNT3-like phenotype similar to that resulting from the hCNT1 Ser319 mutation (22).

Such studies, however, did not indicate which other amino acid residues might be critical for hCNT1 function. We therefore performed a bioinformatic analysis of CNT1-like transporters from a range of animal species, with the aim of identifying conserved amino acid sequences that might highlight other domains of functional significance. This sequence alignment revealed two highly conserved amino acid positions,
F316 and G476, in TM7 and TM11, respectively, that (along with 6 others) are invariant across 17 concentrative transporters drawn from 8 different species. These amino acid residues therefore became a focal point for our initial studies examining the impact of conservative mutations on the transport function and membrane localization of hCNT1. To this end, we engineered a series of single conservative amino acid changes at the F316 and G476 positions of hCNT1 and generated fusion proteins between such CNT1 mutants and green fluorescent protein (GFP). The fusion proteins were then expressed in MDCK cells and characterized for their kinetic properties and plasma membrane expression. In addition, a panel of DNA samples derived from an ethnically diverse group of individuals was genotyped for variants at the F316 position.

**EXPERIMENTAL PROCEDURES**

**Chemicals.** [3H]uridine (17.7 Ci/mmol), [3H]guanosine, [3H]inosine, and [3H]thymidine were purchased from Moravek Biochemicals (Brea, CA). NBMPR, adenosine, inosine, guanosine, uridine, and cytidine were purchased from Sigma (St. Louis, MO).

**Gene construction and site-directed mutagenesis.** As described previously, an ~2.0-kb hCNT1 fragment cloned from human intestine was subcloned into GFP vector pEGFP-C1 (Clontech, Palo Alto, CA) (6). Mutants with single amino acid substitution (G476A, G476L, G476E, and G476N) were generated by means of the QuickChange site-directed mutagenesis kit (Stratagene) using the hCNT1-GFP construct as a template. The nucleotide sequences of the primers used to generate the above mutants were as follows: 1) G476A: 5′-TGTTAGCCCTTTGATGCGTG-3′, 2) G476L: 5′-TGTTAGCCCTTTGATGCGTG-3′, 3) G476E: 5′-TGTTAGCCCTTTGATGCGTG-3′, and 4) G476N: 5′-TGTTAGCCCTTTGATGCGTG-3′. Mutants with single amino acid substitution (G476A, G476L, G476E, and G476N) were then expressed in MDCK cells and characterized for their kinetic properties and plasma membrane expression. In addition, a panel of DNA samples derived from an ethnically diverse group of individuals was genotyped for variants at the F316 position.

**Selection of MDCK cells stably expressing hCNT1 and the mutants.** All cell lines were routinely maintained in MEM with l-glutamine containing 10% fetal bovine serum (FBS), 100 U of penicillin, and 100 µg/mL of streptomycin (GIBCO, Grand Island, NY) at 37°C in 95% air:5% CO2 at 95% humidity. hCNT1-GFP and the mutants were transfected into MDCK cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The transfected cells were subsequently selected by G418 as described previously (6). High-expressing transfectants were identified by the GFP fluorescence intensity using a fluorescence microscope (Carl Zeiss, Thornwood, NY), subsequently isolated by means of cloning cylinders, and then propagated.

**Na+ -dependent [3H]nucleoside transport.** The Na+ -dependent [3H]nucleoside transport experiments were conducted in Na+-containing buffer (in mM: 20 Tris·HCl, 3 K2HPO4, 1 MgCl2, 2 CaCl2, 5 glucose, and 130 NaCl, pH 7.4) or Na+-free buffer in which NaCl was replaced by 130 mM N-methyl-d-glucamine (pH 7.4). Cells grown on 24-well plates were washed three times with Na+-free buffer and then preincubated with Na+-free buffer containing 10 µM NBMPR for 15 min at 37°C. When necessary, transport substrates and inhibitors were dissolved in DMSO at a maximum concentration of 0.1% DMSO because previous experiments have shown that DMSO concentrations of up to 0.1% have no effect on total [3H]nucleoside transport (6). [3H]nucleoside (1 µM, 2 Ci/µL) in either Na+ or Na+-free buffer containing 10 µM NBMPR was added to each well [to inhibit endogenous (canine) ENT1 equilibrative transporter activity]. After incubating at 37°C for different time intervals, the plates were rapidly washed three times with ice-cold Na+-free buffer containing 10 µM NBMPR. The cells were solubilized with 0.3 mL of N NaOH by shaking for 15 min at room temperature and then neutralized with 0.3 mL of 1 N HCl. Next, 0.5 mL of the cell lysates was counted on a scintillation counter. The protein content of the cell

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**Transmembrane 7**

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**Fig. 1.** ClustalW alignment of the transmembrane domain (TM7) and TM11 of 17 concentrative nucleoside transporters from 8 different species, ranging from mammals to nematodes. The alignment shows that Phe180 and Gin189, the subjects of the present study, along with 6 others, are conserved across all 8 species. The amino acid sequences were from Homo sapiens (hCNT1a: GenBank accession no. AAB53837, hCNT2: GenBank accession no. NP_004203, and hCNT3: GenBank accession no. NM_071410), Mus musculus (GenBank accession no. AF079853), Rat mus (rCNT1: GenBank accession no. NP_446315), rCNT2: GenBank accession no. NP_113852, and rCNT3: GenBank accession no. NP_543184), Drosophila melanogaster (DmCG8083-PA: GenBank accession no. NP_610446), DmCG11778-PA: GenBank accession no. NP_610447) and Caenorhabditis elegans (CeF27E11.1: GenBank accession no. AAB62525, CeF27E11.2a: GenBank accession no. AAB65256).
lysates was determined spectrophotometrically using the bicinchoninic acid protein assay kit (Pierce Biochemicals, Rockford, IL) with bovine serum albumin (BSA) used as the standard.

Visualization of hCNT1-GFP and GFP-tagged mutant proteins. Stably expressing cells were grown in two-well Lab-Tek borosilicated coverglass chambers (Nalge Nunc International, Naperville, IL) for 2–3 days (undifferentiated) or for 6–8 days after reaching confluence (differentiated). 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 488 nm and emission at 540 nm, a wavelength suitable for GFP imaging.

Inhibition profile and data analysis. [3H]nucleoside transport was performed in the presence of the unlabeled nucleoside at concentrations varying from 0 to 5 mM. IC50 values of [3H]nucleoside transport were estimated using nonlinear regression (WinNonlin; Pharsight, Mountain View, CA) and the model $E = E_{\text{max}} - \frac{E_{\text{max}} - E_0}{(C/C_{\text{IC50}})}$, where $E$ and $E_{\text{max}}$ are the transport of [3H]nucleoside in the presence and absence of unlabeled nucleoside, respectively. C is the unlabeled nucleoside concentration, and $E_0$ is the transport of [3H]nucleoside obtained in the presence of excess (10 mM) nucleosides. Data are expressed as means ± SD of transport values obtained in three wells. Data shown are representative of a minimum of two experiments performed on different days with different batches of cells.

 Sequencing analysis for exons 11 and 12 of hCNT1. Exon 11 was amplified from genomic DNA using the forward primer 5′-ACTGTGTAAGGTCTTCCACTCCAGC-3′ and the reverse primer 5′-AGATATCCCTGAGGCAAGAATTTGATG-3′. Exon 12 was amplified using the forward primer 5′-TCTATCTCAGAAGGGTGTTGGTAAAC-3′ and the reverse primer 5′-AGAGAGAGATCTGGGCTGTCTG-3′. A 316- or 528-bp DNA fragment flanking exon 11 or 12 of hCNT1 was amplified by performing PCR. To increase the fidelity of PCR, Pfu DNA polymerase (Stratagene) was used. The PCR products were purified by 1.5% agarose gel filtration and then cycle sequenced using BigDye sequencing reagent, version 2 (ABI). To ascertain that the mutant did not arise as an artifact of PCR amplification, we repeated the PCR and reanalyzed the product by cycle sequencing.

TaqMan genotyping assay for human hCNT1 F316H mutant. Genotyping was performed using the ABI Prism Sequence Detection System 7000 instrument in a 96-well format. Dual-labeled TaqMan/TAMRA probes bearing either FAM (6-carboxyfluorescein) or VIC as reporter was custom synthesized by ABI, while PCR primers were made by Invitrogen. The sequences of the TaqMan probes were as follows: 1) hCNT1F316Htg-FAM (mutant allele on complementary strand): 5′-6FAM-CTGGTCCTA CATgGATGTITCCAGC-TAMRA-3′, and 2) hCNT1F316HAA-VIC (wild-type allele on complementary strand): 5′-VIC-CCTGGCTCACAAAAAGATGTITCCAGC-TAMRA-3′. The underlined letters indicate the polymorphism. The sequences for the PCR primers were hCNT1F316H-tg, 5′-GGCACCACACG-CACGTGAGA-3′; and hCNT1F316H-tg-R, 5′-GAGGGTAGAGGAGGTC-TCATACC-3′. These sequences generated a 79-bp amplicon. Sequence-verified, cloned genomic PCR products of the 316 bp containing the F316H polymorphism, derived from the HK114B kidney sample, were used as positive controls for the wild-type AA/AA and mutant CA/CA genotypes in the TaqMan experiments. The heterozygous TT/CA genotype was simulated using an equimolar mixture of the TT and CA genomic PCR products. Each experiment included three no-template controls.

Genotyping. A total of 260 DNA samples were genotyped for this study, of which 19 were isolated from human kidney tissue samples from anonymous organ donors (tissue samples kindly provided by Drs. Kenneth E. Thummel and Evan D. Kharasch, University of Washington, Seattle, WA). The remainder were purchased from the Coriell cell repositories (Camden, NJ) as follows: African-American (87 tissue samples), Caucasian (87), Indo-Pakistani (9), Middle Eastern (10), South American (Andean) (10), South American (Brazil) (10), Russian (Krasnodar) (9), Russian (Moscow) (10), and Ashkenazi Jew (9).

RESULTS AND DISCUSSION

Expression of hCNT1-wt-GFP, F316 (F316Y-GFP, F316A-GFP), and G476 mutants (G476L-GFP and G476A-GFP) in MDCK cells. On the basis of an alignment of 17 CNT-type sequences from 8 different species, we identified 8 amino acid
residues that were conserved across all of the species examined. We hypothesized that these conserved residues were likely critical to the function and/or localization of the transporter. As part of our analysis of the structural and functional impact of these residues, we chose to focus initially on Phe\textsuperscript{316} of TM7 (exon 11) and Gln\textsuperscript{476} of TM11 (exon 15) (Fig. 1). The question we wished to address was whether these residues are critical to the transport characteristics of hCNT1 and/or to the membrane targeting or intracellular processing of the transporter. To this end, we mutated phenylalanine at residue 316 to nonpolar amino acid residues with smaller (alanine) or larger (tyrosine) side chains to generate the fusion proteins F316A-GFP and F316Y-GFP. Similarly, Gln476 was substituted with nonpolar amino acid residues with smaller (alanine) or bulkier (leucine) side chains to generate G476A-GFP and G476L-GFP. Like wild-type (wt)-hCNT1-GFP (6), F316Y-GFP and F316A-GFP exhibited an even distribution on the plasma membrane in undifferentiated MDCK cells (Fig. 2 A) and on the apical membrane when differentiated (Fig. 2, G and H). In contrast, however, G476A-GFP and G476L-GFP were not localized on the plasma membrane; instead, they were diffusely distributed in the cell, much like GFP itself (G476A-GFP; Fig. 2 C), with the exception of greater localization around the nuclei (G476L-GFP, Fig. 2 D). These data show that Phe\textsuperscript{316} is not important for membrane targeting of hCNT1, while Gln\textsuperscript{476} is critical for intracellular processing, folding, or targeting of hCNT1 to the plasma membrane. The results are consistent with previous findings at our laboratory (15) that a single amino acid residue can drastically affect membrane targeting or sorting of a nucleoside transporter.

[^3]H]nucleoside transport by MDCK cells expressing the hCNT1-wt-GFP and mutant transporters. To confirm that F316A-GFP and F316Y-GFP were functional, we measured their ability to transport[^3]H]uridine. Like hCNT1-wt-GFP, MDCK cells expressing the F316A-GFP or F316Y-GFP transporters demonstrated robust, Na\textsuperscript{+}-dependent[^3]H]uridine transport (Fig. 3). In contrast, MDCK cells expressing G476A-GFP or G476L-GFP did not demonstrate any Na\textsuperscript{+}-dependent[^3]H]uridine transport (Fig. 3). This lack of function was not caused by the presence of GFP, because these data were reproducible when the mutants were expressed in MDCK cells without GFP ([^3]H]uridine transport by G476A and G476L mutants not fused with GFP, with or without Na\textsuperscript{+}, averaged at 0.25 ± 0.02 pmol/mg of protein/5 min, respectively). All transport experiments measured initial transport rates of[^3]H]uridine that were linear up to 20 min (Fig. 4).

Nucleoside-inhibitory profiles of hCNT1-wt, F316Y-GFP, and F316A-GFP-expressing MDCK cells. To determine whether F316 mutants demonstrate any functional difference from hCNT1-wt, we compared the ability of various natural nucleosides to inhibit the transport of[^3]H]uridine by hCNT1-wt-GFP, F316A-GFP, or F316Y-GFP. As expected, 0.1 and 1.0 mM of the nucleosides cytidine, uridine, and adenosine (but not inosine) significantly inhibited[^3]H]uridine transport by hCNT1-GFP-, F316A-GFP-, or F316Y-GFP-expressing MDCK cells (Fig. 5). Surprisingly, unlike hCNT1-GFP,[^3]H]uridine transport by F316A-GFP and F316Y-GFP was inhibited by the purine nucleoside guanosine (Fig. 5). Similar data were obtained when[^3]H]thymidine transport was measured in the absence or presence of this same set of nucleosides (data not shown). Next, we determined the capacity of guanosine (IC\textsubscript{50}) to inhibit[^3]H]uridine transport by hCNT1-GFP- or F316Y-GFP-expressing cells. F316Y was remarkably sensitive to inhibition by guanosine (IC\textsubscript{50} = 380 ± 70 \text{mM}), while, as
expected, hCNT1-GFP was not (IC_{50} > 5 mM). IC_{50} values are expressed as means ± SE, where SE denotes the confidence in the estimates of these parameters (Fig. 6). Both transporters were insensitive to inhibition by inosine (Fig. 5). Although F316Y was sensitive to inhibition by guanosine, it did not significantly transport this nucleoside over a 20-min incubation period in Na^+ free medium (data not shown). Because aspects of the functional characteristics (guanosine sensitivity) of F316Y were similar to those described for the N4 transport system, we hypothesized that the N4 transporter might possibly be an F316-type variant of hCNT1 rather than being the product of another distinct gene. To test this hypothesis, we conducted genotyping studies on a panel of DNA samples from anonymous donors to determine whether F316 variants of hCNT1 exist in the human population.

**Genotyping of hCNT1.** We initially sequenced exon 11 encoding TM7 of hCNT1 in an anonymous collection of genomic DNA derived from human kidney (n = 19). One variant, F316H (1/19), resulting from a two-nucleotide change (TT → CA), was found. Online SNP databases of DNA sequence variation, PharmGKB (http://www.pharmgkb.org/index.jsp) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), were searched for previous reports of F316H. However, the search results were negative. Furthermore, no naturally occurring nonsynonymous substitutions were found within TM7 (exon 11). To confirm our findings, we genotyped for the F316H variant within a larger test population (n = 260 individuals). This survey determined that the original carrier was heterozygous for F316H (TT → CA) but yielded no further examples of this variant. This places an upper limit (≤ 0.2%) on the allele frequency for F316H, suggesting that it is likely to be a rare mutation rather than a stable polymorphism.

**Characterization of F316H-GFP-expressing cells.** To determine whether the hCNT1 F316H variant demonstrates the same inhibition profile as F316Y-GFP, we used site-directed mutagenesis to create an F316H-GFP fusion construct and expressed it stably in MDCK cells. Tracking the variant by its green fluorescence, we found that F316H-GFP was distributed evenly on the plasma membrane in undifferentiated MDCK cells but was sorted to the apical membrane upon differentiation (data not shown). Like F316Y-GFP-expressing cells, transport of [3H]uridine by F316H-GFP-expressing MDCK cells was inhibited by guanosine (Fig. 6). Cells were incubated for 10 min with [3H]uridine (1 μM; 2 μCi/ml) in the presence of varying concentrations of guanosine (0.1–5 mM). IC_{50} of guanosine was derived by performing nonlinear regression.
cells was not inhibited by 1.0 mM inosine but was significantly inhibited by the purine nucleoside guanosine (Fig. 7A). The IC₅₀ of guanosine inhibition of [³H]uridine transport by F316H-GFP-expressing MDCK cells was 148 ± 31 μM (mean ± SE). This value is comparable to the data of Gutierrez and Giacomini (5) showing ~40% inhibition of [³H] thymidine uptake at a guanosine concentration of 100 μM. The guanosine sensitivity of the F316 mutants was reminiscent of that of the N4 nucleoside transport system, previously observed in brush-border membrane vesicles prepared from human kidneys. There is currently no evidence that the N4 transporter is encoded by an additional, distinct nucleoside transporter gene apart from the three known examples, hCNT1–3. In fact, with the completion of the Human Genome Project, it appears that all orthologs of the human concentrative nucleoside transporter gene family (SLC28) have been accounted for. It is thus more plausible that the reported N4 transporter activity corresponds to the phenotype of some naturally occurring variant of hCNT1, perhaps at the F316 position.

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The low allele frequency of the F316H in the general population suggests that its discovery was indeed a fortuitous occurrence. However, it is known that rare SNP in particular can frequently display marked ethnicity-dependent distribution or founder effects within specific population subgroups (16). It is possible that our study and that of Gutierrez and Giacomini (5), who examined a small (<8) but indeterminate number of kidneys, might have sampled such a subgroup in which the F316H mutant is present in relatively higher abundance.

In summary, the experiments conducted in the present study demonstrate that the highly conserved residues F316 (TM7) and G476 (TM11) have strong structural and functional significance with regard to the membrane localization and guanosine sensitivity, respectively, of the hCNT1 nucleoside transporter. F316Y and F316H mutants were more sensitive to guanosine inhibition of [³H]uridine transport than the wild type. Guanosine sensitivity of the F316H mutant was comparable to that of the hCNT1 nucleoside transport system, previously observed in brush-border membrane vesicles prepared from human kidneys. There is currently no evidence that the N4 transporter is encoded by an additional, distinct nucleoside transporter gene apart from the three known examples, hCNT1–3. In fact, with the completion of the Human Genome Project, it appears that all orthologs of the human concentrative nucleoside transporter gene family (SLC28) have been accounted for. It is thus more plausible that the reported N4 transporter activity corresponds to the phenotype of some naturally occurring variant of hCNT1, perhaps at the F316 position.

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transporter. Of particular novelty is the discovery of the natural variant F316H, hitherto unobserved in previous surveys (1). This variant occurred in TM7, within which no other mutations, coding or otherwise, have been reported. Thus the natural paucity of F316H, coupled with the functional impact of the variations at both of the conserved residues studied, suggests that the remaining conserved sites within TM7/TM11 are likely to play important functional roles as well. Indeed, the F316 residue may well be important in nucleoside transport by hCNT2 and hCNT3. Mutational analysis of conserved residues within TM7 and TM11 will likely yield additional insights into the workings of the CNT transporters.

GRANTS

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REFERENCES


