A Single Glycine Mutation in the Equilibrative Nucleoside Transporter Gene, hENT1, Alters Nucleoside Transport Activity and Sensitivity to Nitrobenzylthioinosine†

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ABSTRACT: The human equilibrative nucleoside transporter, hENT1, which is sensitive to inhibition by nitrobenzylthioinosine (NBMPR), is expressed in a wide variety of tissues. hENT1 is involved in the uptake of natural nucleosides, including regulation of the physiological effects of extracellular adenosine, and transports nucleoside drugs used in the treatment of cancer and viral diseases. Structure−function studies have revealed that transmembrane domains (TMD) 3 through 6 of hENT1 may be involved in binding of nucleosides. We have hypothesized that amino acid residues within TMD 3−6, which are conserved across equilibrative transporter sequences from several species, may have a critical role in the binding and transport of nucleosides. Therefore, we explored the role of point mutations of two conserved glycine residues, at positions 179 and 184 located in transmembrane domain 5 (TMD 5), using a GFP-tagged hENT1 in a yeast nucleoside transporter assay system. Mutations of glycine 179 to leucine, cysteine, or valine abolished transporter activity without affecting the targeting of the transporter to the plasma membrane, whereas more conservative mutations such as glycine to alanine or serine preserved both targeting to the plasma membrane and transport activity. Similar point mutations at glycine 184 resulted in poor targeting of hENT1 to the plasma membrane and little or no detectable functional activity. Uridine transport by G179A mutant was significantly lower ($p < 0.05$) and less sensitive ($p < 0.05$) to inhibition by NBMPR when compared to the wild-type transporter (IC_{50} 7.7 ± 0.8 nM versus 46 ± 14.6 nM). Based on these data, we conclude that when hENT1 is expressed in yeast, glycine 179 is critical not only to the ability of hENT1 to transport uridine but also as a determinant of hENT1 sensitivity to NBMPR. In contrast, glycine 184 is likely important in targeting the transporter to the plasma membrane. This is the first identification and characterization of a critical amino acid residue of hENT1 that is important in both nucleoside transporter function and sensitivity to inhibition by NBMPR.

Nucleoside permeation across cell membranes is a complex process mediated by multiple transporters. These transporters can be classified into two broad categories: the Na^+-independent equilibrative (or facilitative) transporters that mediate both influx and efflux of nucleosides, and the Na^+-dependent concentrative (energy-requiring) transporters, which physiologically mediate the influx of nucleosides (*1*). Besides their role in the flux of natural nucleosides, nucleoside transporters play a critical role in the transmembrane flux of a variety of nucleoside drugs used in the treatment of various diseases such as cancer [e.g., 5-fluorouridine (*2*)] and diseases of viral origin [e.g., ribavirin (*3*)].

The equilibrative transporters *es* (hENT1)[1] and *ei* (hENT2) have broad substrate selectivity, transporting both purines and pyrimidines (*4*). hENT1 is sensitive to inhibition by low nanomolar concentrations of nitrobenzylthioinosine (NBMPR; IC_{50} 0.4 nM), while hENT2 is relatively insensitive to inhibition by NBMPR (IC_{50} 2.8 μM) (*4*). hENT1 appears to be expressed ubiquitously in all human tissues, suggesting that it fulfills a crucial role in maintaining intracellular nucleoside availability. In addition, hENT1 plays a critical role in regulating the physiological effects of extracellular adenosine (*5*, *6*). Despite this crucial role, little progress has been made in elucidating structure−function relationships of hENT1. Through differences in the sensitivity of hENT1 and its rat counterpart (rENT1) to inhibition by dipyridamole (*1*) and hENT1/rENT1 chimera studies, Sundaram et al. (*7*) have identified transmembrane domains (TMDs) 3−6 of hENT1 as important for interaction with dipyridamole. Since

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[1] Abbreviations: ENT, equilibrative nucleoside transporter; NBMPR, *S*-(*p*-nitrobenzyl)-6-thioinosine; SEM, standard error of the mean; DP, dipyridamole; TMD, transmembrane domain; GFP, green fluorescent protein; PCR, polymerase chain reaction.

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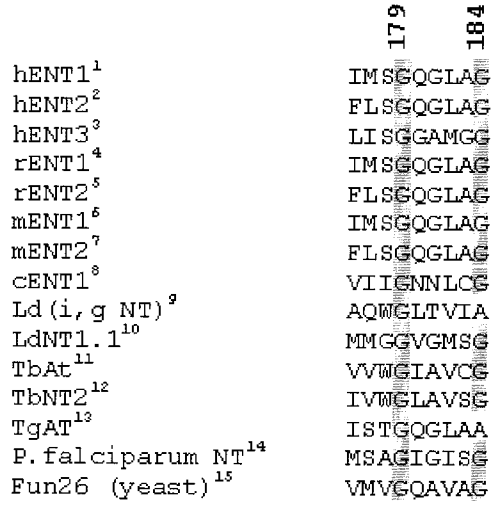

FIGURE 1: Alignment of portions of TMD 5 of 15 nucleoside transporters of the equilibrative transporter family from different species. This alignment (see below) shows that glycine 179 (position in hENT1) is conserved across all species, whereas glycine 184 (position in hENT1) is conserved across all species except in the inosine/guanosine transporter of *Leishmania inosine guanosine* [(Ld (i, g NT)] and the adenosine transporter of *Toxoplasma gondii* (TgAT) where it is replaced by alanine. Amino acid sequences of equilibrative transporters from [1,2,3]human (*18*−*20*), [4,5]rat (*21*), [6,7]mouse (*22*), and related transporters from [8]*Caenorhabditis elegans* (*20*), [9,10]*Leishmania donovani* (*23*, *24*), [11,12]*Trypanosoma brucii* (*25*, *26*), [13]*Toxoplasma gondii*, [14]*P. falciparum* (*27*), and [15]*Saccharomyces cerevisiae* (*14*) were aligned using ClustalW multiple alignment program.

dipyridamole is a competitive inhibitor of nucleoside uptake by hENT1 (*1*), it is likely that these domains are also important in the binding and transport of nucleosides. Although all equilibrative nucleoside transporters identified to date have some differences in affinity toward the natural nucleosides, they all transport both purines and pyrimidines. Therefore, we hypothesized that amino acid residues important in the binding and translocation of nucleosides may be conserved across all equilibrative nucleoside transporters identified to date. As a first step toward identification of these nucleoside binding sites, we aligned 15 nucleoside transporter sequences from different species (Figure 1). Within TMDs 3−6, we identified one glycine residue at position 179 that was conserved across all the aligned sequences and one at position 184 that was conserved in all sequences except two, i.e., the inosine/guanosine transporter of *Leishmania inosine guanosine* [(Ld (i, g NT)]) and the adenosine transporter of *Toxoplasma gondii* (TgAT), where the glycine is replaced by alanine. By using a rapid functional transporter assay based on a yeast (*Saccharomyces cerevisiae*) expression system and site-directed mutagenesis of a GFP-tagged hENT1 gene, we have determined that glycine 179 is critical for the function of hENT1, while glycine 184 appears to be important in targeting hENT1 to the plasma membrane. This is the first identification and characterization of a critical amino acid residue of hENT1 that is important in both nucleoside transporter function and sensitivity to inhibition by NBMPR.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. The cDNA for hENT1 was cloned from a human intestinal cDNA library by PCR (*8*) (GenBank

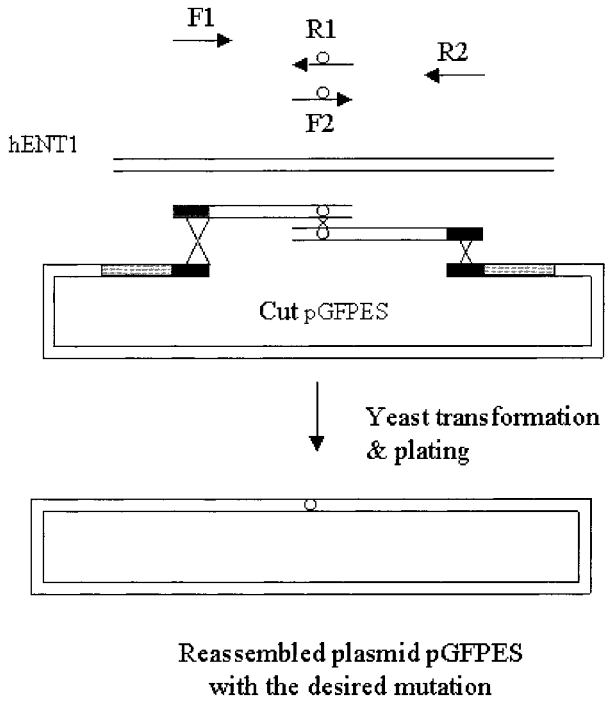

FIGURE 2: Site-directed mutants of hENT1 in yeast were generated using a gapped plasmid and in vivo homologous recombination. Two overlapping PCR products were used to repair a gap within the hENT1 gene created by *Bam*HI and *Hin*dIII digestion of pGFPES. The two sets of PCR products were obtained by using two pairs of primers (F1, R1 and F2, R2) as shown in the diagram. All the primers were 33 bases long. Three bases in the middle of the R1 and F2 primers contributed to the coding for the mutant amino acid. Yeast strain YPL1 was transformed with the cut vector and the pair of PCR products and plated on SD-URA plates. The complete hENT1 gene was assembled by homologous recombination within *Saccharomyces cerevisiae*, resulting in the desired mutation.

accession no. AF079117). To construct the plasmid pES, a PCR fragment of the whole coding sequence of this intestinal hENT1, except for the stop codon, was cloned into pYES2.1V5-His-TOPO vector (Invitrogen) using the topo-cloning procedure. The sequence of the construct was verified by direct sequencing. The plasmid pGFPES was constructed by first cutting pES with *Pvu*II and *Xba*I (both cut outside hENT1 gene within the vector sequence) and then transforming it with two PCR products, one corresponding to green fluorescent protein (GFP-S65T) and the second corresponding to hENT1 coding sequences (with stop codon). The ends of the PCR products were designed such that when these PCR products are transformed into yeast along with pES cut with *Pvu*II and *Xba*I, the endogenous homologous recombination system assembled the whole N-terminal GFP fusion gene within the vector (*9*).

Site-Directed Mutagenesis. Specific amino acid changes were rapidly generated by using a PCR gap-repair method that utilizes the ability of *Saccharomyces cerevisiae* to generate efficient homologous recombination even over short stretches of homology (Figure 2). Briefly, pGFPES was cut with *Bam*HI and *Hin*dIII such that it produced a deletion of ~500 bp within the hENT1 gene encompassing 3−6 TMD. Two PCR products were generated using pES as the template such that a 5′ 33 bp sequence of one overlaps with the 33 bp region at the 3′ end of the other. The other ends of the PCR products were designed such that they had specific

sequences that would target to the ends of the cut pGFPES as shown in Figure 2. Yeast strain YPL1 (*MATa fui1*Δ*::HIS3, ura3*−*52, lys2*−*801, HIS3* Δ) was created from strain RW 128 (*trp1*Δ, *his3*Δ*200*, *fur4*Δ, Δ*fui1::HIS3*; kindly donated by Dr. Wagner of Université Louis-Pasteur/CNRS, Strasbourg, France). The strain YPL1, deficient in uridine permease gene product (pfui1), was transformed with the cut pGFPES along with the pair of PCR products designed for individual mutations and plated on SD-Ura plates containing yeast nitrogen base (6.7 g/L; Difco), synthetic media CSM-URA (0.77 g/L; BIO 101 Inc.), and 2% glucose. The transformation with the cut vector alone was used as a negative control to get an estimate of the number of colonies produced independent of the gap-repair event. The ratio of the number of colonies on plates transformed with cut vector plus the PCR products to cut vector alone varied from 10:1 to 20:1. DNA was isolated from individual yeast colonies, and each mutation was confirmed by sequencing.

Yeast Plate Assay for hENT1 Transporter Activity. We found that the yeast strain YPL1 is resistant to 125 μM 5-fluorouridine or 125 μM tubercidin toxicity due to its inability to transport these nucleoside analogues (see results below). However, this deficiency is complemented by the expression of human hENT1 gene under a GAL promoter control, resulting in a sensitive phenotype when grown in the presence of 2% galactose and 1% raffinose. We have used this to rapidly assay the transporter activity of hENT1 mutants. Agar plates were prepared with the following constituents: yeast nitrogen base (6.7 g/L), synthetic media CSM-URA (0.77 g/L) containing 2% galactose (Sigma) and 1% raffinose (Sigma). 5-Fluorouridine (125 μM; Sigma), tubercidin (125 μM; Sigma), and NBMPR (75 μM; Sigma) were added to the plates where indicated. The mutants were patched onto the plate along with wild-type and vector-alone controls. After 2 days of incubation at 30 °C, the plates were scanned to record the growth of the patches in the presence of different drugs using a Hewlett-Packard ScanJet 5200C.

Fluorescence Microscopy. Yeast carrying the hENT1 mutants were induced for protein production for 15 h at 30 °C in the presence of 2% galactose and 1% raffinose, prior to fluorescence microscopy with a Zeiss microscope. Images were digitally captured using IPlab software.

Preparation of Yeast Membrane Fraction. Cells from the same batch examined under the fluorescent microscope were pelleted, and the yeast pellet was resuspended in ice-cold lysis buffer [0.3 M sorbitol, 0.15 M NaCl, 5 mM $MgCl_2$, 10% glycerol, 10 mM sodium phosphate, pH 7.4, and protease inhibitor cocktail from Roche (Complete tablets)]. The yeast cells were lysed by the glass-bead method following a protocol described by Parrish et al. (*10*). Total protein quantity in the membrane fraction was estimated using the BCA protein assay reagent from Pierce Inc., using the vendor's recommended protocol.

Western Blot. Polyacrylamide gels containing SDS were electrophoresed and blotted to a nitrocellulose membrane using a BioRad apparatus. The blots were developed with JL-8 (Clontech), an anti-GFP monoclonal antibody (0.01 μg/mL), and rabbit anti-PMA polyclonal antibody (A_{280} = 0.4 × 10^{-3} unit; a kind gift from Carolyn Slayman, Yale University). Signals were detected using the ECL kit (Amersham), a chemiluminescence-based western blotting kit.

[^{3}H]Uridine Transport Assay in Yeast. Yeast cells (YPL1) harboring hENT1 or hENT1 mutants were grown overnight in 5 mL of SD-Ura media. These cells were then washed 3 times in an equal volume of galactose media [yeast nitrogen base (6.7 g/L), synthetic media CSM-URA (0.77 g/L) containing 2% galactose (Sigma) and 1% raffinose (Sigma)] to remove any glucose from the media. The cells were then resuspended in the galactose media at 0.5 ODU and incubated for 15 h for induction of hENT1 protein at 30 °C. At the end of induction, the cells were washed to remove the galactose media and resuspended in transport buffer (100 mM choline chloride, 10 mM HEPES, pH 7.4, 2 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). The uptake of [^{3}H]uridine (1 μM; Moravek Chemicals) by yeast cells (~10^7 cells) at 20 min was determined at room temperature, in a total volume of 200 μL, by the rapid filtration method described previously (*8*). The transport of [^{3}H]uridine into the yeast cells was terminated by rapid filtration and by washing the cells 3 times with 4 mL of ice-cold transport buffer containing 10 μM NBMPR. All uptake experiments were conducted in triplicate. The Michaelis−Menten constants, V_{max} and K_m, of [^{3}H]uridine transport into yeast cells were determined by measuring the transport of [^{3}H]uridine into cells in the presence of increasing concentrations of nonradioactive uridine (0−10 mM). Kinetic parameters of transport were estimated by nonlinear regression analysis (WinNONLIN) of tracer displacement curves as described previously (*11*). The ability of various nucleosides to inhibit the transport of [^{3}H]uridine into yeast cells was also measured. The inhibitory capacity (IC_{50}) of NBMPR was determined by measuring the uptake of [^{3}H]uridine by yeast at varying concentrations of NBMPR (0−10 mM). The data were analyzed by fitting the following equation to the data by nonlinear regression:

$$E = E_{max} - (E_{max} - E_0)*[C/(C + IC_{50})]$$

where E_{max} is the uptake in the absence of NBMPR, E_0 is the uptake not inhibitable by NBMPR, and C is the concentration of NBMPR. All uptake measurements were conducted in at least triplicate. All experiments shown in the figures are representative results of three independent experiments.

Experiments in MDCK Cells. For experiments in MDCK cells, the DNA fragment containing the coding sequence for human intestinal ENT1 was amplified from the plasmid pES such that *Bgl*II and *Kpn*I sites were added using the following primers: sense, 5′ tga tga aga tct ***atg*** aca acc agt cac cag c 3′; antisense, 5′ tag tag ggt acc tca cac aat tgc ccg gaa ca 3′. After digesting with restriction enzymes, the fragment was subcloned into pEYFP expression vector (Clontech, Palo Alto, CA). G179A and G179L mutants in pEYFP expression vector were constructed using the megaprimer method (*12*). Briefly, the first round of PCR amplified the megaprimer by hENT1 sense/mutant primers: G179A (5′ to 3′) GCCTGCTAGGCCCTGGAGACTCATCATGATGGGGGC; G179L (5′ to 3′) GCCTGCTAGGCCCTGGGCACTCATGATGGGGGC. The second round of PCR amplified the hENT1 mutant fragment by hENT1 antisense/megaprimer pairs. All constructs were confirmed by automated sequence analysis using the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA).

MDCK cells were cultured in MEM medium with Earle's salts and L-glutamine containing 10% FCS, 100 units of penicillin, and 1% streptomycin (Gibco). The expression vector was transfected into MDCK cells by the SuperFect Transfection Reagent (Qiagen) according to the manufacturers' instructions. The transfectants were selected by cycling the cells for a period of 3−5 days through varying concentrations of G418 (GIBCO-BRL), ranging from 300 to 1000 μg/mL. For the [^{3}H]uridine uptake assay, cells resistant to 500 μg/mL G418 were seeded on 24 well plates and cultured to reach confluence in the presence of 500 μg/mL G418. The cells were washed 3 times in Na^+-free buffer solution (20 mM Tris-HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2$, 5 mM glucose, 130 mM *N*-methyl-D-glucamine), with or without 10 μM NBMPR, followed by 15 min preincubation in the same buffer conditions. Preincubation buffer was discarded, and 0.2 mL of Na^+-free buffer containing 1 μM [^{3}H]uridine (1 mCi/mL, 17.7 Ci/mmol) with or without NBMPR (dissolved in DMSO) was added to each well. After 10 min of incubation, the wells were rapidly washed 3 times with ice-cold Na^+-free buffer containing 10 μM NBMPR. Then, 1 mL of 5% Triton X-100 was added to lyse the cells. Twenty-five microliters of solublized protein was kept for protein estimation. The radioactivity in the rest of the lysate was measured on a scintillation counter. The protein content was determined spectrophotometrically using BCA protein assay kit (Pierce Biochemicals, Rockford, IL). For fluorescence microscopy, the transfectants were grown as above and visualized under a Zeiss microscope using a yellow filter.

RESULTS AND DISCUSSION

To explore the functional role of the two conserved glycine residues, we have expressed an N-terminal GFP-tagged clone of the hENT1 in the yeast strain YPL1, which lacks the uridine permease gene, FUI1 (*13*). The FUI1 gene product has been shown to exhibit nucleoside transporter activity (*14*). Although the YPL1 strain is resistant to 125 μM 5-fluorouridine or tubercidin, expression of hENT1 in this strain confers sensitivity to these drugs (Figure 3). As expected, this sensitivity was reversed by NBMPR (75 μM; only reversal of 5-fluorouridine toxicity is shown in Figure 3). The sensitivity of hENT1 to toxic nucleosides was present even when the transporter protein was not tagged with GFP ([^{3}H]uridine uptake by untagged hENT1 and GFP-tagged was similar; data not shown). However, this plate assay for nucleoside transporter activity has the advantage that it is amenable to rapid and high-throughput assay of the functional activity of mutated hENT1 transporters.

As outlined in Figure 2, we exploited the highly efficient homologous recombination system in *Saccharomyces cerevisiae* to rapidly create point mutations in the GFP-tagged hENT1 expressed in the yeast strain YPL1. The mutants were confirmed by sequencing and then directly assayed for sensitivity to 125 μM 5-fluorouridine or tubercidin toxicity. We mutated glycine 179 and glycine 184 to amino acids with varying sizes of side chains. Substitution of glycine 179 with larger side chain amino acids such as leucine (G179L), valine (G179V), or cysteine (G179C) resulted in lack of nucleoside transport activity as evidenced by lack of sensitivity to 125 μM 5-fluorouridine or tubercidin toxicity (Figure 3). In contrast, substitution of glycine 179 with amino acids

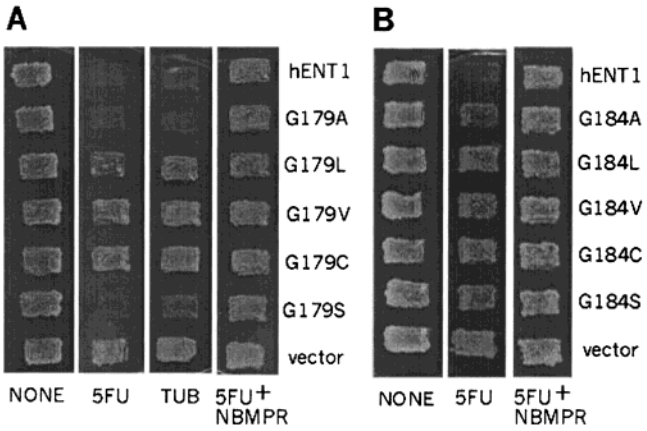

FIGURE 3: Transport activity of wild-type and mutant hENT1 transporters was measured by their sensitivity to 5-fluorouridine and tubercidin toxicity on agar plates. GFP-tagged wild-type hENT1 and hENT1 mutants at glycine 179 and glycine 184 positions were patched on −ura plates containing 2% galactose and 1% raffinose (without nucleoside drugs) or containing 2% galactose and 1% raffinose plus either 125 μM 5-fluorouridine (5FU), 125 μM tubercidin (TUB), or 125 μM 5-fluorouridine plus 75 μM NBMPR (5FU+NBMPR). All glycine 179 mutants were resistant to 5-fluorouridine or tubercidin toxicity except G179A and G179S. G179S mutant, however, showed only modest sensitivity to tubercidin. Of the glycine 184 mutants, only G184A showed modest sensitivity to 125 μM 5-fluorouridine, and none of the mutants showed sensitivity to 125 μM tubercidin (data not shown). This toxicity of nucleoside analogues was reversed by NBMPR (only reversal of 5-flourouridine toxicity is shown).

with smaller side chains such as alanine (G179A) or serine (G179S) did not affect the sensitivity of these mutant hENT1 transporters to these toxic nucleoside analogues. Further, like wild-type hENT1, the sensitivity of the G179A and G179S mutants to 5-fluorouridine toxicity was reversed by 75 μM NBMPR. These data suggest that the transport activity of G179A and G179S is inhibitable by NBMPR. Similar results were obtained when glycine 184 was substituted with the same amino acids except that G184A showed only partial sensitivity to 5-fluorouridine or tubercidin and G184S was found to be resistant to the toxicity of 125 μM 5-fluorouridine and tubercidin (Figure 3; data not shown for tubercidin).

To ascertain if the reduced or lack of activity of the glycine 179 mutants is due to reduced or lack of expression of mutant transporter protein, we made total membrane preparations of yeast cells expressing the wild-type or mutant hENT1 genes. The membrane proteins were separated by 4−15% SDS−polyacrylamide gel electrophoresis and immunoblotted with anti-GFP antibody. The predicted molecular mass for GFP-tagged hENT1 protein is about 68 kDa. All the glycine 179 mutants expressed a band at ~68 kDa, similar in intensity to that expressed by the wild-type protein, suggesting that similar amounts of wild-type and mutant proteins are expressed in these yeast cells (Figure 4). Bands of size 130 kDa (likely representing dimers; data not shown) and of smaller sizes (most likely representing degradation products of the fusion protein) were also detected. Such high molecular mass bands have been observed by others when hENT1 is expressed in yeast (*15*). Immunoblotting with an antibody against a resident plasma membrane protein, the plasma membrane ATPase (anti-PMA), confirmed that an equivalent amount of total protein was loaded on the gel. These data suggest that mutations at glycine 179 did not

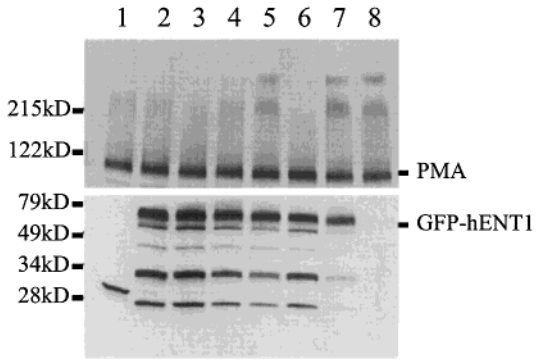

FIGURE 4: Western blot analysis of membrane expression of wild-type and glycine 179 hENT1 mutants in yeast cells. Total membrane-associated protein was loaded onto a 4−15% SDS−polyacrylamide gel. The proteins were detected by the ECL kit (Chemiluminescence kit from Amersham) using an anti-GFP monoclonal antibody (JL-8, Clontech). A polyclonal antibody against yeast plasma membrane ATPase (PMA) was used to confirm equivalent loading of the samples. GFP−hENT1 fusion protein should be ~68 kDa. Lower molecular mass bands likely represent specific degradation products of the GFP−hENT1 protein. Lane 1, GFP alone; lane 2, GFP−hENT1 (wild-type) fusion protein; lane 3, GFP−hENT1 G179A; lane 4, GFP−hENT1 G179L; lane 5, GFP−hENT1 G179V; lane 6, GFP−hENT1 G179C; lane 7, GFP−hENT1 G179S; lane 8, pYES2.

change the total quantity of the membrane-associated hENT1 protein. We further examined the plasma membrane localization of our GFP-tagged mutants by fluorescence microscopy of whole yeast cells. The wild-type hENT1 when fused to GFP predominantly exhibited a uniform plasma membrane localization discernible as a continuous ring around the yeast cell. Diffuse GFP fluorescence was also detected inside the cell with occasional bright spots. The plasma membrane and the intracellular localization of the various GFP-tagged G179 mutants of hENT1 were similar to those of wild-type hENT1 (Figure 5). These data suggest that the mutations at glycine 179 do not alter the localization of the transporter at the plasma membrane, and thus defective or poor localization cannot explain the lack of functional activity of the G179L, G179V, or G179C mutants. In contrast, all glycine 184 mutants showed altered localization. While G184A, G184L, and G184S exhibit some ring-like GFP distribution, the fusion protein appears to reside primarily in bright patches close to the plasma membrane (see G184L). Despite an altered amount of plasma membrane localization of G184A compared with the wild-type or G179L, G179V, and G179C, this level of plasma membrane localization was sufficient to render this mutant sensitive to 5-fluorouridine. The altered localization of glycine 184 mutants suggests that this amino acid residue may be important in the normal targeting or distribution of hENT1 in the plasma membrane.

To confirm the above-described transport activities of the mutants, we determined the uptake of [^{3}H]uridine (1 μM) by yeast cells expressing these mutants. As expected from our plate assays, G179L, G179V, G179C, G184L, G184V, G184C, and G184S mutants did not demonstrate any significant [^{3}H]uridine uptake when compared with the uptake activity in the presence of NBMPR. Mutants G179A, G184A, and G179S exhibited significant NBMPR (10 μM)-inhibitable uridine uptake, although much reduced compared to the wild type (Figure 6). Only G179A exhibited sufficient activity to determine if this lower [^{3}H]uridine uptake was

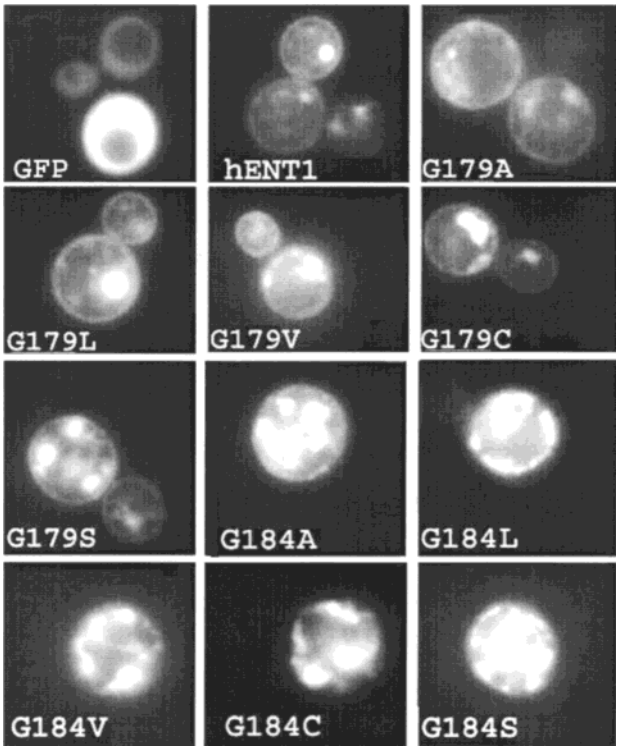

FIGURE 5: Localization of GFP-tagged wild-type and mutant hENT1 on the plasma membrane of yeast cells. Wild-type hENT1 and all glycine 179 mutants, G179A, G179L, G179V, G179C, G179S, show good transporter localization at the plasma membrane. In contrast, glycine 184 mutants show mostly intracellular localization and poor or patchy targeting to the plasma membrane, while expression of GFP vector alone (GFP) shows diffuse cytoplasmic localization.

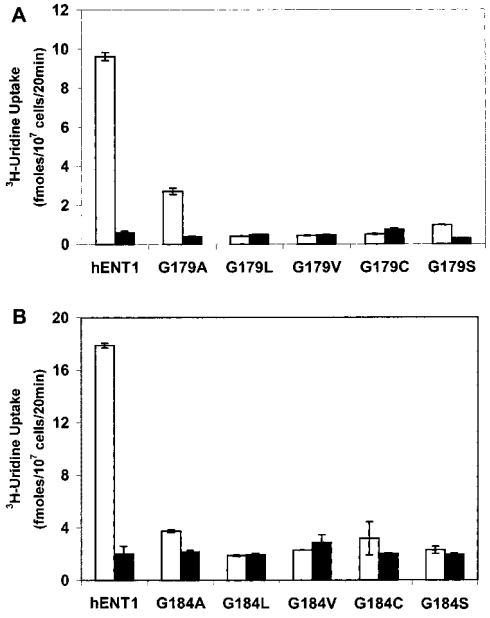

FIGURE 6: Uptake [fmol (10^7 cells)$^{-1}$ (20 min)$^{-1}$; mean ± SEM] of 1 μM [^{3}H]uridine by yeast cells expressing G179 (A) and G184 (B) hENT1 transporter mutants in the absence and presence of NBMPR (10 μM). The open bars correspond to uptake in the absence of NBMPR (but presence of 1% DMSO), and the filled bars correspond to uptake in the presence of 10 μM NBMPR (dissolved in 1% DMSO). Only G179A, G179S, and G184A show uptake significantly inhibitable by NBMPR ($p < 0.05$).

due to changes in the affinity (K_m) or maximal capacity (V_{max}) of the transporter. (The uptake by G179S and G184A was

Table 1: Michaelis−Menten Constants ($n = 3$) for the Uptake of [^{3}H]Uridine by Wild-Type and Mutant (G179A) hENT1 Transporters

	hENT1	G179A	p value
V_{max} [fmol (10^7 cells)$^{-1}$ (20 min)$^{-1}$]	239.7 ± 64.7	169.4 ± 97.5	>0.05
K_m (μM)	18.7 ± 10.1	39.2 ± 30	>0.05
V_{max}/K_m	14.8 ± 5.7	5 ± 3.2	<0.05

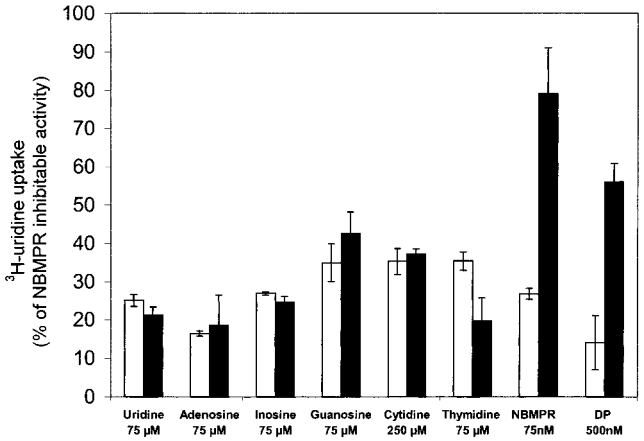

FIGURE 7: Inhibition of hENT1-mediated [^{3}H]uridine (1 μM) uptake (mean ± SEM) by various nucleosides and nucleoside analogues in yeast cells expressing wild-type hENT1 (open bars) or G179A mutant (filled bars). Values are expressed as percent of uptake inhibitable by 10 μM NBMPR. A statistically significant difference in inhibition capacity was exhibited by NBMPR and dipyridamole ($p < 0.05$).

too low to determine their Michaelis−Menten constants of uptake.) Although statistically the K_m and V_{max} of uridine uptake by this mutant are not significantly different from those obtained for the wild-type hENT1 (Table 1), they show a trend toward being lower (V_{max}) or higher (K_m) than those exhibited by the wild-type transporter. Combined, these effects result in a decrease in the uptake of uridine (V_{max}/K_m) at tracer concentrations. These results suggest that substitution of glycine 179 with alanine results in altered transport activity at tracer or at physiologically relevant concentrations of uridine. The lack of significant change in V_{max} is consistent with the fluorescent microscopy data that the wild-type and mutant hENT1 (in particular G179A) proteins were both produced and targeted to the plasma membrane in equal or similar amounts. To determine if the interaction of G179A mutant with other nucleosides was similar to that of the wild type, we conducted inhibition studies with nucleosides and nucleoside transport inhibitors such as NBMPR and dipyridamole (Figure 7). While we did not detect a significant difference ($p > 0.05$) in the ability of the natural nucleosides to inhibit the transport of 1 μM [^{3}H]uridine by G179A compared to that of the wild type, the inhibitory effects of NBMPR and dipyridamole on G179A were significantly lower than those on the wild type ($p < 0.05$). To characterize this diminished inhibition by NBMPR, a nucleoside analogue, we determined the IC_{50} values of this inhibitor on the uptake of [^{3}H]uridine (1 μM) by the wild-type and G179A hENT1 transporters. As expected, the NBMPR IC_{50} value for inhibiting uridine uptake by G179A hENT1 transporter was approximately 6-fold larger than that for the wild-type hENT1 transporter (Table 2). Since NBMPR is itself a nucleoside analogue and

Table 2: NBMPR IC_{50} Values for Wild-Type and G179A hENT1 Transporters

	E_{max}[a] [fmol (10^7cells)$^{-1}$ (20 min)$^{-1}$]	IC_{50} (nM)	E_0[a] [fmol (10^7cells)$^{-1}$ (20 min)$^{-1}$]
hENT1	10.3	8.2	1.6
	9.8	7.1	1.8
(mean ± SD)	10 ± 0.3	7.7 ± 0.8	1.7 ± 0.14
G179A	2.5	36.3	0.84
	2.2	57	1.2
(mean ± SD)	2.35 ± 0.21	46.6 ± 14.6	1.02 ± 0.25

[a] E_{max} is the uptake in the absence of NBMPR; E_0 is the uptake not inhibitable by NBMPR.

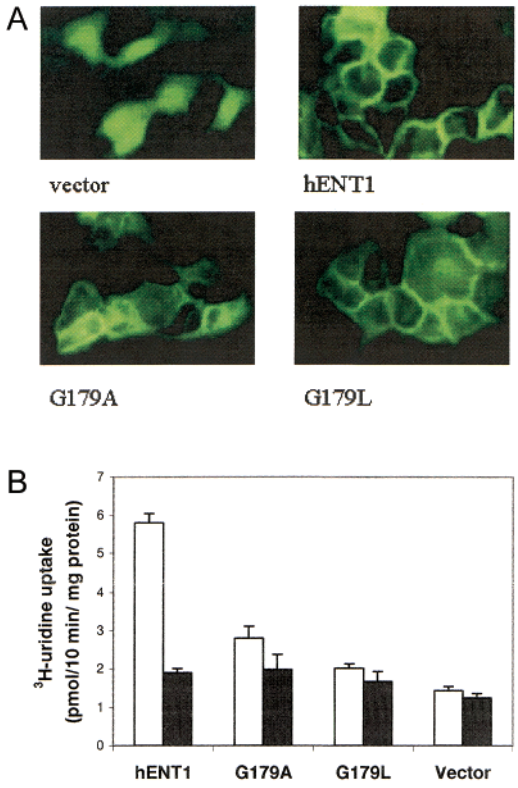

FIGURE 8: (A) Localization of YFP-tagged wild-type hENT1, G179A, or G179L mutants in MDCK cells shows similar localization at the plasma membrane. In contrast, the localization of YFP is diffuse. (B) [^{3}H]Uridine uptake (mean ± SEM, $n = 3$) by MDCK cells expressing wild-type hENT1, G179A, G179L, or vector alone in the absence (open bars) or presence (filled bars) of 10 μM NBMPR. Only hENT1 and G179A mutants show uptake significantly inhibitable by NBMPR ($p < 0.05$).

a competitive inhibitor of nucleoside transport by hENT1, these data support the notion that glycine 179 is important in the binding of nucleosides and nucleoside analogues.

Although yeast has been used as a reliable host for assaying the functional activity of human transporters, we confirmed in mammalian cells the reduction in hENT1 transporter activity due to mutations at glycine 179. Stable cell lines were created by transfecting MDCK cells with vectors expressing hENT1, G179A, or G179L mutants fused to yellow fluorescent protein (YFP). The cellular distribution of hENT1, G179A, or G179L mutants was identical with all the transporters showing a predominantly plasma membrane localization (Figure 8, panel A). However, as was observed in yeast, the transport activity of G179A mutant was significantly lower than the wild type and the G179L

mutant failed to show any significant transporter activity (Figure 8, panel B). These data show that localization and transport activity of hENT1 mutants at glycine 179 position behave similarly whether expressed in yeast or mammalian cells.

Several pieces of evidence presented above support our conclusion that glycine 179 is critical to the transport of nucleosides across plasma membranes by hENT1. First, the fact that this amino acid is conserved across 15 equilibrative nucleoside transporters of different origin indicates that it fulfills a crucial role in the function of these transporters. Arguably, this conservation of glycine 179 could be for other than transporter activity such as for the correct targeting and folding of the transporter. However, our data showing that the localization of glycine 179 mutant proteins at the plasma membrane of yeast and MDCK cells was similar to that of the wild-type protein clearly indicate that this glycine is not critical for targeting the transporter to the plasma membrane. Nevertheless, we cannot discount the possibility that glycine 179 may have an indirect but critical role in the permeation pathway of hENT1, such as through a subtle effect on protein folding. Clearly, our data in MDCK cells show that these effects of point mutations at glycine 179 on the activity and localization of the transporter are independent of whether the host expression system is yeast or mammalian cells. Finally, our inhibition data show that glycine 179 is critical to binding of the nucleoside analogue, NBMPR, to the transporter. Uridine uptake by G179A hENT1 transporter was less sensitive to inhibition by NBMPR when compared with the wild-type transporter. The exact mechanism(s) by which glycine 179 interacts with nucleosides or NBMPR is (are) yet to be elucidated. We speculate from a predicted helical wheel map of TMD 5 (constructed using normal helical parameters) that NBMPR, a hydrophobic nucleoside analogue, interacts with glycine 179, as this residue is located in a hydrophobic area of this TMD with other nonpolar residues (e.g., A183, F186, A190) clustered in its vicinity. If, as our data suggest, glycine 179 is important in determining the inhibitory capacity of NBMPR, glycine 179 must make critical contact with NBMPR, or this residue must affect the interaction of NBMPR with the neighboring hydrophobic residues. Likewise, substitution of glycine 179 with other amino acids of longer side chains (such as leucine, cysteine, or valine) likely affects the binding of natural nucleosides to this residue or indirectly affects the permeation pathway of the nucleoside. To test the possibility that the effect of glycine 179 mutations was due to an indirect steric effect on binding of uridine or NBMPR to either or both of the flanking polar residues, serine 178 and glutamine 180, we mutated these residues individually to cysteine, a nonpolar amino acid. Both of these mutations exhibited sensitivity to 5-fluorouridine and a [^{3}H]uridine transporter activity comparable to the wild-type protein (data not shown). These data suggest that the two polar residues flanking glycine 179 are not critical to nucleoside transporter activity and mutations at glycine 179 do not produce their effect due to steric effects on these flanking residues. In contrast to mutations at glycine 179, mutations at glycine 184 appear to affect localization of hENT1 at the plasma membrane. For the nucleoside transporter of *Leishmania donovani*, LdNT1.1, this glycine residue (position 183 for LdNT1.1) has been shown to confer substrate specificity (*16*). Notably, glycine residues have been implicated in substrate binding in several proteins with transmembrane domains. For example, glycine 313 in hCNT2 is known to contribute to the nucleoside transport specificity of this transporter (*17*). In summary, we conclude that glycine 179 is critical to the function of hENT1, while glycine 184 is important in the proper targeting and distribution of hENT1 to the plasma membrane when hENT1 is expressed in yeast. This is the first set of studies to identify and characterize a critical amino acid residue of hENT1 that is important in both transporter activity and sensitivity to inhibition by NBMPR.

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