The first mammalian examples of the equilibrative nucleoside transporter family to be characterized, hENT1 and hENT2, were passive transporters located predominantly in the plasma membranes of human cells. We now report the functional characterization of members of a third subgroup of the family, from human and mouse, which differ profoundly in their properties from previously characterized mammalian nucleoside transporters. The 475-residue human and mouse proteins, designated hENT3 and mENT3, respectively, are 73% identical in amino acid sequence and possess long N-terminal hydrophilic domains that bear typical (DE)XXX(L/L) endosomal/lysosomal targeting motifs. ENT3 transcripts and proteins are widely distributed in human and rodent tissues, with a particular abundance in placenta. However, in contrast to ENT1 and ENT2, the endogenous and green fluorescent protein-tagged forms of the full-length hENT3 protein were found to be predominantly intracellular proteins that co-localized, in part, with lysosomal markers in cultured human cells. Truncation of the hydrophilic N-terminal region or mutation of its dileucine motif to alanine caused the protein to be relocated to the cell surface both in human cells and in Xenopus oocytes, allowing characterization of its transport activity in the latter. The protein proved to be a broad selectivity, low affinity nucleoside transporter that could also transport adenine. Transport activity was relatively insensitive to the classical nucleoside transport inhibitors nitrobenzylthioinosine, diprydamole, and dilazep and was sodium ion-independent. However, it was strongly dependent upon pH, and the optimum pH value of 5.5 probably reflected the location of the transporter in acidic, intracellular compartments.

Nucleoside and nucleobase transporters play key roles in the uptake of precursors for nucleotide synthesis by salvage pathways in a number of cell types, in particular in the bone marrow and brain (1). They are similarly required for the efficient cellular uptake of hydrophilic anticancer and antiviral nucleoside drugs such as gemcitabine and zidovudine (3’-azido-3’-deoxythymidine, AZT) (2, 3). By regulating the concentration of adenosine available to cell surface purinoreceptors, transporters also influence many physiological processes, including coronary blood flow, inflammation, and neurotransmission (4, 5).

Nucleoside transport processes in mammalian cells are mediated by two families of unrelated nucleoside transporter proteins. Active, sodium-dependent nucleoside transport is found primarily in specialized epithelial tissues such as small intestine, kidney, and liver and is mediated by members of the concentrative nucleoside transporter family, also classified as the SLC28 family (6, 7). In contrast, passive nucleoside transport processes are almost ubiquitous and are mediated by members of the equilibrative nucleoside transporter (ENT) family, also classified as the SLC29 family (5, 6). This family is widely distributed in eukaryotes and, despite its name, includes examples of active, proton-linked transporters, such as those of the kinetoplastid protozoa (8). Family members are predicted to share a common topology of 11 transmembrane (TM) α-helices, with a cytoplasmic N terminus and extracellular C terminus, and typically possess a large cytoplasmic loop linking TM6 and -7 (9). Direct experimental evidence for this topology has been obtained in the case of the archetypal family member hENT1 (10).

The human and rodent genomes encode four ENT isoforms, designated ENT1–4 (5). The best characterized of these isoforms, ENT1 and ENT2, are broad selectivity equilibrative nucleoside transporters that have been classified, on the basis of their sensitivity to inhibition by nitrobenzylthioninosine (nitrobenzylmercaptopurine riboside; NBMPR), as es (equilibriative-sensitive) or ei (equilibriative-insensitive), respectively (6). The two human isoforms also differ in their sensitivities to inhibition by coronary vasodilators such as diprydamole, dilazep, and drafazine, with hENT1 being ~100–1000-fold more sensitive than hENT2 (5, 11). Although the transporters exhibit similar selectivities for natural nucleosides, hENT2 dif-

1 The abbreviations used are: AZT (zidovudine), 3’-azido-3’-deoxythymidine; ENT, equilibrative nucleoside transporter; hENT, human ENT; mENT, mouse ENT; GFP, green fluorescent protein; NBMPR, nitrobenzylthioninosine (6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine); PBS, phosphate-buffered saline; TM, putative transmembrane helix; TRITC, tetramethylrhodamine isothiocyanate.
fers from hENT1 in that it transports antiviral 3'-deoxynucleosides, in particular AZT (12), and a wide range of purine and pyrimidine nucleobases (13, 14).

We have previously reported the cloning of cDNAs encoding mouse and human ENT3 (9), and a fourth human ENT isoform, hENT4, has been identified by genome data base analysis (15). Interestingly, the latter has recently been characterized as a low affinity monamine, rather than a nucleoside, transporter and has been alternatively designated plasma membrane monamine transporter, PMAT (16), although we have shown that the mouse homologue is in fact capable of adenosine transport (5). In contrast to hENT4, the functional properties of hENT3 have not yet been described. In light of the presence of a long (51 residues), hydrophilic N-terminal region preceding TM1, which possesses a putative dileucine-based targeting motif, we previously suggested that hENT3 might function intracellularly, as is the case for the yeast homologue FUN26 (9, 17). In the present study we have confirmed this hypothesis by examining the effect of mutating the targeting motif on the subcellular location of hENT3 and have shown that the transporter is indeed intracellular and partially colocalizes with lysosomal markers. Relocation of the hENT3 to the cell surface in the absence of the motif has allowed detailed characterization of its functional properties when expressed in *Xenopus* oocytes.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors Encoding hENT3 and mENT3**—We have previously reported the identification and sequencing of cDNA clones encoding hENT3 (GenBank™ accession code AF326887) and mENT3 (GenBank™ accession code AF326896) (9). For *Xenopus* expression of the latter, a 2055-bp region of mENT3 cDNA (nucleotides 31–2085) was amplified using primers bearing 5'-EcoRI and HindIII sites, respectively, and subcloned using these sites into the *Xenopus* expression vector pGEMHE (18). The resultant construct, pGEMHE-mENT3, contained the entire coding region of the mENT3 cDNA flanked by 29 bp of untranslated 5'-nucleotide sequence and 598 bp of untranslated 3'-sequence. A construct (pGEMHE-mENT3AA) encoding a mutant in which the putative dileucine motif residues Leu-31 and Leu-32 were mutated to alanine was produced from pGEMHE-mENT3 using the QuikChange method (Stratagene). To generate an expression construct lacking the putative dileucine motif and directing expression of a frame shift product, a 2141-bp segment containing residues 1868–2085 of the mENT3 cDNA was amplified using primers bearing 5'-EcoRI and HindIII sites, respectively, and subcloned using these sites into the *Xenopus* expression vector pGEMHE. The forward primer used for this amplification, 5'-GCTTGGATCCATATGAGATCCATATAAACGGATCGG-3', also included a Kozak translation initiation sequence and an ATG initiation codon (underlined). The resultant construct, pGEMHE-mENT3A3N, encoded residues 37–475 of mENT3 plus 598 bp of untranslated 3'-sequence. For *Xenopus* expression of hENT3, a 1433-bp region of hENT3 cDNA (nucleotides 1–1433) was amplified using primers bearing 5'-EcoRI and HindIII sites, respectively, and subcloned using these sites into pGEMHE. The resultant construct, pGEMHE-hENT3, contained the entire coding region of hENT3 cDNA plus 5 bp of untranslated 5'-nucleotide sequence. A construct (pGEMHE-hENT3AA) encoding a mutant in which the putative dileucine motif residues Leu-31 and Leu-32 were mutated to alanine was produced from pGEMHE-hENT3 using the QuikChange method (Stratagene). To generate an expression construct lacking the putative dileucine motif and directing expression of a frame shift product, a 1918-bp segment containing residues 168–1918 of the hENT3 cDNA was amplified using primers bearing 5'-EcoRI and HindIII sites, respectively, and subcloned using these sites into the *Xenopus* expression vector pGEMHE. The resultant construct, pGEMHE-hENT3A3N, and pGEMHE-hENT3A3N containing insertions into pEGFP-C1 that had been restricted using the same enzymes, yielding constructs pSF4, pSF5, and pKB1, respectively. For expression of the wild-type hENT3 and the dialanine mutant in GFP-tagged form in *Xenopus* oocytes, AgeI/XbaI fragments of pSF4 and pSF5 containing the complete coding regions of the GFP/hENT3 fusions, were subcloned into pGEMHE that had been restricted with XmaI and XbaI, yielding the constructs pSF6 and pSF7, respectively.

**Transient Transfection of *HeLa* Cells**—Cells grown on coverslips in 6-well plates were transfected with 1 µg of GFP-hENT3 constructs using Lipofectamine™ (Invitrogen) according to the manufacturer’s instructions. After 48 h, the post-transfection cells were fixed for 20 min in 4% paraformaldehyde, washed twice with phosphate-buffered saline, and mounted in Vectashield® medium.

To examine their subcellular distributions when expressed in cultured cells, the coding regions of wild-type hENT3 or of mutant transporters were inserted into the green fluorescent protein (GFP) fusion vector pEGFP-C1 (Clontech). The resultant constructs expressed transporters with the GFP moiety fused to their N termini. For expression of wild-type protein, the L31A,L32A mutant, and the N-terminal-truncated protein, Smal/XbaI fragments of pGEMHE-hENT3, pGEMHE-hENT3AA, and pGEMHE-hENT3A3N were inserted into pEGFP-C1 that had been restricted using the same enzymes, yielding constructs pSF4, pSF5, and pKB1, respectively. For expression of the wild-type hENT3 and the dialanine mutant in GFP-tagged form in *Xenopus* oocytes, AgeI/XbaI fragments of pSF4 and pSF5, containing the complete coding regions of the GFP/hENT3 fusions, were subcloned into pGEMHE that had been restricted with XmaI and XbaI, yielding the constructs pSF6 and pSF7, respectively.

**Tissue Distribution of hENT3 mRNA**—Using Northern blotting techniques with 32P-labeled antisense RNA probes corresponding to residues 267–285 of hENT3 and residues 276–294 of hENT3 that had been preincubated for 2 h with a 2-fold excess by weight of synthetic peptide. The subcellular distributions of endogenous hENT3 and of heterologously expressed GFP fusion proteins were assessed by simultaneously incubating cells with mouse monoclonal antibodies against CD63 (2 µg/ml, Serotec) or β-1,4-galactosyltransferase (GLT2, 1 µg/ml) (20) or with affinity-purified sheep antibodies against TGN46 (1 µg/ml) (20). After subsequent washing with PBS, cells were incubated for 1 h with a goat anti-rabbit IgG fluorescein isothiocyanate conjugate plus goat anti-mouse IgG tetramethylrhodamine isothiocyanate conjugate or goat anti-mouse IgG tetramethylrhodamine isothiocyanate (TRITC) conjugate of mouse monoclonal 0KTA antibodies (20) against the transferrin receptor, as appropriate, in antibody buffer. After being washed twice in PBS, cells were mounted in Vectashield® medium. For imaging, 20 optical sections were captured at 0.2-µm intervals with a Delta Vision® system (Applied Precision, Issaquah, WA) comprising an Olympus 1×70 microscope linked to a charge-coupled device camera and plate reader, using the maximum intensity projection software Vision® system (Applied Precision, Issaquah, WA) comprising an Olympus 1×70 microscope linked to a charge-coupled device camera and plate reader, using the maximum intensity projection software.
ENT3 proteins represent a distinct subfamily of the vertebrate equilibrative nucleoside transporters. A, phylogenetic tree constructed from a multiple alignment of the following Homo sapiens (h), Mus musculus (m), Rattus norvegicus (r), Gallus gallus (g), Fugu rubripes (f), Xenopus laevis (x), and Ciona intestinalis (c) sequences using ClustalX version 1.83 (35) and the neighbor-joining method of Saitou and Nei (36): hENT1 (gi: 1845045), mENT1 (gi: 85658088), rENT1 (gi: 2656137), gENT1 (gi: 38014788), hENT2 (gi: 2754821), mENT2 (gi: 85658092), rENT2 (gi: 2656139), fENT2 (predicted from F. rubripes genome scaffold_967, nucleotides 58710–61475), hENT3 (gi: 12656639), mENT3 (gi: 12656637), rENT3 (gi: 31745142), cENT (predicted from F. rubripes genome scaffold_1216, nucleotides 41822–39991), hENT4 (gi: 25418480), mENT4 (gi: 2212849), cENT (C. intestinalis genome data base accession ci0100132971). The three chicken (Gallus gallus) ENT sequences were predicted using a combination of GENSCAN predictions from the ENSEMBL chicken genome data base (37) and expressed sequence tag sequences. B, aligned N-terminal regions of vertebrate ENT isoforms showing the predicted locations of the first transmembrane region (TM1) and the dileucine-containing lysosomal targeting motif uniquely found in the ENT3 sequences. Black boxes indicate residues conserved in all the sequences and the characteristic acidic and hydrophobic positions within the motif. Protein sequences are as indicated in panel A, except for the Sus scrofa (s) amino acid sequence, which was predicted from a 5'-EST nucleotide sequence (gi: 14198595).

RESULTS

Amino Acid Sequences of hENT3 and mENT3—The human and mouse genomes each contain four ENT family genes, designated ENT1–4 (5, 15). The 475-residue hENT3 and mENT3 proteins were found to be 73% identical in amino acid sequence and exhibited between 22 and 33% identity to the other three human and mouse ENT isoforms. Although only a single ENT isoform has so far been identified in the genome of the primitive chordate Ciona intestinalis (Fig. 1A), genes encoding close homologues (51–60% amino acid sequence identity) of the two ENT3 proteins are also present in the chicken and pufferfish genomes, indicating that divergence of the ENT1, ENT2, and ENT3 isoforms was an early event in vertebrate evolution (Fig. 1A). Divergence of the ENT4 isoforms from the other three mammalian isoforms appears to predate divergence of the vertebrate ENT family and are predicted to adopt the same 11-TM topology as the other mammalian isoforms, with a cytoplasmic N terminus and extracellular C terminus (9). However, they differ from the other mammalian isoforms in possessing a typical (DE)XXX(L/I) endosomal/lysosomal targeting motif (22) in the hydrophilic region of the sequence that precedes the first TM region. A significant function for this motif is suggested by its conservation in ENT3 sequences from pig and chicken (Fig. 1B). A dileucine motif is also found at the same position in the ENT3 homologue from the pufferfish Fugu rubripes, although this is separated from the upstream aspartic residue by 6 rather than 3 other residues (Fig. 1B).

The presence of a putative lysosomal targeting motif suggested that ENT3 might function as an intracellular rather than as a cell surface transporter. To investigate this hypothesis, antipeptide antibodies against hENT3 were used to examine the distribution of the endogenous transporter in HeLa cells, which preliminary experiments had shown to contain hENT3 transcripts. On Western blots of cell lysates the affinity-purified antibodies stained a major band of apparent molecular mass ~60 kDa (supplemental Fig. S1A), consistent with an N-glycosylated form of the transporter (predicted protein mass 51.9 kDa). Additional bands of higher and lower molecular mass probably represented oligomeric and non-glycosylated precursor forms of the transporter, respectively, as we have previously described for rENT1 (23). None of these bands was stained by nonspecific rabbit IgG (supplemental Fig. S1B).

Treatment of fixed, permeabilized HeLa cells with affinity-purified anti-hENT3276–294 yielded a punctate intracellular staining pattern with no discernable cell surface staining (Fig. 2A). The specificity of the staining was confirmed by the profound reduction in intensity resulting from preincubation of the antibodies with synthetic peptide (Fig. 2B). The pattern of staining did not coincide with that obtained using antibodies against Golgi or endoplasmic reticulum markers (data not shown), but some co-localization with the late endosomal/lysosomal marker CD63 (24) was observed (Fig. 2A). HeLa cells...
transiently transfected with an expression construct encoding GFP-tagged hENT3 exhibited a similar pattern of punctate, intracellular GFP fluorescence with no co-localization with early endosomal (Fig. 2C), trans-Golgi network (Fig. 2D), or trans-Golgi (Fig. 2E) markers and substantial co-localization with the lysosomal marker CD63 (Fig. 2F). Each image corresponds to one representative deconvolved optical section. Scale bar, 10 μm.

To examine the role of the N-terminal dileucine motif in the subcellular distribution of the transporters, a comparison was made of the distribution of GFP fusion proteins bearing wild-type hENT3 (GFP/hENT3), hENT3 lacking the first 36 residues of the N-terminal region (GFP/hENT3ΔN), or hENT3 in which the dileucine motif at positions 31 and 32 had been replaced by alanine residues (GFP/hENT3AA). In contrast to the intracellular distribution of fluorescence seen in HeLa cells expressing GFP/hENT3 (Fig. 3A), substantial cell surface fluorescence was evident in cells expressing either GFP/hENT3ΔN or GFP/hENT3AA (Fig. 3B and C). Similarly, although only intracellular fluorescence was evident in Xenopus oocytes that had been injected with RNA transcripts encoding GFP/hENT3 (Fig. 3D), strong cell surface fluorescence was evident in oocytes injected with transcripts encoding GFP/hENT3AA (Fig. 3E). Oocytes injected with water alone showed no fluorescence (Fig. 3F).

Functional Expression and Permeant Selectivity of Recombinant hENT3 and mENT3—The experimental conditions chosen for assessment of the transport activity of the ENT3 proteins reflected the predominantly intracellular distribution of wild-type hENT3 and its partial co-localization with the acidic late endosome/lysosome compartments (25). Transport experiments were performed in Xenopus oocytes both at physiological (7.5) and acidic (5.5) pH, using both the wild-type proteins and mutants lacking the putative intracellular targeting motif. At pH 7.5 the transport activities of the wild-type and mutant human (Fig. 4) and mouse proteins (supplemental Fig. S2) were extremely low, such that the rates of adenosine uptake in RNA-injected oocytes were essentially equal to those seen in oocytes injected with water alone. In the case of wild-type mENT3, a similarly low activity was apparent at pH 5.5 (supplemental Fig. S2), but for hENT3 the rate of uptake was substantially greater than at pH 7.5 (Fig. 4). For both proteins, still larger rates of adenosine uptake were seen when the putative intracellular targeting signal was removed either by N-terminal truncation of the protein or mutation of the leucine residues of the motif to alanine (Fig. 4 and supplemental Fig. S2). The greatest activity was seen for the hENT3AA mutant, and this activity was essentially identical for the non-tagged form of the protein (Fig. 4) and for the form of the protein bearing an N-terminal GFP tag (GFP/hENT3AA, data not shown). The non-tagged form of the mutant was therefore used for further characterization of the pH dependence of the transporter. In the experiment of Fig. 5, adenosine uptake in oocytes producing the non-tagged form of hENT3 exhibited a strong dependence on pH; maximal activity was seen at pH 5.5, whereas no significant transport activity was apparent at pH 8.0 and above. In contrast to this effect of proton concentration, no dependence of transport on sodium ions was detectable.
either for the wild-type or for the two mutant forms of hENT3, adenosine uptake rates measured in sodium-free buffer containing choline chloride being similar to those measured in sodium-containing buffer (Fig. 4).

Because hENT3AA yielded the highest transport activity, this form of the transporter was used for subsequent investigations of permeant and inhibitor selectivity, all of which were performed at the optimal pH value of 5.5. Under these conditions, the uptake of adenosine was essentially linear for at least 10 min (Fig. 6A), and a period of 5 min was therefore used to estimate initial rates of permeant uptake in subsequent experiments. The permeant selectivity of the transporter was assessed by measuring mediated uptake of natural purine and pyrimidine ribonucleosides and nucleobases. The results shown in Fig. 7A demonstrate that hENT3 resembled hENT1 and hENT2 in possessing a broad permeant selectivity for nucleosides. Like ENT2, it also exhibited the ability to transport adenine but, unlike ENT2, did not transport hypoxanthine. The hENT3AA-mediated influxes of adenosine and uridine, defined as the difference in uptake between RNA-injected and water-injected oocytes, were saturable and conformed to simple Michaelis-Menten kinetics (Fig. 6, B and C) with apparent $K_m$ values, respectively, of 1.86 ± 0.35 and 2.02 ± 0.37 mM.
The $V_{\text{max}}$ value for adenosine transport, $263 \pm 18 \text{ pmol/oocyte-5 min}^{-1}$, was ~2-fold greater than that for uridine transport, $140 \pm 10 \text{ pmol/oocyte-5 min}^{-1}$.

The broad permeant selectivity of hENT3AA included a structurally diverse group of nucleoside analog drugs, including three used in cancer chemotherapy (Fig. 7B). The adenosine analogs cladribine (2-chloro-2'-deoxyadenosine), cordycepin (3'-deoxyadenosine), and tubercidin (7-deazaadenosine) were as efficiently transported as adenosine when tested at a concentration of $20 \mu M$, whereas fludarabine (9-D-arabinosyl-2-fluoroadenine), which differs from adenosine in its configuration at the 2'-hydroxyl position in addition to bearing a fluorine-substituted adenine ring, was a less efficient permeant than adenosine. Mediated transport of pyrimidine nucleoside analogs such as zebularine (2-pyrimidine-1-D-riboside) was also apparent, although for some, such as the anticancer drug gemcitabine (2',2'-difluorodeoxycytidine), the apparent transport efficiency was substantially less than that for the corresponding natural pyrimidine (Fig. 7, compare panels B and A).

The antiviral purine and pyrimidine nucleoside analogs 2',3'-dideoxynosine, 2',3'-dideoxycytidine, and, in particular, AZT were also efficiently transported, whereas the acyclic guanosine analog ganciclovir (9-(1,3-dihydroxy-2-propoxymethyl)guanine), used for treatment of cytomegalovirus infections, was a poor permeant (Fig. 7C).

**Interaction of hENT3AA with Nucleoside Transport Inhibitors**—As described in the Introduction, hENT1 is potently inhibited by the inosine analog NBMPR, with a $K_i$ of $5 \text{nM}$ (5), and by the coronary vasodilators dipyridamole and dilazep, which exhibit $K_i$ values of 48 and 19 nM, respectively (11). hENT2 is much less potently inhibited, with $K_i$ values for NBMPR, dipyridamole, and dilazep of $>1, 6.2, \text{ and } 134 \mu M$, respectively (11, 26).

Fig. 8 shows that hENT3AA-mediated transport of adenosine was unaffected when these compounds were used at 1 $\mu M$ and was only partially inhibited at 10 $\mu M$, with the maximum inhibition of 70% being achieved with dipyridamole.
**DISCUSSION**

Mammalian ENT1, ENT2 and ENT4 proteins are thought to be primarily located at the cell surface, although some ENT1 has also been detected in liver mitochondria (27). Both ENT1 and ENT2 have been identified in the nuclear envelope of cultured cells (28). In contrast, in the present study we have shown that the endogenous hENT3 proteins of cultured HeLa cells are predominantly located in intracellular membranes, with little or none of the transporter present at the cell surface (Fig. 2). Examination of the subcellular distribution of a GFP-tagged form of the human protein expressed in oocytes and/or in HeLa cells confirmed the intracellular location of the protein (Figs. 2 and 3). Lack of cell surface targeting probably contributed to the very low or negligible transport activities observed for the human and mouse ENT3 proteins, respectively, when these were heterologously produced in Xenopus oocytes (Fig. 4 and supplemental Fig. S2). Substantial co-localization was found between the lysosomal marker CD63 and GFP-tagged hENT3 in HeLa cells, whereas partial co-localization was apparent between the lysosomal marker and endogenous hENT3 (Fig. 2). No co-localization was seen between hENT3 and the Golgi, trans-Golgi network, or endoplasmic reticulum. It is therefore likely that the wild-type transporter functions, at least in part, in the transport of nucleosides across lysosomal membranes. Studies on isolated lysosomes have previously revealed a low affinity uptake system for nucleosides in such membranes (29). Its likely physiological function is the release of nucleosides produced by nucleic acid breakdown in the lysosomal interior; lysosomes have been shown to be the main site for basal and induced cytoplasmic RNA degradation in liver (30).

In contrast to the other equilibrative nucleoside transporter isoforms, the ENT3 proteins of human, mouse, rat, pig, and chicken all contain a typical (DEXXX/LL) endosomal/lysosomal targeting motif (22). The importance of this motif in determining the intracellular targeting of ENT3 was revealed by the consequences of mutating its 2 leucine residues to alanine or of removing the motif in its entirety by truncation of the protein N terminus. For both the human and mouse proteins, these changes substantially increased the rate of adenosine uptake into Xenopus oocytes injected with transcripts encoding the mutants (Fig. 4 and supplemental Fig. S2). In the case of hENT3, replacement of the dileucine motif by alanine residues was also shown to result in the relocation of a GFP-tagged form of the transporter from the oocyte and HeLa cell interior to the plasma membrane (Fig. 3).

Deletion or mutation of the dileucine motif in hENT3, which resulted in localization of the transporter at the oocyte surface, enabled a detailed characterization to be made of its permeant and inhibitor selectivity. Interestingly, the transporter activity exhibited a strong dependence on pH, optimal uptake into oocytes occurring from an extracellular medium buffered at pH 5.5 (Fig. 5). It is unclear whether such pH dependence reflects proton-nucleoside co-transport activity, such as is seen for kine-toplastid ENT family members (8). However, the optimum pH value corresponds to that of late endosomes/lysosomes and probably reflects an evolutionary adaptation to the acidic interior of these organelles: lysosomes are known to contain other proton-linked solute exporters, such as amino acid transporter LYAAT1/PAT1 (31). Previous studies of adenosine uptake into

**FIG. 8.** Inhibition of hENT3AA-mediated adenosine influx by NBMPR, dipyridamole, and dilazep. Uptake of 14C-labeled adenosine (20 μM, 20°C, 5 min) in oocytes injected with the hENT3AA RNA transcripts or water alone (control, water) of the indicated inhibitors for 1 h prior to addition of permeant.

**FIG. 9.** Tissue distribution of ENT3. Samples (100 μg) of membrane fractions prepared from rat tissues were resolved by SDS/10% (w/v) polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membranes, and stained with affinity-purified anti-mENT3237-255. The mobilities of standard proteins of known molecular mass are indicated on the left.
isolated rat liver lysosomes showed no similar dependence on the pH of the medium (29), but the dependence of activity on the intraorganelle pH was not studied. In the present studies, the extracellular medium was topologically equivalent to the lysosomal interior.

Characterization of the transport activity of hENT3AA at its optimum pH of 5.5 revealed that the transporter had low affinities for adenosine and uridine, the measured $K_m$ values for these permeants being, respectively, ~40- and 10-fold greater than those previously reported for hENT1 (32, 33). Although the kinetic properties of nucleoside efflux from lysosomes have not been reported, these low apparent affinities are consistent with the $K_m$ value of 9 mM reported for uptake of adenosine into isolated lysosomes (29). Transport mediated by hENT3AA also resembled that reported for lysosomes in that it was much less sensitive to inhibition by NBMPR and coronary vasodilator drugs than is the case for the plasma membrane transporter hENT1 (Fig. 8) (29). Another striking difference between hENT1 and hENT3AA was that the latter efficiently transported 3’-deoxyxynucleosides. For example, at a concentration of 20 μM, the rate of AZT uptake was similar to those of thymidine and uridine (Fig. 7, A and C). In contrast, we have previously shown that, when tested in oocytes at the same permeant concentration, hENT1 was unable to transport AZT, whereas the rate of AZT uptake mediated by hENT2 was only about one third that of uridine (12). Thus, the 3’-hydroxyl group is unlikely to be involved in interaction between nucleoside permeants and hENT3.

In conclusion, we have characterized novel members of the ENT family that appear to function intracellularly rather than at the plasma membrane and may be involved in the export of nucleosides from the lysosomal interior. Although the transporters exhibit an acidic pH optimum, further investigations will be required to establish whether protons are co-transported with the nucleoside permeant as is the case for some other lysosomal transporters. We have so far been unable to detect proton fluxes by electrophysiological approaches in oocytes expressing hENT3AA (data not shown). Similarly, the physiological roles of the transporter remain to be established by gene knock out or other approaches; the importance of other lysosomal transporters is evident from investigations of mutations in lysosomal cystine and sialic acid transporter genes, which cause nephropathic cystinosis and Salla disease, respectively (34). It is currently unclear whether the ability of hENT3 to transport the antileukemic drugs fludarabine and cladribine or antiviral 3’-deoxyxynucleoside analogs is of clinical relevance, but further investigations of structure-function relationships in the transporters may throw more light on the mechanisms by which these drugs interact with nucleoside transporters per se and thus assist in the development of improved therapies.

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