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Lysine Uptake by Cloned hCAT-2B: Comparison with hCAT-1 and with Trophoblast Surface Membranes

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Abstract. To study the cationic amino-acid transporter hCAT-2B of human placenta, total RNA was harvested from primary cultured trophoblast and from the BeWo choriocarcinoma cell line (b30 clone) and used for reverse transcription (RT) and polymerase chain reaction (PCR). Primers based on published sequences identified expression of mRNA for hCAT-2B. RT-PCR yielded a 2.06 kb hCAT-2B cDNA, which was cloned. hCAT-2B cRNA injection into *Xenopus laevis* oocytes stimulated saturable lysine uptake ($K_m \sim 125$ μM). In the presence of Na^+, uptake was completely inhibited by L-arginine but only partially by neutral amino acids. To compare directly the interaction of hCAT-1 and hCAT-2B with neutral amino acids and sodium, we examined the inhibition of these transporters by L-leucine and L-alanine over a wide concentration range. L-Alanine and L-leucine inhibit uptake by hCAT-2B substantially less completely than uptake by hCAT-1. The interaction of hCAT-2B resembles that of system y^+ in the microvillous membrane of human placenta, while that of hCAT-1 is more comparable to that of system y^+ in basal membrane. The identification and characterization of the various cationic amino-acid transporters of the human placenta have the potential to increase the understanding of the cellular mechanism of transplacental transfer.

Key words: Fetal nutrition — System y^+ — Placenta — *Xenopus laevis* oocytes — Amino-acid transporters — Kinetics — Cationic amino-acid transport

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Introduction

Fetal growth and development depends on the continuous transfer of amino acids to the fetal blood by the placental syncytiotrophoblast [28]. Cationic amino acids are more concentrated in fetal than in maternal blood and the lysine concentration ratio is approximately 3:1 [27].

The movement of cationic amino acids across the microvillous (maternal-facing) and basal (fetal-facing) plasma membranes is mediated by a group of sodium-independent mechanisms [13, 14, 17, 19, 30–32]. In human placental membranes and related cell culture models, we and others have identified the high-capacity system y^+ and the low-capacity, high-affinity systems $b^{0,+}$ and y^+L [15–19]. Functional differences in the processes by which cationic amino acids interact with these two membranes are likely to provide the basis for vectorial maternal/fetal transfer. In particular, the differential interaction of the high-capacity system y^+ transport mechanisms of these membranes with neutral amino acids in the presence of sodium may be expected to enhance uptake at the microvillous membrane and facilitate transplacental transfer to the fetus [18].

Recently we identified three hCAT cDNAs [21] in trophoblast directly cultured from human placenta and the analogous b30 BeWo choriocarcinoma cell line [34, 36]. These cDNAs code for system y^+ transporters, which have the potential to transport cationic amino acids in a manner similar to human placenta [20]. We characterized the transport of hCAT-1 *Xenopus laevis* oocytes and observed that its characteristics were similar to transport by isolated placental basal membrane [23].

We now describe hCAT-2B-stimulated uptake in *X. laevis* oocytes and compare it to the transport activity of hCAT-1 and that of isolated placental membranes. This investigation was designed to pro-

vide information on the polarity and characteristics of the hCAT isoforms and their function in trans-syncytial transport.

Materials and Methods

Materials

L-[^{3}H]Lysine (specific activity 87.4 Ci/mmol) and L-[^{3}H] alanine (specific activity 66 Ci/mmol) were obtained from NEN-DuPont (Boston, MA). Opti-Fluor was from Packard Instruments (Downers Grove, IL). Chemically defined fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, Utah). Essential ammo acids were obtained from Hazleton Biologics (Lenexa, KS). Minimal essential medium, vitamins and DH5 α cells were from Gibco BRL (Gaithersburg, MD). Gentamicin and nonessential amino acids were from the Washington University Tissue Culture Support Center. ABI Prism Dye (or Big Dye) Terminator Cycle Sequencing Ready Reaction Kit were from Perkin Elmer (Foster City, CA). PGEM-T Easy vector and restriction enzymes were from Promega Corporation (Madison, WI). MMessage-Mmachine kit was from Ambion (Austin, TX). Nucleotide primers were synthesized by Oligos Etc. (Wilsonville, OR). Collagenase (Type 1) was obtained from Worthington Biochemical (Freehold, NJ) and other chemicals were from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI). *Xenopus laevis* frogs were purchased from *Xenopus* I (Ann Arbor, MI). The Washington University School of Medicine Review Boards have approved all human and animal studies.

Cell Preparation and Culture

The b30 BeWo clone was derived from the original parent BeWo line by limiting dilution [36]. Cells were maintained in 25-cm^2 flasks and cultured in MEMB media [20, 25] containing 10% FBS, gentamicin (0.5 mg/ml) and penicillin (100 units/ml)/streptomycin (100 mg/ml).

Cellular trophoblasts were isolated by trypsin digestion followed by separation on a Percoll gradient as described previously [20]. Cells were immediately frozen in 10% dimethyl sulfoxide after isolation and stored in liquid nitrogen for later culture [20].

Cloning of hCAT-2B

The hCAT-2B sequence in BeWo and trophoblast cells was identified in our previous publication [23]. This fragment has a unique *Bam*H I site (nt 1257), which is present only in isoform 2B and not in 2A [10, 23].

A 2.06 kb hCAT-2B cDNA encompassing the entire coding sequence was cloned from BeWo cells essentially as described previously for hCAT-1 [23]. Total RNA, isolated from BeWo cells cultured for 24 hours, was subjected to reverse transcription (RT) using random hexamers and then to polymerase chain reaction (PCR). PCR was performed using an annealing temperature of 57.7°C and a primer set of a forward primer: nt 119–137 (GTCGAGGGTTTATTGGAAC) and reverse primer: nt 2178–2158 (CCTGCAAGTGTTAGAATTCAC) [10]. The use of two other sets of primers for the same sequence regions (set 1 forward primer: nt 126–144 and reverse primer: nt 2185–2165 and set 2 forward primer: nt 125–143 and reverse primer: nt 2184–2165) with the same and alternative PCR protocols resulted in an hCAT-2B cDNA of the same size. However, yields were markedly poorer, as seen in lanes 1 and 2 in Fig. 1*A* for the PCR protocol described above.

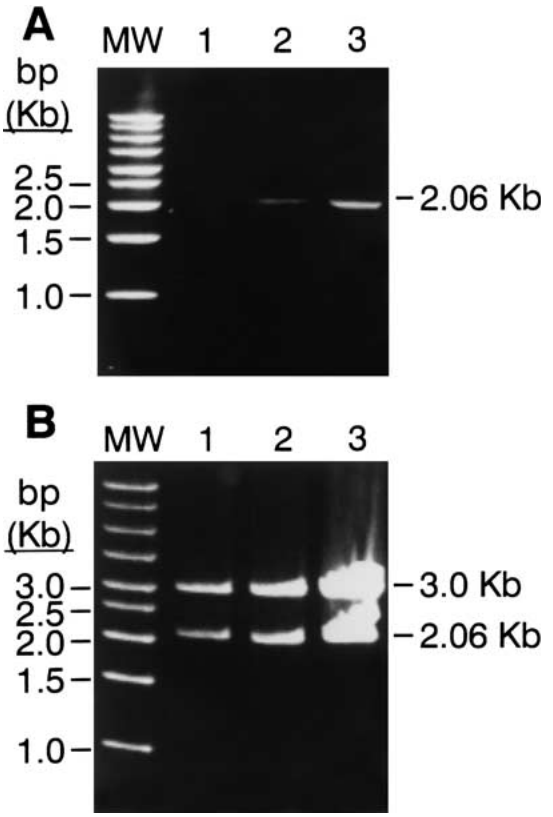

Fig. 1. Cloning of hCAT-2B. (*A*) Reverse Transcription (RT)-PCR product representing a full-length human cationic amino-acid transporter (hCAT-2B) from BeWo RNA using various sets of PCR primers (lane *1*: 126–2185, lane *2*: 125–2184, and lane 3: 119–2178; *see* Methods and Materials). The major 2.06 kb product (from lane *3*), was subjected to gel purification for cloning. (*B*) Gel electrophoresis of cloned hCAT-2B after *Eco*R I digestion. Lanes *1* through *3* show representative clones. The bands visualized represent an insert cDNA (2.06 kb) and the residual vector DNA (3 kb).

The 2.06 kb cDNA hCAT-2B sequence generated by RT-PCR was purified and then ligated to pGEM-T Easy vector. DH5-α *E. coli* cells, transformed with the ligation mixture, were grown on agar plates. Selected colonies were amplified, and plasmid DNA was isolated and evaluated for presence of hCAT-2B insert by excision with *Eco*RI. Correct orientation of the insert was determined by *Nco* I restriction enzyme digestion.

Appropriate clones were sequenced with ABI Prism Dye (or Big Dye) Terminator Sequencing kit. The resultant sequences were analyzed using initially the GCG program [12], then later, Vector NTI software (InforMax, No. Bethesda, MD). The sequence of the initially cloned BeWo cDNA used in this study was identical to that of the human hepatoma, HepG2, hCAT-2B [10], except for the following three nucleotide differences: a substitution of G for an A at base pair #330 yielding a glycine instead of a serine, a substitution of a G for an A at base pair #1004, which results in "silent" glycine-to-glycine change and a substitution of a G for an A at base pair #1785, which yields an alanine instead of a threonine. The substitution at #1785 has been reported for hCAT-2B clones derived from other sources [21, 22]. The small differences in nucleotide and amino-acid sequences between the clone used in this study and that of Closs et al. [10] are well within the variations reported among clones from different sources [10, 21, 22].

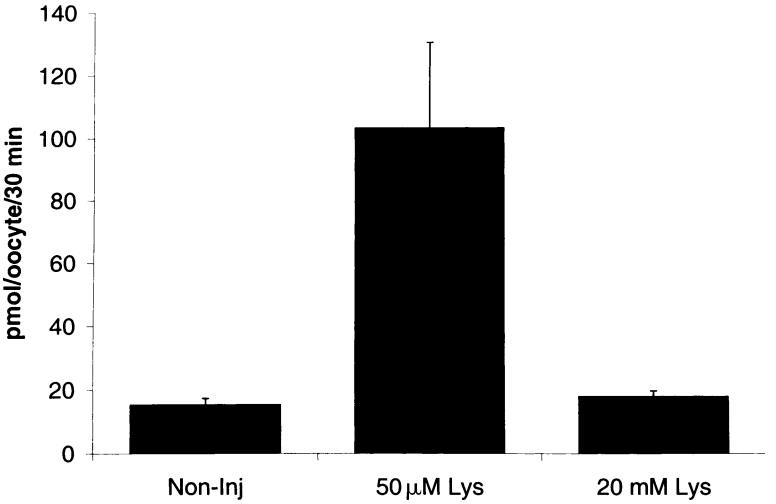

Fig. 2. Lysine uptake in *Xenopus* oocytes injected with placental hCAT-2B cRNA (~70 ng). Oocytes were injected with cloned BeWo hCAT-2B cRNA (50 μM or 20 mM lysine) or not injected. Uptake of 50 μM L-[^{3}H] lysine was measured at 22°C for 30 min. hCAT-2B cRNA injection produced a mean stimulation of approximately 5-fold (n = 6). Data are means ± SE of 6 experiments.

Most of the experiments described utilized this clone. A modified clone with A at position 330 as reported by others [10, 21, 22] was also prepared for comparison and used in some of the inhibition studies.

In Vitro Transcription

Plasmid DNA containing either 2.06 kb hCAT-2B cDNA or hCAT-1 cDNA [23] was linearized with *Sal* I leaving the hCAT gene under the control of T7 RNA polymerase promoter. The cRNA was synthesized by in vitro transcription using the mMessage-mMachine kit as described previously [23].

Expression in *Xenopus* Oocytes

A small piece of ovarian lobe was dissected from *Xenopus laevis* under anesthesia [11] and incubated in Ca^{2+}-free medium (in mM: 92 NaCl, 1 KCl, 2 $NaHCO_3$ and 15 HEPES/Tris, pH 7.4) containing 2 mg/ml collagenase type I for 90 min at room temperature. After removing the oocytes from the medium that contained collagenase they were placed in sterile modified Barth's saline, supplemented with gentamicin and albumin. The oocytes were treated and injected with cRNA in the same manner as in our previous study [23]. Injected oocytes were maintained at 18°C for the desired number of days in sterile Barth's saline [1, 4, 6].

Uptake of [^{3}H]lysine into oocytes was measured at room temperature [3, 16]. Six to 10 oocytes were incubated for 30 min [4, 16, 24] in the desired medium. The medium, the washing of oocytes and the determination of radioactivity were described previously by our laboratory [23] and others [11, 16]. Control (nonstimulated) uptake was determined using noninjected oocytes which we [23] and others found to give equivalent values to water-injected oocytes [1, 9]. Stimulated uptake was calculated by subtracting control from total uptake.

Kinetic and Statistical Analyses

The Henri-Michaelis-Menten equation describing the relationship of saturable activity to concentration with an added diffusion term was fit to uptake data using the RS/1 program [5] on a personal computer. Inhibition data were analyzed similarly to determine K_i value for competitive inhibition [19]. The program finds the least-squares solution by the Marquardt-Levenberg method of iteration.

Results

Cloning of Trophoblast hCAT-2B

The hCAT-2B mRNA was identified in both trophoblast and BeWo cell models in our previous paper [23]. The pair of primers for hCAT-2B specified as most effective in Materials and Methods yielded a 2.06 kb cDNA product (Fig. 1*A*, lane *3*) from BeWo RNA. Clones were digested with *Eco*RI (Fig. 1*B*, lanes *1* through *3*) to confirm the presence of the 2.06 kb insert.

Stimulation of Transport by Cloned Protein

Purified BeWo cRNA (~70 ng in 46 nl) was injected into *X. laevis* oocytes. Optimal transporter expression was obtained with a 3- to 5-day period of incubation after RNA injection and a 30-min incubation for uptake measurement. Under these conditions the cloned placental transporter stimulated L-lysine (50 μM) uptake fivefold (Fig. 2). Addition of 20 mM L-lysine demonstrated complete saturability of the stimulated uptake.

Concentration Dependence of hCAT-2B Stimulated L-Lysine Uptake in *Xenopus* Oocytes

Uptake of L-[^{3}H]lysine (1 μM) in *Xenopus* oocytes was inhibited by increasing concentrations of nonradioactive L-lysine. A one-system model fitted to concentration-dependence data yielded K_m and V_{max} values of 124 ± 29 μM and 458 ± 87 pmol/oocyte/30 min (Fig. 3). The K_m value agrees with that of system y^+ (124 ± 28 μM) previously reported in human placental microvillous membrane by our laboratory [18]. Attempts to fit the data with a 2-site model failed to improve the quality of the fit.

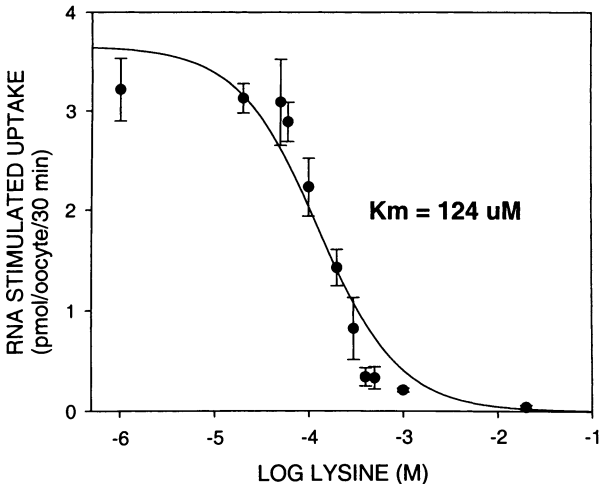

Fig. 3. Concentration dependence of cloned BeWo hCAT-2B uptake in *Xenopus* oocytes. *Xenopus* oocytes were injected with cRNA (~70 ng) and the concentration dependence of 1 μM L-[^{3}H] lysine uptake was measured in a medium containing NaCl. Each point is the mean of hCAT-2B cRNA-stimulated lysine uptake in 6 to 10 oocytes (uptake in noninjected oocytes has been subtracted). A model with one saturable component was fit with K_m = 124 ± 29 μM and V_{max} = 458 ± 87 pmol/oocyte/30 min. Data are means ± SE of 4 experiments.

Inhibition of hCAT-2B-stimulated L-Lysine Uptake by Neutral Amino Acids

Inhibition by neutral amino acids in the presence of Na^+ is an established characteristic of system y^+ in placental and other membranes [35] but had not been investigated in various cloned hCAT transporters. We therefore studied the effects of several amino acids (2 mM) on L-lysine (20 μM) uptake in the presence and absence of Na^+. In the absence of Na^+, uptake was not inhibited by the neutral amino acids L-leucine, L-homoserine and L-alanine (Table 1). In the presence of Na^+, uptake was inhibited completely by L-arginine but only partially by L-leucine, L-alanine and L-homoserine (Table 1). L-Valine, L-glutamate and D-leucine did not inhibit. Similar partial inhibition by alanine and leucine occurred when oocytes were injected with a cRNA prepared from the modified clone with A at position 330 (*data not shown*). Thus, the partial inhibition is independent of the minor sequence variation in the cDNA initially cloned.

Because of the apparent difference between these results and the interaction of hCAT-1 with neutral amino acids [21], we investigated leucine and alanine interaction over a wider concentration range. Leucine inhibited hCAT-1-stimulated lysine (1 μM) uptake completely with a K_i value of 442 ± 115 μM (Fig. 4*A*). In contrast, leucine inhibited hCAT-2B-stimulated lysine (1 μM) uptake only partially even at extremely high concentrations (Fig. 4*B*). In similar experiments, alanine inhibited hCAT-1-stimulated

Table 1. Inhibition of hCAT-2B-stimulated L-Lysine (20 μM) uptake in *Xenopus laevis* oocytes

Inhibitor (2 mM)	Percent Inhibition	
	+Na	−Na
Control	(100)	(100)
Lysine	(0)	(0)
Leucine	41 ± 10	98 ± 10
Homoserine	19 ± 5	91 ± 14
Alanine	49 ± 11	102 ± 7
Valine	81 ± 3	ND
Glutamate	94 ± 5	ND
D-Leucine	102 ± 8	ND
Arginine	5 ± 3	ND

Inhibition of lysine uptake in *Xenopus* oocytes stimulated by hCAT-2B cRNA both in the absence and presence of Na^+. Oocyte uptake was measured with 20 μM L-[^{3}H] lysine at 22°C for 30 min in hCAT-2B cRNA-injected and control oocytes. Values are means ± SE in 5 experiments for 7 to 10 oocytes. ND = not determined.

lysine (1 μM) uptake completely in the presence of Na^+ with a K_i value of 4 ± 1 mM (Fig. 5*A*) but inhibited hCAT-2B-stimulated lysine uptake only partially (Fig. 5*B*). Preliminary experiments demonstrated that alanine is a substrate of hCAT-2B and that its uptake is saturable and sodium-stimulated (*data not shown*).

Discussion

This paper describes the expression of the mRNA of hCAT-2B, a cationic amino-acid transporter in human placental trophoblast, and details the interaction of the transporter with substrate and neutral amino acids. We have previously demonstrated that three cationic amino-acid transporters, hCAT-1, hCAT-2B and hCAT-4 are expressed in trophoblast [23]. hCAT-1 and hCAT-2B have similar K_m and V_{max} values, but differ substantially in their interaction with neutral amino acids in the presence of Na^+. The presence of multiple CAT transporters gives the trophoblast the potential to vary transporter interaction with substrates and inhibitors that interact with its two surface membranes.

Cationic amino-acid uptake mediated by the cloned hCAT-2B transporter expressed in *X. laevis* oocytes resembles that of system y^+ in microvillous membrane isolated from term human placenta [18]. The K_m value of the cloned transporter, 124 ± 29 μM (Fig. 3), is essentially the same as that of uptake by placental microvillous membrane (124 ± 28 μM) [18]. Additionally, uptake by both the microvillous membrane [18] and the expressed hCAT-2B transporter (Table 1) is only partially inhibited by certain neutral amino acids in the presence of sodium. In contrast, in basal membrane, inhibition by alanine, leucine and

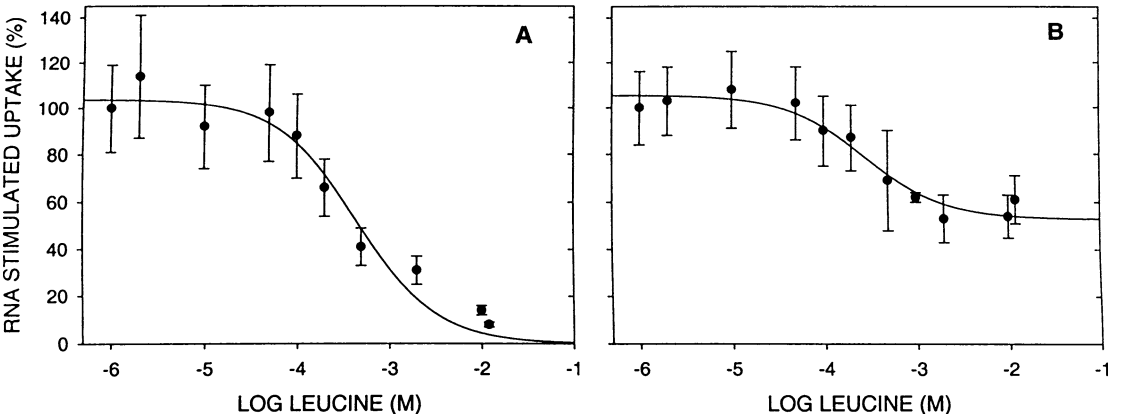

Fig. 4. Concentration dependence of inhibition by L-leucine of L-[^{3}H] lysine (1 μM) uptake in the presence of Na^+ in *Xenopus* oocytes stimulated by hCAT-1 (*A*) or hCAT-2B (*B*) cRNA. The K_m value used in the fit was from reference 21 (*A*) or Figure 3 (*B*). Each point is the mean of CAT-stimulated lysine uptake in 6 to 10 oocytes (uptake in noninjected oocytes has been subtracted). A model with one saturable component was fit with K_i = 442 ± 115 μM and V_{max} = 1180 ± 55 pmol/oocyte/30 min (*A*) and the same was done for hCAT-2B data (*B*) yielding a K_i = 266 ± 87 μM and V_{max} = 530 ± 40 pmol/oocyte/30 min. Data are means ± SE of 4 experiments. 100 percent uptake = 11.46 ± 2.18 pmol/oocyte/30 min (*A*) and 8.13 ± 1.34 pmol/oocyte/30 min (*B*).

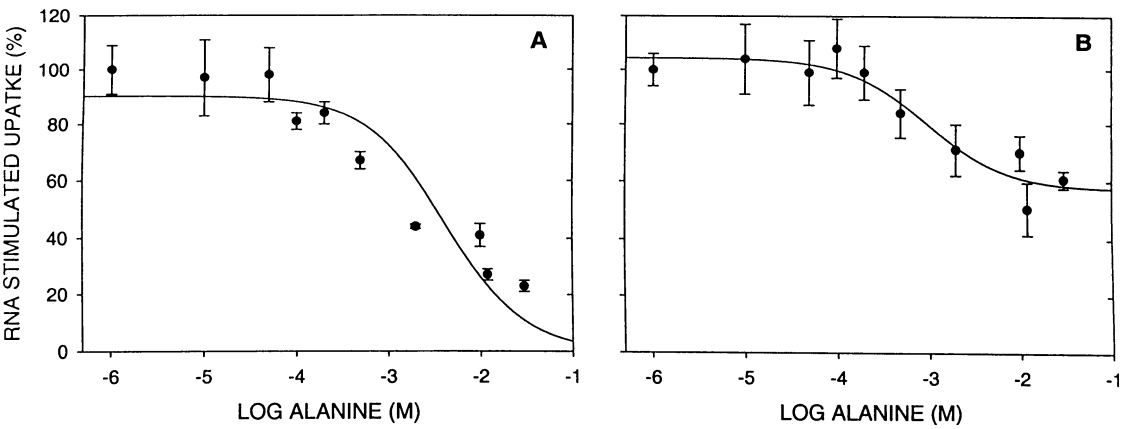

Fig. 5. Concentration dependence of inhibition by L-alanine of L-[^{3}H] lysine (1 μM) uptake in the presence of Na^+ in *Xenopus* oocytes stimulated by hCAT-1 (*A*) or hCAT-2B (*B*) cRNA. The K_m value used in the fit was from reference 21 (*A*) or Figure 3 (*B*). Each point is the mean of CAT-stimulated lysine uptake in 6 to 10 oocytes (uptake in noninjected oocytes has been subtracted). A model with one saturable component was fit with K_i = 4 ± 1 mM and V_{max} = 1061 ± 49 pmol/oocyte/30 min (*A*) and the same was done for hCAT-2B data (*B*) yielding a K_i = 908 ± 545 μM and V_{max} = 626 ± 78 pmol/oocyte/30 min. Data are means ± SE of 4 experiments (for *A*, n = 3). 100 percent = 12.40 ± 1.20 pmol/oocyte/30 min (*A*) and 10.57 ± 0.65 pmol/oocyte/30 min (*B*).

other neutral amino acids in the presence of sodium was essentially complete [17, 18], and closely resembled the inhibition of hCAT-1-stimulated uptake in oocytes [23] as well as uptake in the BeWo cell model of undifferentiated syncytiotrophoblast [34].

The interaction of hCAT-1 and hCAT-2B with alanine and leucine was investigated over a wide concentration range. Inhibition of hCAT-2B-mediated uptake by both amino acids was clearly only partial and strikingly different from the interaction with hCAT-1 (Figs. 4 and 5) [23]. It should be noted that microvillous membrane and hCAT-2B transport do differ somewhat in their interaction with neutral amino acids in the presence of sodium. Leucine inhibits hCAT-2B-mediated uptake only partially (Fig. 4*B*) and microvillous membrane uptake apparently more completely [18]. This difference may result from activity of another transporter (perhaps hCAT-4) in microvillous membrane or from some other unidentified cause. Although hCAT-1 and hCAT-2B transporters share similar K_m values (100 μM and 125 μM, respectively), they clearly differ in interaction with neutral amino acids and the characteristics of their transport resemble transport by basal and microvillous membrane.

The mechanism of the apparent partial interaction of hCAT-2B with neutral amino acids in the presence of sodium is not fully known. Since alanine

uptake by that transporter is saturable and sodium stimulated, its interaction may, at least to some extent, be described as competitive. There is some evidence that the CAT transporters can exist in more than one form with differential substrate interaction [33]. As yet unknown protein modifiers may also play a role [8]. Clearly, however, the functional mechanism, and the structural basis, of the differential interaction of the hCAT transporters with cationic and neutral amino acids remain to be further elucidated.

A recent study reported that system y^+ is not present in isolated placental basal membrane and that uptake is entirely attributable to system y^+L [2]. This conclusion was based on the strong inhibition of arginine uptake by glutamine and sodium in that study. The transport activity we attribute to system y^+ in basal membrane has a K_m of ~200 μM [17], and is sensitive to inhibition by the sulfhydryl-binding reagent N-ethylmaleimide (NEM) [19], which has been shown to discriminate between system y^+ and other cationic amino acid transporters [13]. The NEM-sensitive transport activity was inhibited by neutral amino acids in the presence of sodium [16–18], an established property of system y^+ [35] and of the cloned trophoblast hCAT-1 transporter expressed in *Xenopus* oocytes [23].

While system y^+L also interacts with leucine in the presence of sodium, that interaction, and the overall properties of system y^+L, differ in important respects from those of the hCAT transporters. System y^+L is a high-affinity transporter (K_m values are 17 μM and 44 μM in placental microvillous membrane and erythrocytes), whereas the K_m values of hCAT-1 and -2B transporters are 125 μM [14, 18, 23]. In the same two membranes, in the presence of sodium, system y^+L is inhibited by leucine at low micromolar concentrations ($K_i = 10$ μM in placental microvillous membrane and 22 μM in erythrocytes) [14, 18, 19]. Inhibition of the cloned hCAT-1 and hCAT-2B transporters in the presence of sodium requires substantially higher concentrations of leucine (K_i values were 442 μM and 266 μM for hCAT-1 and hCAT-2B, respectively). Even at these higher concentrations inhibition of hCAT-2B was only partial (Fig. 4*B*). The molecular characteristics of the two systems also differ. System y^+L has been reported to be related to a heterodimer of the 4F2hc antigen protein and a recently cloned "y^+LAT" cDNA [26, 29]. Coexpression of those two cDNAs in oocytes produces a transporter that interacts sensitively with leucine. System y^+ activity is produced by the expression of a single cDNA [10] with a sequence substantially different from that of y^+LAT (nucleotide sequence similarity 35%).

The vectorial transport of amino acids across the syncytiotrophoblast (and other polarized epithelia) is likely to be mediated in part by differing interactions of the hCAT transporter isoforms at the apical and basal or basolateral surfaces. The virtually complete inhibition by neutral amino acids in the presence of sodium (present in hCAT-1 and isolated basal membrane) in combination with the weaker or partial inhibition (present in hCAT-2B and microvillous membrane) may facilitate uptake at the maternal-facing microvillous membrane for transfer to the fetus. To our knowledge this is the first report of apparently different interactions of hCAT-1 and hCAT-2B with neutral amino acids in the presence of sodium. The direct localization of the hCAT isoforms in the trophoblast surface membranes is hindered by the apparent difficulties in preparing isoform-specific antibodies in various laboratories. Using fluorescent-tagged transporters, we have recently observed differential localization in a model epithelial cell [7]. It is apparent that more detailed investigation of the properties of transporter isoforms and their cellular localization is likely to increase understanding of the cellular mechanisms of epithelial transport.

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