Human Cationic Amino Acid Transporters hCAT-1, hCAT-2A, and hCAT-2B: Three Related Carriers with Distinct Transport Properties[†,‡]

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ABSTRACT: In this study, we aimed at analyzing the human homologues of the murine cationic amino acid transporters mCAT-1, mCAT-2A, and mCAT-2B. cDNAs encoding hCAT-1 had been previously reported by two independent groups [Albritton, L.M., et al. (1993) *Genomics 12*, 430; Yoshimoto, T., et al. (1991) *Virology 185*, 10]. We isolated cDNAs encoding hCAT-2A and hCAT-2B from a human liver cDNA library and from cDNA derived from the human hepatoma cell line HepG2, respectively. Analyses of the deduced amino acid sequences of both carriers demonstrated 90.9% identity with the respective murine proteins. In their functional domains (42 amino acids), both hCAT-2A and hCAT-2B differ only by one residue from the respective mouse proteins. Thus, CAT-2 proteins demonstrate a higher interspecies conservation than CAT-1 proteins that are overall 86.5% identical between mouse and human and differ by seven residues in the functional domain. The high degree of sequence conservation was reflected by the functional similarity of the human carriers with their mouse homologues. When expressed in *Xenopus* oocytes, hCAT-1 and hCAT-2B demonstrated transport properties consistent with y^+. Unlike the mouse CAT-1 and CAT-2B, whose transport properties could hardly be distinguished, the transport properties of the human CAT-1 and CAT-2B isoforms showed clear differences: hCAT-1 had a 3-fold higher substrate affinity and was more sensitive to trans-stimulation than hCAT-2B. In contrast to the y^+ carriers, hCAT-2A exhibited a 10−30-fold lower substrate affinity, a greater maximal velocity, and was much less sensitive to trans-stimulation at physiological substrate concentrations.

Three related carrier proteins that catalyze the exchange of the cationic amino acids L-arginine, L-lysine, and L-ornithine between the two sides of the plasma membrane have been characterized in murine cells [mCATs,[1] for mouse cationic amino acid transporters; for review, see Closs (1996) and MacLeod & Kakuda (1996)]. mCAT-1, the first member of this protein family has been identified as a virus receptor (Albritton et al., 1989). Its cellular function as an amino acid carrier was suggested by similarities in the predicted secondary structure with the L-histidine and L-arginine permease of the yeast *Saccharomyces cerevisiae* and verified by expression in *Xenopus* oocytes and subsequent studies of amino acid uptake (Kim et al., 1991; Wang et al., 1991). mCATs are members of a larger family of integral membrane proteins found in yeast, fungi, and plants (Frommer et al., 1995; Kim et al., 1991; Reizer et al., 1993; Sophianopoulou & Diallinas, 1995). Depending on the algorithm used, mCATs are predicted to have 12 or 14 trans-membrane spanning domains (TM) connected by short intra- and extracellular loops (Albritton et al., 1989; Reizer et al., 1993). The three proteins are encoded for by two different genes, *mCAT-1* and *mCAT-2* (Finley et al., 1995). Differential splicing of the primary *mCAT-2* transcripts leads to two different gene products, mCAT-2A and mCAT-2B, that differ only in a stretch of 42 amino acids located at the intracellular loop between TM VIII and IX, according to the 14 TM model (Closs et al., 1993a,b; Kavanaugh et al., 1994).

The mCAT proteins have been found to be differentially expressed in murine tissues and cultured cells. Their expression can be altered by a variety of external stimuli such as mitogens, bacterial lipopolysaccharide (LPS), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), insulin, or angiotensin II [for review, see MacLeod & Kakuda (1996)]. Indeed, all three CAT isoforms can be coexpressed in the same cell (Simmons et al., 1996). The complex regulation of CAT expression suggests distinctive roles of each carrier depending on the requirement of different cells for cationic amino acids. With the exception of the liver, mCAT-1 is ubiquitously expressed, whereas mCAT-2A is constitutively expressed in hepatocytes. mCAT-2B expression can be induced in a variety of cells. It remains to be elucidated if mCAT-2B plays a specific role in biological processes that consume basic amino acids, such as NO- or polyamine synthesis.

Most studies on the transport properties of the mCAT proteins were performed in *Xenopus* oocytes, where each carrier can be expressed individually at a high level against a low background of endogenous transport activity [for

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[‡] The sequences of the complete hCAT-2A and the partial hCAT-2B cDNAs have been deposed in GenBank; accession numbers: U76368 and U76369.

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[1] Abbreviations: CAT, cationic amino acid transporter (m, mouse; h, human; r, rat); TM, transmembrane spanning domain; PCR, polymerase chain reaction; RT, reverse transcription; K_M, Michaelis Menten constant; V_{max}, maximal velocity; LPS, bacterial lipopolysaccharide.

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review, see Closs (1996)]. These studies have demonstrated that the three mCAT proteins share the same substrate specificity. Their transport activity is independent of the presence of sodium ions and stimulated by substrate at the trans-side of the membrane (trans-stimulation). Recognition of basic amino acids by the mCATs is stereoselective. Both, mCAT-1 and mCAT-2B demonstrate high substrate affinity and sensitivity to trans-stimulation (Closs et al., 1993b; Kakuda et al., 1993; Kim et al., 1991). These properties are consistent with system y^+, the principal mechanism for cellular uptake of cationic amino acids [for review, see White (1985)]. The K_M values for L-arginine of mCAT-1 (70−250 μM) and mCAT-2B (38−380 μM) reported by independent groups overlap. Therefore, the two carriers are difficult to distinguish when coexpressed in the same cell. mCAT-2A differs from mCAT-2B only in a stretch of 42 residues, but it exhibits a 10-fold lower substrate affinity, a greater maximal velocity, and is much less sensitive to trans-stimulation (Closs et al., 1993a; Kavanaugh et al., 1994). Interestingly, the exchange of the mCAT-2A- and mCAT-2B-specific domains with the corresponding region in mCAT-1 led to chimeras with transport properties of the donor of that region (Closs et al., 1993b).

CAT-1 cDNAs have also been cloned from human and rat cells (Albritton et al., 1993a; Puppi & Henning, 1995; Wu et al., 1994; Yoshimoto et al., 1991). In the optimal alignment, the deduced amino acid sequence of mCAT-1 is 95.8 and 86.5% identical to rCAT-1 and hCAT-1, respectively. The high degree of conservation suggests that also the functional properties of the CAT-1 proteins are conserved between mammalian species. In order to further characterize the human CAT proteins, we cloned cDNAs encoding for hCAT-2A and hCAT-2B. Here, we report a comparison of the deduced amino acid sequences of the mouse and the human CAT-2A and CAT-2B as well as a detailed analysis of the transport properties of all three hCAT-proteins.

EXPERIMENTAL PROCEDURES

Isolation and Sequencing of hCAT-2A cDNAs. A human liver cDNA library in λ gt10 (Clontech, Palo Alto, CA) was screened under stringent conditions using as a hybridization probe the ^{32}P-labeled insert of pMH305 (Closs et al., 1993a), a plasmid containing the full-length mCAT-2A cDNA. Three different *Eco*RI restriction fragments were identified in six independent phages isolated in this screen. These fragments were subcloned into pBluescript (Stratagene, La Jolla, CA) and sequenced. Analyses of the DNA sequences suggested that the three fragments overlapped at two *Eco*RI sites with the second (more 3′) *Eco*RI restriction site containing the last two codons of a long open reading frame. To insure that no small internal *Eco*RI fragments present in the phage were lost during subcloning, Polymerase chain reaction (PCR) was performed on phage DNA using primers that flank the two *Eco*RI sites. Analyses of the nucleotide sequence of the reaction product verified the presence of only two *Eco*RI sites.

A 2 kb DNA fragment containing the complete open reading frame was synthesized by PCR (with Taq polymerase) using the phage DNA as template and a sense primer, CCGGAGCTCAGATCTGACGTCAGAATGATTCCT, which included a *Sst*I and a *Bgl*II restriction site 5′ to the codon for the putative initiator methionine (all three underlined), and an antisense primer, CCGAAGCTTAGATCTGCTCCTGCAAGTGTT, which included a *Hin*dIII and a *Bgl*II restriction site 3′ of the two last bases of the TAA stop codon (all three underlined). The PCR product was digested with *Bgl*II and subcloned into pSP64T (Melton et al., 1984). The resulting plasmid was named phCAT-2A 118. The sequence of the complete hCAT-2A cDNA was determined from both strands.

Isolation and Sequencing of hCAT-2B cDNA. RNA isolated from the human hepatoma cell line HepG2 was reverse transcribed using the Gibco/BRL Superscript reverse transcriptase kit (Life Technologies, Gathersburg, MD) and hexa oligonucleotides with random sequences as primers. The cDNA served as a template in a PCR using the oligonucleotides listed above (deduced from the hCAT-2A sequence) as primers. To distinguish DNA fragments containing hCAT-2B and hCAT-2A sequence, PCR products were digested with *Bam*HI, assuming that the *Bam*HI restriction site found in the mCAT-2B (but not mCAT-2A or hCAT-2A) cDNA would be conserved in the human CAT-2B cDNA. Three independent PCR products that could be restricted with *Bam*HI were digested with *Bgl*II and subcloned into pSP64T. Four hundred base pairs of these clones were sequenced using oligonucleotides deduced from the hCAT-2A sequence flanking the cDNA region divergent between CAT-2A and -2B. The plasmid utilized in the subsequent experiments was named phCAT-2B 181.

Expression of cRNAs in Xenopus Laevis Oocytes and Measurement of Amino Acid Uptake. A cDNA containing the complete coding sequence of hCAT-1 was obtained by PCR using single-stranded cDNA from the colon carcinoma cell line DLD-1, the oligonucleotide GGAGGATCCTGAACAGCAACATGGGGT (containing a *Bam*HI restriction site 5′ of the codon for the putative start methionine, both underlined) as sense primer and the oligonucleotide GGAAGATCTCACTTGCACTGGTCCAAG (containing a *Bgl*II restriction site 3′ of the stop codon, both underlined) as antisense primer. The PCR fragment was inserted into the *Bgl*II restriction site of pSP64T, and the resulting plasmid was named pSPhCAT-1AB1.

pSPhCAT-1AB1, phCAT-2A 118, and phCAT-2B 181 were linearized with *Sal*I and cRNA was prepared by *in vitro* transcription from the SP6 promoter of pSP64T (mMessage mMachine in vitro transcription kit, Ambion, Austin, TX). cRNA (25 ng in 25 nL of H_2O) were injected into each *X. laevis* oocyte (Dumont stage VI−VII). Oocytes injected with 25 nL of water were used as controls. Amino acid uptake was determined 3−5 days after injection of cRNA, as previously described (Kim et al., 1991). Briefly, oocytes were incubated at 20 °C in uptake solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 50 mM Tris, pH 7.5) containing various concentrations of unlabeled L-arginine and 5 μCi/mL [2,3-^{3}H]L-arginine (Dupont NEN, Bad Homburg, Germany), respectively. After a 30 min incubation, oocytes were washed five times in ice-cold uptake solution, solubilized individually in 1% sodium dodecyl sulfate (SDS), and their incorporated radioactivity was determined in a liquid scintillation counter.

For trans-stimulation experiments, three oocytes were injected with 3.6 nmol of [2,3-^{3}H]L-arginine (3.6 nCi) in 36 nL of water each. The oocytes were then transferred into an uptake solution containing a defined concentration of unlabeled L-arginine. After a 1 h incubation at 20 °C, the [^{3}H]L-arginine that had accumulated in the uptake solution was determined by liquid scintillation counting.

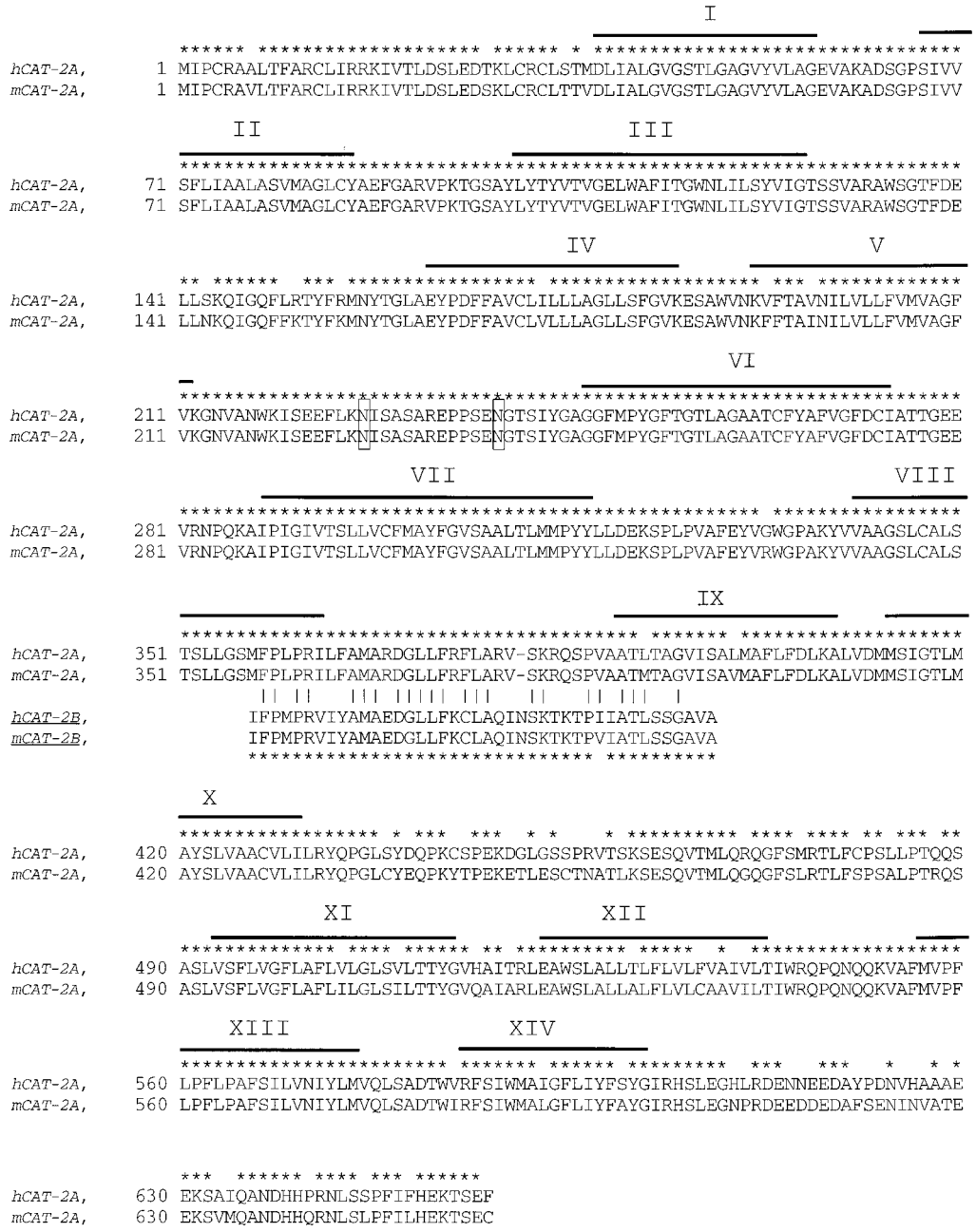

FIGURE 1: Alignment of the deduced amino acid sequences of the human and mouse CAT-2 proteins. The amino acid sequences of hCAT-2A and hCAT-2B were deduced from the respective cDNAs (isolated as described in the text) and aligned with the published sequence of mCAT-2A (Closs et al., 1993a) and mCAT-2B (Reizer et al., 1993). Lines on top of the sequence indicate the putative transmembrane domains I−XIV. The putative N-linked glycosylation sites in the third extracellular loop are boxed. For the CAT-2B proteins, only the regions divergent from CAT-2A are shown. Stars mark residues identical in the human and mouse proteins, vertical lines mark residues identical in CAT-2A and CAT-2B. The sequences of the complete hCAT-2A and the partial hCAT-2B cDNAs have been deposed in GenBank; accession numbers: U76368 and U76369.

HPLC Analysis of Oocyte Amino Acid Content. Oocytes were incubated 0, 3, or 6 h at 20 °C in uptake solution containing 10 mM L-arginine. Following the incubation, the oocytes were washed five times in ice-cold uptake solution. Then six oocytes were pooled and lysed in 150 μL of methanol/0.5 M boric acid, pH 7.7, 9:1, v/v. γ-Aminobutyric acid (GABA) was added as the internal standard (1 nmol/40 μL). Cell debris were sedimented at 14000*g* for 5 min and 5−40 μL of the supernatant was used for precolumn derivatization with *o*-phthaldialdehyde (OPA). Amino acid derivatives were separated on a Superspher 60 RP-select B HPLC column (Merck, Darmstadt, Germany) using a two solvent gradient (methanol/50 mM sodium acetate, pH 7.0). The flow rate was 1 mL/min. Fluorescence (excitation wavelength, 330 nm; emission wavelength, 450 nm) was monitored with a Shimadzu RF-530 fluorimeter connected to a Shimadzu C-R3A integrator.

RESULTS

Isolation of hCAT-2A and hCAT-2B cDNAs and Analyses of the Deduced Amino Acid Sequences. Using a mCAT-2A cDNA as a hybridization probe, we isolated several independent phages from a human liver cDNA library. Sequence analyses of *Eco*RI subclones from these phages revealed three overlapping *Eco*RI fragments with an open reading frame coding for 657 amino acids. Alignment with the mouse CAT-2A amino acid sequence demonstrated 90.9% identity in the amino acid sequences of both species (Figure 1). Similar to other known CAT proteins, two consensus

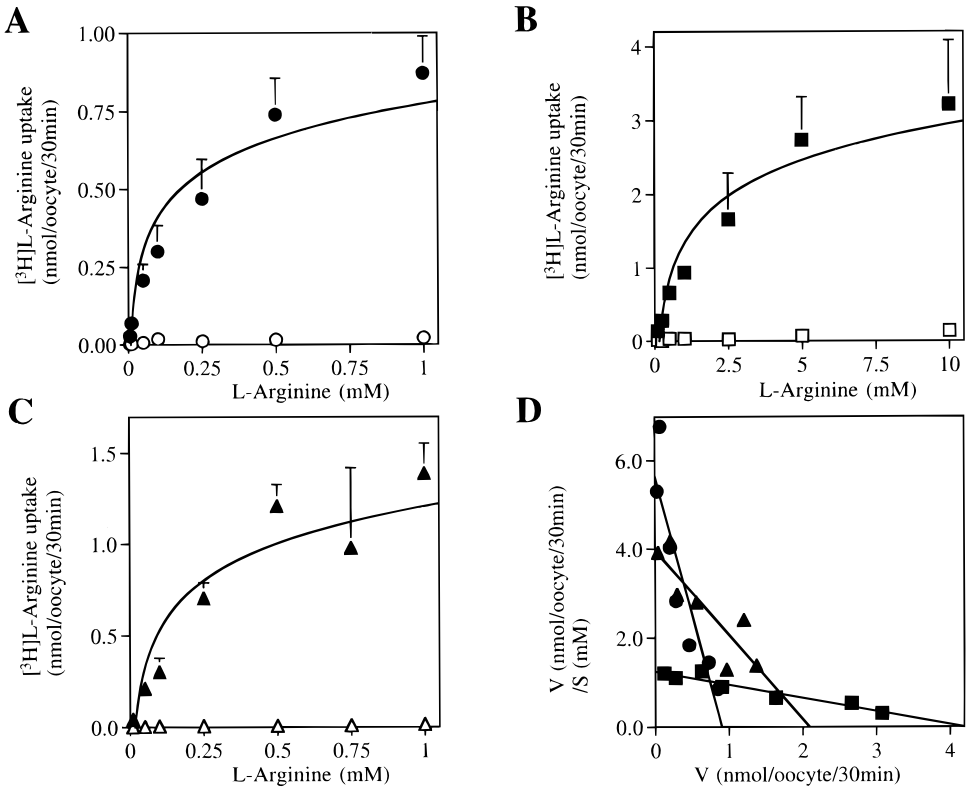

FIGURE 2: L-Arginine uptake by oocytes expressing hCAT-proteins. *Xenopus laevis* oocytes were injected with 25 ng of cRNA (in 25 nL of water) encoding one of the three hCAT-carriers or 25 nL of water alone (open symbols in panels A−C). Three days after injection, the uptake of L-arginine was measured in oocytes incubated for 30 min in uptake solution containing the indicated concentrations of [^{3}H]L-arginine. Panel A, hCAT-1-injected oocytes (closed circles); Panel B, hCAT-2A-injected oocytes (closed squares); Panel C, hCAT-2B-injected oocytes (closed triangles). Data points represent means ± SEM, $n = 4-6$. Note the different scales of the ordinates in the individual panels. Panel D, Eadie Hofstee plots of the data shown in panel A−C. Here, the values obtained from water-injected oocytes were deduced from the respective values obtained from cRNA-injected oocytes.

sequences for N-linked glycosylation were found in the third extracellular loop. A cDNA clone containing the hCAT-2B sequence was obtained by RT/PCR using oligonucleotides with sequences deduced from hCAT-2A as primers. Four hundred base pairs of this cDNA containing the region divergent between CAT-2A and CAT-2B were sequenced. As for the mouse cDNAs, the differences between the sequences of the human CAT-2A and CAT-2B cDNAs were confined to a short stretch of only 133 nucleotides. The remaining sequence was 100% identical between CAT-2A and CAT-2B. In the divergent region, the deduced amino acid sequences of hCAT-2A and hCAT-2B differ in 19 residues (Figure 1). Interestingly, in this region, hCAT-2A and hCAT-2B differ only by one residue from the corresponding mouse proteins.

Characterization of the Transport Properties of the hCAT Proteins. To analyze the transport properties of the hCAT proteins, cRNAs transcribed *in vitro* from plasmids containing the complete coding sequence of hCAT-1, hCAT-2A, or hCAT-2B were injected into *Xenopus* oocytes and transport activity was assayed 3−5 days later. Transport studies performed with oocytes injected with either cRNA demonstrated at least a 15−20-fold higher uptake of L-arginine than control oocytes injected with water (Figure 2). Accumulation of L-arginine was linear over a 60 min period (data not shown). Measurements of L-arginine uptake over 30 min revealed a concentration-dependent transport activity (Figure 2, panels A−C). The apparent half-saturating substrate concentrations (K_M) for each carrier were determined by fitting the data according to the Eadie Hofstee equation (after subtraction of the values obtained from water-injected oocytes) (Figure 2, panel D). The apparent K_Ms determined in several independent experiments for each carrier were 0.11−0.16 mM for hCAT-1, 3.36−3.90 mM for hCAT-2A, and 0.32−0.73 mM for hCAT-2B (Table 1). The apparent maximal velocities (V_{max}s) were 1.6−1.8, 2.2−8.4, and 1.2−4.0 nmol/oocyte/h in oocytes expressing hCAT-1, hCAT-2A, and hCAT-2B, respectively. The uptake activities of each carrier measured at about half-saturating L-arginine concentrations were not significantly different

Table 1: Comparison of the Transport Properties of hCAT and mCAT Proteins[a]

carrier	K_M (mM L-Arg)	V_{max} (nmol L-Arg/ oocyte/h)	trans-stimulation[b]	accumulation of L-Arg (nmol/oocyte)[c]
hCAT-1	0.11−0.16	1.6−1.8	9.8	2.5
hCAT-2A	3.36−3.90	2.2−8.4	1.1	14.0
hCAT-2B	0.32−0.73	1.2−4.0	1.8	7.9
mCAT-1	0.14− 0.25	1.1−1.6	8.3	1.0
mCAT-2A	2.10−5.20	3.9−7.1	1.5	11.0
mCAT-2B	0.25−0.38	1.1−3.4	2.9	2.5

[a] Data for K_M and V_{max}, accumulation of L-arginine, and trans-stimulation were determined for the hCATs as described in Figures 2, 4, and 5, respectively. For K_M and V_{max}, the data of 3−4 independent experiments were pooled. Data for the mCAT proteins are from (Closs et al., 1993b). [b] Trans-stimulation: fold transport activity at high (0.25 mM) versus zero trans-substrate (L-arginine). [c] Accumulation of L-Arg: nanomoles of L-arginine taken up per oocyte after incubation in 10 mM L-arginine for 6 h. Significant differences (as calculated by factorial ANOVA followed by Fisher's protected least-significant-difference test) were found when comparing the K_M data of the hCAT proteins: for hCAT-1/hCAT-2A and hCAT-1/hCAT-2B with $P < 0.001$, for hCAT-2A/hCAT-2B with $P < 0.01$.

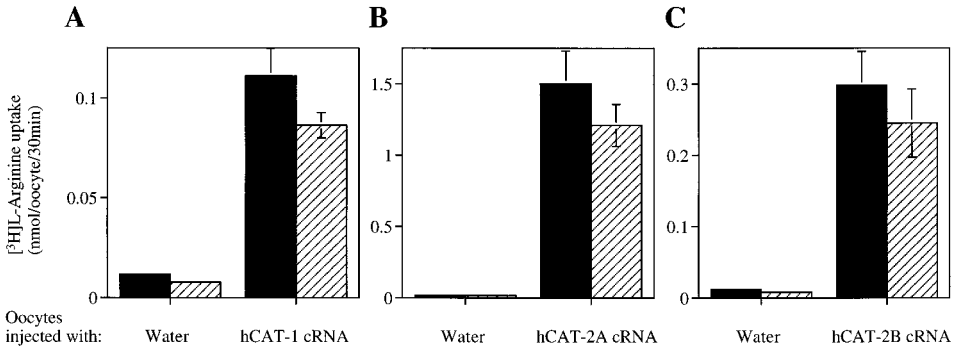

FIGURE 3: Sodium-independent transport of L-arginine mediated by hCAT-1, hCAT-2A, and hCAT-2B. Each transporter was expressed in *Xenopus* oocytes as described in Figure 2. Uptake of L-arginine was measured for 30 min in oocytes incubated in uptake buffer containing 154 mM NaCl (closed bars) or 154 mM choline chloride (hatched bars). Panel A, oocytes expressing hCAT-1, incubated in 0.1 mM L-arginine. Panel B, oocytes expressing hCAT-2A, incubated in 1 mM L-arginine. Panel C, oocytes expressing hCAT-2B, incubated in 0.1 mM L-arginine. Data points represent means ± SEM, $n = 4-6$. Note the different scales of the ordinates in the individual panels.

when choline chloride was substituted for sodium chloride in the uptake buffer (Figure 3).

In an attempt to determine the relative substrate affinity of the three CAT isoforms at the inner side of the membrane, we measured accumulation of tritiated L-arginine over 6 h in oocytes incubated in an isotonic salt solution containing 10 mM [^{3}H]L-arginine. Under these conditions, all three CAT isoforms are saturated and, therefore, influx should occur at a maximal rate (V_{max}). Using tritiated substrate, initially only influx is monitored, as no significant amount of labeled substrate is transported out of the cell. An apparent steady state is reached when the labeled substrate is transported at the same rate in and out of the cell. The apparent steady state will be reached sooner, the faster the V_{max} for efflux is reached. In hCAT-1-expressing oocytes, an apparent steady state was observed after a 1 h incubation in 10 mM L-arginine (Figure 4A). About 2.5 nmol of L-arginine accumulated during this period. In contrast, in hCAT-2A-expressing oocytes incubated in 10 mM L-arginine, steady state was only reached after 5 h and an accumulation of 14 nmol of L-arginine in the cell (Figure 4A). Incubation of hCAT-2B-expressing oocytes in 10 mM L-arginine led to an accumulation of 7.9 nmol of L-arginine over 4 h (Figure 4A). To determine the actual L-arginine content of the oocytes, HPLC analyses were performed on lysates of oocytes incubated in culture medium (containing 1.42 mM L-arginine and 0.5 mM L-lysine) for several days and then either lysed directly or transferred for 3 or 6 h to uptake solution containing 10 mM unlabeled L-arginine (Figure 4B). The average L-arginine content of an oocyte-expressing hCAT-1 and incubated in culture medium was 0.23 ± 0.03 nmol. On the basis of an estimated oocyte volume of 0.5−1 μL, this corresponds to an intracellular L-arginine concentration of about 0.25−0.5 mM. After incubation in 10 mM L-arginine for 3 or 6 h, the L-arginine content was 0.47 ± 0.01 and 0.41 ± 0.08 nmol per hCAT-1-expressing oocyte, respectively. The average L-arginine content of an oocyte-expressing hCTA-2A or hCAT-2B was 1.2 ± 0.2 and 1.8 ± 0.3 nmol in culture medium, 3.3 ± 0.6 and 2.3 ± 0.6 nmol after 3 h in 10 mM L-arginine, and 4.1 ± 1.1 and 2.4 ± 0.1 after 10 h in 10 mM L-arginine, respectively (Figure 4B).

Carrier-mediated transport is often stimulated by substrate at the trans-side of the membrane, a property referred to as trans-stimulation. All three hCATs were assayed for trans-stimulation in efflux experiments. To this end, oocytes were injected with cRNA of hCAT-1, hCAT-2A, or hCAT-2B and 3 days later reinjected with tritiated L-arginine (3.6 nCi, 3.6 nmol). Efflux into isotonic salt solutions with defined L-arginine concentrations (trans-substrate) were measured immediately after the second injection (Figure 5). hCAT-1-mediated efflux was strongly dependent on extra-

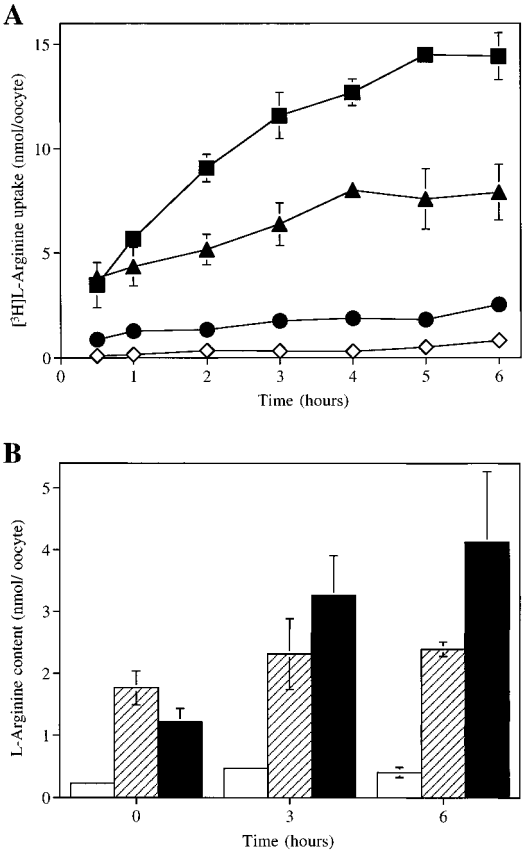

FIGURE 4: Accumulation of L-arginine in oocytes exposed to 10 mM extracellular L-arginine. Each transporter was expressed in *Xenopus* oocytes as described in Figure 2: Panel A, three days after injection with cRNA, the accumulation of [^{3}H]L-arginine was measured over 6 h in oocytes incubated in uptake buffer containing 10 mM [^{3}H]L-arginine. Closed circles, hCAT-1; closed squares, hCAT-2A; closed triangles, hCAT-2B; open diamonds, water-injected oocytes. Data points represent means ± SEM, $n = 4-6$. Panel B, The contents of L-arginine were determined in oocytes incubated in culture medium for several days (containing 1.42 mM L-arginine and 0.5 mM L-lysine) and then either lysed directly or transferred for 3 or 6 h to uptake solution containing 10 mM unlabeled L-arginine (Figure 4B). The amino acids were separated by HPLC. The amounts (peaks) of L-arginine were quantified using respective external standards. Open bars, hCAT-1; hatched bars, hCAT-2B; filled bars, hCAT-2A. The data shown represent means ± SEM, $n = 2-3$.

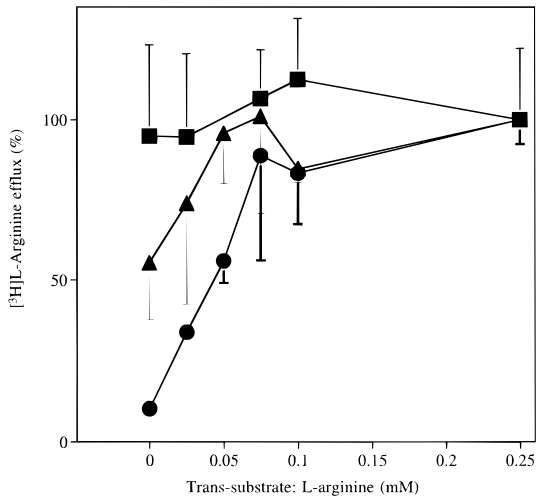

FIGURE 5: Trans-stimulation of hCAT-mediated L-arginine transport. Each transporter was expressed in *Xenopus* oocytes as described in Figure 2: closed circles, hCAT-1; closed squares, hCAT-2A; closed triangles, hCAT-2B. Three days after injection with cRNA, oocytes were injected with 36 nL of [^{3}H]L-arginine (3.6 nCi, 3.6 nmol). Immediately after the second injection, L-arginine efflux was measured by transferring oocytes (3 oocytes/200 μL) into isotonic salt solution containing the indicated concentrations of unlabeled L-arginine (= trans-substrate). The amount of [^{3}H]L-arginine (counts per minute) in the incubation solution accumulated over 1 h was determined. Values obtained with water-injected oocytes were subtracted (no L-arg, 634 ± 155; 50 μM L-arg, 872 ± 126; 75 μM L-arg, 652 ± 133; 100 μM L-arg, 889 ± 51; 250 μM L-arg; 1000 ± 253 cpm). Data points were calculated as a percentage of the mean of the transport activity observed at 0.25 mM (100%; hCAT-1, 2748 cpm; hCAT-2A, 7580 cpm; hCAT-2B, 4824 cpm). Each point represents the mean ± SEM, $n = 3$.

cellular L-arginine. In two independent experiments, about a 10-fold higher L-arginine efflux was observed from hCAT-1-expressing oocytes when incubated in 0.1 mM compared to zero extracellular L-arginine. In contrast, efflux from hCAT-2A-expressing oocytes did not differ significantly at extracellular L-arginine concentrations between zero and 0.25 mM L-arginine (Figure 5). At 10 mM extracellular L-arginine, hCAT-2A-mediated efflux was about 2-fold higher compared to efflux with zero trans-substrate (data not shown). hCAT-2B-mediated efflux also increased between zero and 0.1 mM extracellular L-arginine, however to a lesser extent than for hCAT-1 (about 1.8-fold, Figure 5, Table 1). At 0.25 mM, trans-substrate the radioactivity measured in the extracellular solution of hCAT-2A-expressing oocytes was about 2.7 times higher than from hCAT-1-expressing oocytes and 1.6 times higher than from hCAT-2B-expressing oocytes (Figure 5, see legend).

To further characterize the transport properties of the hCATs, we measured the transport activity of each isoform at different extracellular pH. Between pH 5.5 and 8.5, no significant difference in the transport activity of hCAT-1-mediated L-arginine uptake was observed. In contrast, maximal uptake activity of hCAT-2A was only seen between pH 7 and 8.5. At pH 5.5, hCAT-2A-mediated uptake was only 45 ± 13 % (mean ± SEM) of the uptake at pH 7.5. Similarly, the activity of hCAT-2B at pH 5.5 was 57 ± 12% (mean ± SEM) of the activity at pH 7.5.

DISCUSSION

The characterization of the human cationic amino acid transporters hCAT-1, hCAT-2A, and hCAT-2B reported in this study demonstrates a high degree of similarity between the human and the homologous mouse carriers. However, in contrast to the murine carriers, where the transport properties of the constitutive (CAT-1) and the inducible (CAT-2B) y^+ carrier could not be distinguished unambiguously, we found a more pronounced difference in the transport properties of the human CAT-1 and CAT-2B isoforms. Compared to hCAT-2B, hCAT-1 had a higher apparent affinity for L-arginine, its transport activity was more dependent on substrate at the trans-side of the membrane, and oocytes expressing hCAT-1 accumulated less L-arginine when incubated in 10 mM L-arginine. Similar to the murine carriers, the largest difference was observed between the transport properties of hCAT-1 and hCAT-2A. hCAT-2A had a 20−30-fold higher apparent K_M compared with hCAT-1, exhibited no dependence on trans-substrate concentrations within the physiological range and accumulated about 6-fold more L-arginine when incubated in 10 mM L-arginine. In addition, the transport activities of hCAT-2A and hCAT-2 B were sensitive to changes of the extracellular pH between 5.5 and 7.5 whereas the transport activity of hCAT-1 did not change in this pH range. The pH dependence of hCAT-2B may prove useful in distinguishing the transport activities of the two y^+ carriers in human cells. A comparison of the values for the apparent K_Ms of the mouse and human carriers suggests that the two CAT-1 isoforms are more different from each other than either of the two CAT-2A or CAT-2B isoforms. However, as the apparent K_Ms for L-arginine reported by different groups for the three mCATs vary largely, we performed parallel experiments with each pair of mouse and human CATs. Under identical conditions with the same batch of *Xenopus* oocytes, we found the apparent K_M of mCAT-1 was higher than that of hCAT-1 (data not shown). In contrast, the K_M values for the murine and the human CAT-2A or CAT-2B isoforms could not be distinguished (data not shown). Interestingly, a clear difference between the two human y^+ carriers was also seen in experiments designed to monitor the intracellular affinity of the CAT-proteins for L-arginine: When incubated in 10 mM L-arginine, hCAT-1-expressing oocytes reached the apparent steady state earlier and accumulated significantly less L-arginine than did hCAT-2B-expressing oocytes. Also in this respect, hCAT-2A differed significantly from hCAT-1. As CAT-mediated transport is energy independent, long-time incubation of oocytes expressing any CAT protein should lead to an electrochemical equilibrium of the substrate between both sides of the membrane. Assuming that the mean volumes of the oocytes are equal and given that the specific activity of the labeled substrate is the same at the beginning of the experiment, the same amount of radioactivity should be accumulated by each oocyte at the electrochemical equilibrium. However, when incubated at a very high L-arginine concentration over 6 h, different amounts of radioactivity accumulated in sets of oocytes expressing the different carriers. HPLC analyses on oocytes incubated in 10 mM unlabeled L-arginine demonstrated that the L-arginine content of hCAT-2A-expressing oocytes was about 20-fold that of oocytes expressing hCAT-1 and about 2-fold that of hCAT-2B-expressing oocytes. The discrepancy between the absolute values obtained with radioactive labeled and unlabeled L-arginine might be due to metabolism of the radioactive tracer during the 6 h incubation period. The simplest explanation for the different L-arginine contents of

oocytes expressing different hCAT proteins, is a higher affinity of hCAT-1 and hCAT-2B at the intracellular side of the membrane resulting in a maximal efflux rate at low intracellular L-arginine concentrations. This leads to an apparent steady state (where no net influx occurs) before the electrochemical equilibrium is reached in these oocytes. Our efflux experiments demonstrate that the CAT-mediated efflux is larger than the efflux mediated by endogenous transporters (at least 3-fold at high intracellular L-arginine concentrations, more than 10-fold at low intracellular L-arginine concentrations (Closs et al. 1993b). Therefore, efflux mediated by an endogenous transporter should play a minor role for the L-arginine accumulation. Alternatively, our results might be explained by intracellular sequestration of L-arginine by the CAT-2 carriers transporting L-arginine into compartments where it is not freely exchangeable with the extracellular pool. However, thus far, there is no evidence for the existence of such compartments in *Xenopus* oocytes. Finally, a clear difference in trans-stimulation has been observed between all three carriers. The lack of trans-stimulation observed for hCAT-2A at extracellular L-arginine concentrations between zero and 0.25 mM cannot simply be explained by a lack of saturation of the extracellular binding sites of hCAT-2A at these relatively low extracellular L-arginine concentrations. First, the L-arginine efflux from hCAT-2A-expressing oocytes was greater than either hCAT-1 or hCAT-2B, demonstrating the high activity of hCAT-2A. Second, even at 10 mM extracellular L-arginine, trans-stimulation of hCAT-2A was only 2-fold compared to zero trans-substrate. Maximal trans-stimulation of hCAT-2B was also about 2-fold. However, similar to hCAT-1, maximal trans-stimulation of hCAT-2B was observed at about 0.1 mM trans-substrate. The lesser dependence of both CAT-2 carriers on substrate concentration at the trans-side of the membrane might facilitate cationic amino acid uptake into cells with low intracellular concentrations of these amino acids such as hepatocytes and activated macrophages.

In accordance with the transport data, the deduced amino acid sequences of hCAT-2A and hCAT-2B demonstrate a very high degree of identity (90.9%) with the respective mouse carriers. Analyses of the open reading frame and comparison with mCAT-2A strongly suggests that the hCAT-2A cDNA we have isolated contains the complete coding region of hCAT-2A. In the 400 bp fragment analyzed of the hCAT-2B cDNA, sequence differences from hCAT-2A were only found in a stretch of 133 nucleotides, corresponding to the region that is also divergent between the two murine CAT-2 cDNAs. The remainder of the sequence was 100% identical, suggesting that, like the mouse CAT-2 proteins, hCAT-2A and hCAT-2B are the products of the same gene. In the divergent region, that determines the transport properties of the CAT proteins; the mouse and human CAT-2 proteins differ only in one residue. In contrast, in the corresponding protein domain hCAT-1 differs in seven residues from mCAT-1. In this domain, the mouse and rat CAT-2 proteins are identical (Closs, E.I., unpublished observation) whereas the CAT-1 proteins differ in two amino acids. Interestingly, in the third extracellular loop, the amino acid sequences of mouse and human CAT-2 proteins are 100% identical. In the corresponding region of CAT-1, the highest divergence in the amino acid sequences between the mouse and human proteins is found. The third extracellular loop of mCAT-1 contains the binding site for ecotropic murine leukemia viruses and also two asparagine residues shown to be glycosylated in mCAT-1 (Albritton et al., 1993b; Kim & Cunningham, 1993). Two consensus sequences for N-glycosylation are found in all CAT proteins analyzed to date in the third extracellular loop at the same position as in mCAT-1.

Our results establish distinct transport properties for all three hCAT proteins with hCAT-1 and hCAT-2B showing more similarities than either has with hCAT-2A. The functional as well as the structural similarities between the mouse and human CAT proteins demonstrate that these carriers are highly conserved between species. CAT-2 proteins show an even higher conservation than the CAT-1 proteins. The high degree of conservation of these proteins is indicative of significant physiological functions.

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