Human Cationic Amino Acid Transporter hCAT-3 Is Preferentially Expressed in Peripheral Tissues†

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ABSTRACT: At least five distinct carrier proteins form the family of mammalian cationic amino acid transporters (CATs). We have cloned a cDNA containing the complete coding region of human CAT-3. hCAT-3 is glycosylated and localized to the plasma membrane. Transport studies in *Xenopus laevis* oocytes revealed that hCAT-3 is selective for cationic L-amino acids and exhibits a maximal transport activity similar to other CAT proteins. The apparent substrate affinity and sensitivity to trans-stimulation of hCAT-3 resembles most closely hCAT-2B. This is in contrast to rat and murine CAT-3 proteins that have been reported to display a very low activity and to be inhibited by neutral and anionic L-amino acids as well as D-arginine (Hosokawa, H., et al. (1997) *J. Biol. Chem*. **272**, 8717−8722; Ito, K., and Groudine, M. (1997) *J. Biol. Chem*. **272**, 26780−26786). Also, in adult rat and mouse, CAT-3 has been found exclusively in central neurons. Human CAT-3 expression is not restricted to the brain, in fact, by far the highest expression was found in thymus. Also in other peripheral tissues, hCAT-3 expression was equal to or higher than in most brain regions, suggesting that hCAT-3 is not a neuron-specific transporter.

Cationic amino acids are building blocks of proteins but also precursors for important metabolic and signaling pathways in mammalian cells, such as the synthesis of proline, glutamate, urea, creatine, agmatine, polyamines (important regulators of cell growth and differentiation), and nitric oxide (a radical involved in such divergent actions as smooth muscle relaxation, host defense and learning). Evidently, every mammalian cell must be capable of taking up the essential amino acid L-lysine. However, also the semiessential amino acids L-arginine and L-ornithine must be exchanged between cells, as the cells and organs that synthesize these amino acids are different from those that are the major consumers (for review, see ref *1*). Specialized carrier proteins in the plasma membrane mediate the exchange of cationic amino acids. In most cell types, transport of cationic amino acids is energy-independent, with facilitated diffusion of substrate between extracellular and intracellular compartments. This Na^+-independent membrane transport of cationic amino acids was originally assigned to system y^+ first described in fibroblasts (for review, see ref *1*). In addition to the Na^+-independence, system y^+ is characterized by selectivity for cationic amino acids (although small neutral amino acids are recognized in the presence of Na^+, but with very low affinity), half-maximal activity at cationic amino acid concentrations (K_M) of 0.1−0.2 mM, pH independence and strong stimulation of transport by substrate at the trans-side of the membrane (trans-stimulation). The molecular identification of the carrier proteins mediating system y^+ activity revealed a greater diversity than had been anticipated from radiotracer flux experiments. A major focus of current research concerns the expression and physiological role of these carriers in cells with a special need for cationic amino acids, e.g., cells producing nitric oxide. To date, three different carrier proteins have been identified in mammalian cells that exhibit transport activities resembling system y^+. They belong to the family of cationic amino acid transporters (CATs,[1] for review, see refs *2*−*4*). CAT-1 seems to conform best with system y^+, as at least for the human isoforms (hCATs) CAT-2B shows a lower affinity and is less dependent on trans-stimulation and more sensitive to pH changes than CAT-1 (*5*). CAT-3 has so far only been described in rat and mouse. It also mediates the Na^+-independent transport of cationic amino acids with the apparent K_M for L-arginine ranging from 40 to 120 μM (*6*, *7*). However, the maximal transport activity (V_{max}) of mCAT-3 expressed in *Xenopus laevis* oocytes reported in these studies was considerably lower than that of the aforementioned isoforms (*7*). In addition, the substrate selectivity of CAT-3 seems to differ from that of CAT-1 and CAT-2B, e.g., a Na^+-independent recognition of a number of neutral and even anionic amino acids by rat and mouse CAT-3 has been found (*6*, *7*). In contrast to the almost ubiquitous expression of CAT-1, CAT-3 expression has been

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[1] Abbreviations: CAT, cationic amino acid transporter (prefix h, m, and r, human, mouse and rat, respectively); EGFP, enhanced green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; nt, nucleotides; NOS, nitric oxide synthase; nNOS, neuronal NOS; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SDS, sodium dodecyl sulfate

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reported to be confined to the brain in adult mice and rats (*6*, *7*). A special role of CAT-3 for providing substrate for the neuronal NO synthase (nNOS) has been proposed based on the neuron specific expression of CAT-3 in rat brain (*8*). To elucidate the function of CAT-3 in human tissues, we have cloned a cDNA encoding hCAT-3 and investigated the transport properties of hCAT-3 expressed in oocytes from *X. laevis*. To assess if hCAT-3 might be essential for the substrate supply of nNOS, we compared the tissue distribution of nNOS in the central nervous system and peripheral tissues with that of hCAT-3, as well as other hCAT isoforms.

EXPERIMENTAL PROCEDURES

Cell Culture. The human testis teratocarcinoma cell line NT2 was purchased from Stratagene (Heidelberg, Germany). Cells were grown in a 1+1 mixture of Dulbecco's minimal essential medium and Ham's F12 Nutrient mix, supplemented with 2 mM glutamine and 10% fetal bovine serum. The U373 MG glioblastoma cell line was obtained from ATCC, Bethesda, MD. Cells were grown in Iscove's medium supplemented with 10% fetal bovine serum. Cells were regularly tested for mycoplasm infection using 4′,6 diamidine 2′-phenylindole dihydrochloride (DAPI, Roche Molecular Biochemicals, Mannheim, Germany). No contamination was detected.

Cloning of a cDNA Encoding hCAT-3. Reverse transcription (RT) was performed using 2 μg of total RNA from NT2 cells as template, a combination of oligo(dT)$_{12-18}$ and random hexamers as primer and Gibco/BRL Superscript reverse transcriptase (Life Technologies, Karlsruhe, Germany). The obtained cDNA served as template in polymerase chain reactions (PCR) using the oligo nucleotides: GCCTTCAYCACYGGCTGGAA and CCRATDGACATSAKGTCCAC (initial cloning step) or GGAAGATCTCTTCAATTCCTGCTAGGATGC and GGAGATCTGGTGTGACGATGTCAAACTGA (final cloning step, *Bgl*II sites and start and stop codons underlined) as sense and antisense primer, respectively, and Expand polymerase (Roche Diagnostic, Mannheim, Germany). The 911 nucleotide (nt) PCR product from the initial RT/PCR was cloned into pXcmI (*9*), resulting in the plasmid pXcmHC3/1. Filters with human plasmid cDNA libraries from different brain regions (resource center, German human genome project, Berlin, Germany) were screened under stringent conditions using the ^{32}P-labeled insert of pXcmHC3/1 as a hybridization probe (oligo labeling kit, Pharmacia, Freiburg). 5′ RACE (rapid amplification of cDNA ends) was performed using the Marathon cDNA Amplification kit (Clontech, Heidelberg, Germany) and the oligonucleotides CACATCTCTAAGACTCTGCAGGGG and CTCTCCTATGTCATTGGTACAGCCAG as antisense primers in the first and second amplification step, respectively. The PCR product from the second amplification step was cloned into pCR-Script SK(+) (Stratagene, Heidelberg, Germany) and sequenced. Two products from independent final RT/PCRs were cloned into pCR-Script SK(+) (resulting in HC3.pCR.ges I and II) and sequenced.

Expression of cRNAs in X. laevis Oocytes. The insert of HC3.pCR.ges I was subcloned into the *Bgl*II site of pSP64T (*10*) resulting in HC3.pSP64T. The plasmids HC3.pSP64T and CAT-3.EGFP-pSP64T (see below) were linearized with *Sal*I and *Afl*III, respectively, and cRNA was prepared by in vitro transcription from the SP6 promoter (mMessage mMachine in vitro transcription kit, Ambion, AMS Biotechnology Europe, Wiesbaden, Germany). A total of 36 ng of cRNA (in 36 nL of H_2O) was injected into each *X. laevis* oocyte (Dumont stage V−VI). Oocytes injected with 36 nL of water were used as controls.

Transport Studies in X. laevis Oocytes. L-Arginine uptake was determined 2 days after injection of cRNA as previously described (*5*). Briefly, oocytes were equilibrated for 2 h at 20 °C in "uptake solution" (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, and 5 mM Tris, pH 7.5) containing the indicated concentrations of unlabeled L-amino acids. The oocytes were then transferred to the same solution containing in addition ^{3}H- or ^{14}C-labeled L-amino acids [5 μCi/mL; L-[^{3}H]arginine, L-[^{3}H]lysine, L-[^{3}H]serine, L-[^{3}H]leucine, L-[^{3}H]glutamine, L-[^{3}H]proline, L-[^{3}H]phenylalanine, L-[^{3}H]glutamic acid, L-[^{14}C]ornithine (ICN, Eschwege, Germany); L-[^{14}C]citrulline (Dupont NEN, Bad Homburg, Germany); L-[^{3}H]methionine, L-[^{3}H]histidine (Biotrend, Köln, Germany)]. After a 15 min incubation at 20 °C, the oocytes were washed four times in ice cold "uptake solution" and solubilized individually in 2% sodium dodecyl sulfate (SDS). The incorporated radioactivity was determined in a liquid scintillation counter.

For trans-stimulation experiments, three oocytes were injected with 3.6 nmol L-[^{3}H]arginine (3.6 nCi) in 36 nL of water each. The oocytes were then transferred into an uptake solution containing either 1 mM L-arginine or no cationic amino acids. After a 30 min incubation at 20 °C, the L-[^{3}H]-arginine that had accumulated in the uptake solution was determined by liquid scintillation counting.

Dot Blot Analyses. The plasmids pSPhCAT-1AB1, phCAT-2A104, HC3.pSP64T, hCAT-4.pSP64T, and pNO1wt containing the complete coding region of hCAT-1, -2, -3, -4, and human nNOS, respectively, were restricted to release the respective inserts. After gel purification, 25 ng of each insert were labeled with ^{32}P-dCTP (ICN Biomedicals, Eschwege, Germany) by the random prime method (oligo-labeling kit, Amersham Pharmacia Biotech, Freiburg, Germany). A commercial filter containing polyA^+ RNA from 68 human tissues and 8 cell lines (MTE Array, Clontech, Heidelberg, Germany) was hybridized consecutively with the probes for hCAT-3, -4, -1, nNOS, and hCAT-2, using the protocol given by the manufacturer. After each hybridization, the remaining probe was removed by boiling in 0.5% SDS (10 min).

Ribonuclease Protection Analyses. A plasmid containing a 243 nt fragment of hCAT-3 (pXcmHC3/4) was generated by deleting the *Pst*I/*Pst*I and a *Nco*I/*Xba*I fragments from pXcmHC3/1. A plasmid containing a 108 nt cDNA fragment of the human βactin cDNA (pCR_βactin_hu__Δ*Bst*EII_*Hin*dIII) has previously been generated (*11*). To generate radio-labeled antisense RNA probes, pXcmHC3/4 was linearized with *Eco*RI and pCR_βactin_hu_Δ*Bst*EII_*Hin*dIII with *Asp*718. In vitro transcription was performed as previously described (*12*) resulting in α-^{32}P-labeled riboprobes of 292 nt for hCAT-3, and 228 nt for hβactin. Total RNA was isolated from NT2 cells or human tissues using the method of Chomczynski and Sacchi (*13*). Ribonuclease protection analyses were performed with 20 μg of RNA/sample as described (*12*).

Generation and Expression of EGFP Fusion Proteins. A PCR fragment containing the complete coding sequence of hCAT-3 was generated using the oligonucleotides ATGTCCTTGCAGAATATCCAG and GGAGTCGACTGAACTGAGTGGACATAGAGAGT (*Sal*I site underlined) as sense and antisense primers and HC3.pCR.ges I as template, and cloned in the *Sal*I/*Eco*RI sites of pEGFP-N1 (Clontech, Heidelberg, Germany). The plasmid obtained was restricted with *Bgl*II and *Xba*I and the *Bgl*II and *Xba*I fragment from HC3.pCR.ges I was inserted. U373 MG glioblastoma cells were transfected with the resulting plasmid (hCAT-3-pEGFP.N1) using Superfect (Qiagen, Milden, Germany). Two days after transfection, the cells were split and exposed to G418 (400 μg/mL). Cell clones expressing hCAT-3.EGFP were singled out and grown to confluence in G418-containing medium.

For expresssion in *X. laevis* oocytes, hCAT-3-pEGFP.N1 was cut with *Not*I and *Nhe*I, blunted, and inserted in the blunted *Bgl II* site of pSP64T (resulting plasmid, hCAT-3.EGFP-pSP64T).

Protein Lysates and Western Blots. U373 MG glioblastoma cells stably transfected with hCAT-3-pEGFP.N1 and grown to confluence in culture plates (diameter, 10 cm) were washed three times with PBS, scraped from the plates, and pelleted at 180*g*. Cells were then lysed with 2 vol of NP-40 buffer (10 mM Tris/HCl pH 7.5; 10 mM NaCl; 1 mM $MgCl_2$; 0.5% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride; 0.3 μg/mL aprotinin; 1 μg/mL leupeptin; 1 mg/mL pepstatin), and the nuclei pelleted at 720*g*. After determining the protein concentration (using the Bradford reaction) aliquots of the lysates were treated for 1h at 37 °C with N-glycosidase F (Roche Molecular Biochemicals, Mannheim, Germany, 10 units/100μg) and then an equal volume sample buffer containing 6 M urea was added.

Lysates (20 μg of protein) were separated in 8% SDS−polyacrylamide gel electrophoresis (PAGE) and then blotted to nitrocellulose membranes (Protran83, Schleicher and Schuell, Dassel, Germany). Staining for hCAT-3.EGFP protein was achieved by sequential incubations in Blotto (50 mM Tris/HCl pH 8; 2 mM $CaCl_2$; 0.01% antifoam A (Sigma, Deisenhofen, Germany); 0.05% Tween 20; 5% nonfat dry milk) containing 10% goat serum (2 h, room temperature), a 1:300 dilution of an anti-GFP polyclonal antibody (Clontech, Heidelberg, Germany) in PBS containing 1% bovine serum albumin and 0.1% Tween 20 (overnight, 4 °C), three times Blotto (15 min, room temperature), a 1:10000 dilution in Blotto of a peroxidase-conjugated secondary goat anti-rabbit IgG antibody (Calbiochem, Bad Soden, Germany) (1 h, room temperature); three times TBST (10 mM Tris/HCl, pH 8; 150 mM NaCl; 0.05% Tween 20), once in TBS (10 mM Tris/HCl pH 8; 150 mM NaCl) and finally for 1 min with chemiluminescence reagent (Renaissance, Dupont NEN, Bad Homburg, Germany). A X-ray film (Agfa, Leverkusen, Germany) was then immediately exposed to the membranes.

Statistics. Results were compared for statistical differences using either Student's T-test or analysis of variance with the Bonferroni post hoc test. *P*-values of <0.001 were marked by three asterisks, *P*-values of >0.05 were considered not significant and marked by ns.

Protein Analysis. Glycosylation, kinase, and myristoylation sites were identified using the MacVector 6.5 protein sequence analysis program.

Table 1: Comparison of the hCAT-3 Amino Acid Sequence with Other CAT Proteins

	hCAT-3	
	total	functional domain
mCAT-3	82.0 (90.6)	73.8 (92.8)
rCAT-3	83.4 (90.8)	88.1 (95.2)
hCAT-1	61.8 (74.8)	71.4 (85.7)
hCAT-2A	58.6 (73.6)	52.4 (83.3)
hCAT-2B	59.9 (74.1)	71.4 (90.5)
hCAT-4	42.6 (61.0)	42.9 (70.7)

[a] The table compares the deduced amino acid sequence of hCAT-3 with those of mouse and rat CAT-3, as well as with those of the four human CAT isoforms identified to date. Numbers indicate % identity (in parentheses % similarity) after an optimal alignment of the complete amino acid sequences (left column) or of the short 42 amino acid sequence that differs between CAT-2A and CAT-2B (functional domain, right column). The corresponding sequences in mouse, rat and human CAT-3 (pos. 353−394), hCAT-1 (pos. 355−396), and hCAT-4 (pos. 337−378) were identified after a clustal alignment of the complete amino acid sequences of all CAT-isoforms. Sequences were aligned using the NCBI BLASTP program. The sequence of the hCAT-3 cDNA has been deposed in GenBank, accession number: AF320612

RESULTS

Isolation of hCAT-3 cDNAs and Analyses of the Deduced Amino Acid Sequence. Different cloning strategies were necessary to obtain the complete coding region of hCAT-3. First, we performed RT/PCR using RNA from different human neuronal, glia, and teratocarcinoma cell lines and a pair of oligonucleotides with sequences well conserved in all the previously known hCATs. Restriction enzyme analyses of the PCR fragments obtained showed that the fragment amplified from NT2 teratocarcinoma cells did not conform to hCAT-1, -2A, -2B, or -4. Further analysis revealed sequence homology with the recently identified mouse and rat CAT-3. Using the PCR fragment as a probe, we then screened cDNA libraries from different regions of the human brain to obtain a full-length cDNA for hCAT-3. Only one positive clone (from a fetal brain library) was identified screening a total of 11 libraries containing more than 470 000 individual clones. Other libraries used were derived from hypothalamus, hippocampus, total adult brain, subthalamus, substantia nigra, cerebellum, and amygdala. Sequence analyses showed that the positive clone encodes for the 236 C-terminal amino acids of hCAT-3. Subsequently, the missing 5′ sequence of the hCAT-3 cDNA was identified by 5′ RACE using mRNA from NT2 cells. The sequence of the complete coding region of hCAT-3 has been deposed in GenBank (accession number AF320612). The deduced amino acid sequence is 83.4 and 82.0% identical to rat and mouse CAT-3, respectively (Table 1). In the short protein domain (43 amino acids) that has been shown to determine the transport properties of the CAT proteins (*14*), the hCAT-3 sequence differs by 5 and 11 residues from rCAT-3 and mCAT-3, respectively. Like the latter, hCAT-3 contains only one potential glycosylation site (N^{232}) in the third extracellular loop. Seven of nine recognition sites for casein kinase II and all 13 sites for myristoylation in hCAT-3 are also present in rCAT-3 and mCAT-3. In contrast, the recognition sites for cAMP-dependent kinase (T^{18}, N-terminus), protein kinase C (S^{453}, 5th intracellular loop and S^{599}, C-terminus) and protein tyrosine kinase (Y^{224}), found in hCAT-3, are not conserved in rat and mouse. According to the model with

14 membrane spanning domains, the latter is located in the third extracellular loop and therefore unlikely to be phosphorylated.

Characterization of the Transport Properties of hCAT-3 Expressed in X. laevis Oocytes. A cDNA containing the complete coding sequence of hCAT-3 was obtained by RT/PCR using mRNA from NT2 cells. The cDNA was inserted into pSP64T. To analyze the transport properties of hCAT-3, cRNA transcribed in vitro from the resulting plasmid (HC3.pSP64T) was injected into *X. laevis* oocytes and transport activity was assayed 2 days later. Transport studies using radiolabeled tracers demonstrated at least a 10-fold higher uptake of L-arginine, L-lysine, and L-ornithine (each 100 μM) in cRNA-injected oocytes as compared to control oocytes injected with water (Figure 1A). In contrast, no transport activity could be detected when cRNA-injected oocytes were incubated with anionic (Glu) or neutral L-amino acids (Ser, Leu, Gln, Pro, Phe, Met, or Citrulline). To measure uptake of L-histidine in its protonated and zwitterionic form, transport studies were performed at pH 5.5 and pH 7.5, respectively. hCAT-3 did not transport L-histidine under either condition (Figure 1B). In contrast, L-histidine became a substrate for hCAT-1 at pH 5.5. A significant inhibition of hCAT-3-mediated L-arginine uptake (50 μM) could only be observed for L-lysine, but not D-arginine (Figure 1C). Uptake of cationic amino acids was linear over a 60 min period (data not shown). Measurements of cationic amino acid uptake over 15 min revealed a concentration-dependent transport activity (Figure **2**). The apparent half-saturating substrate concentrations determined by fitting the data according to the Eadie Hofstee equation (after subtraction of the values obtained from water-injected oocytes) were 0.45 $\pm$ 0.13 mM for L-arginine, 0.65 $\pm$ 0.23 for L-lysine, and 0.91 $\pm$ 0.26 for L-ornithine. The V_{max} values obtained were 1.4 $\pm$ 0.3, 1.4 $\pm$ 0.7, and 2.5 $\pm$ 0.8 nmol/oocyte/h for L-arginine, L-lysine, and L-ornithine, respectively. The transport activity of hCAT-3 measured at 100 μM L-arginine was not significantly different when choline chloride was substituted for sodium chloride in the uptake buffer (Figure **3**A). Similarly, changes in the pH of the uptake buffer between 5.5 and 8.5 did not alter the transport activity of hCAT-3 (Figure **3**B).

Efflux experiments were performed to assay hCAT-3 for trans-stimulation, defined as the stimulation of transport by substrate at the trans-side of the membrane. To this end, oocytes injected with cRNA of hCAT-3 were reinjected 2 days later with tritiated L-arginine (3.6 nCi, 3.6 nmol). Measurement of efflux immediately after the second injection showed an about 2-fold higher efflux into an isotonic salt solution containing 1 mM L-arginine (trans-substrate) compared to the same solution containing no extracellular cationic amino acids (Figure 4).

Expression of hCAT3 cDNA in Human Tissues and Cell Lines. A commercial filter containing poly(A^+) RNA from 68 different human tissues and 8 cell lines was hybridized with a probe for hCAT-3. This analysis showed that hCAT-3 is not only expressed in different regions of the human brain but also in a number of peripheral tissues. In fact, by far the strongest expression of hCAT-3 was observed in thymus and Burkitt's lymphoma cells (Figure **5**A). Also in uterus, testis, mammary gland, and MOLT-4 leukemia cells, the level of hCAT-3 expression was at least as high as in the brain areas

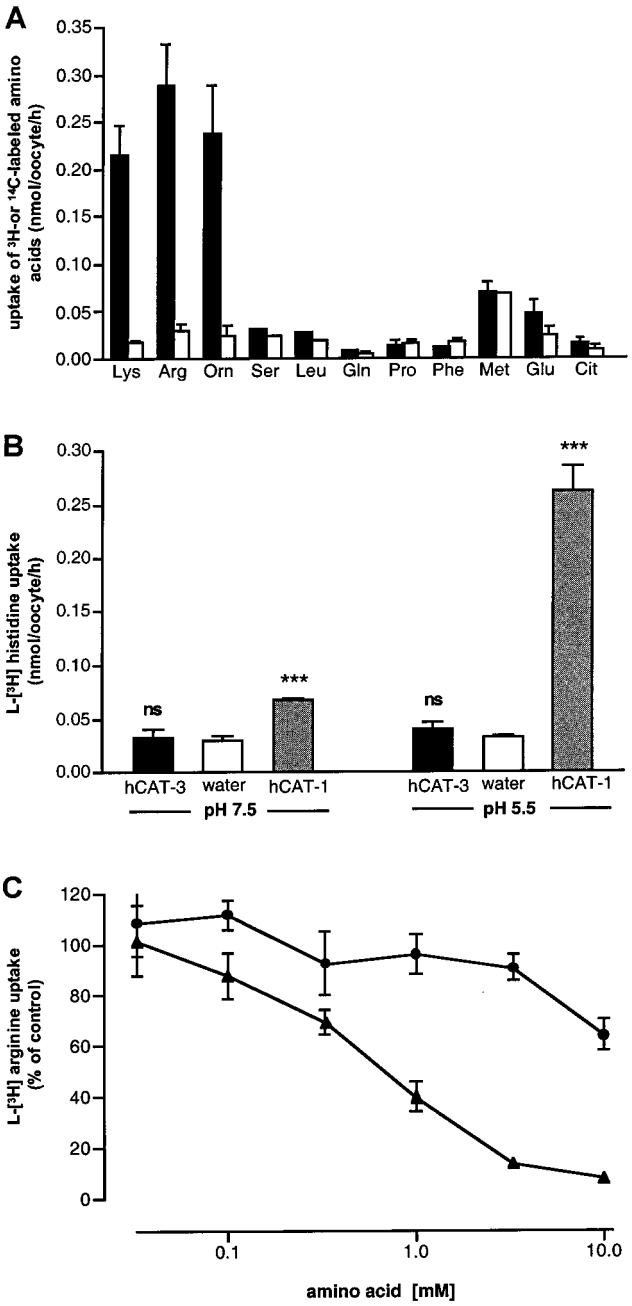


FIGURE 1: Substrate specificity of hCAT-3. (A) *X. laevis* oocytes were injected with 36 ng of hCAT-3 cRNA (in 36 nL of water, black bars) or with 36 nL of water alone (open bars). Two days later, uptake of the indicated ^{3}H- or ^{14}C-labeled amino acids (each 100 μM) was measured (15 min, 20 °C). (B) The uptake of 100 μM L-[^{3}H]histidine in oocytes injected with hCAT-3 (black bars), water (open bars) or hCAT-1 (gray bars) was measured at pH 7.5 and 5.5 as indicated. (C) In oocytes expressing hCAT-3, the uptake of 50 μM L-[^{3}H]arginine was measured in the presence of the indicated concentration of L-lysine (triangles) or D-arginine (circles). Here, the values obtained with water-injected oocytes were subtracted from the respective value obtained with hCAT-3-injected oocytes. Data points represent means $\pm$ SEM, $n = 3$ with 5−10 replicates each.

with the strongest hCAT-3 expression (thalamus, amygdala, and hippocampus). In ovary and stomach, a weak hCAT-3 signal was found, comparable to a number of different brain areas. The expression of hCAT-3 in human uterus, testis, ovary, and mamma was confirmed by RNase protection analysis (Figure **6**). Here, only a low hCAT-3 expression

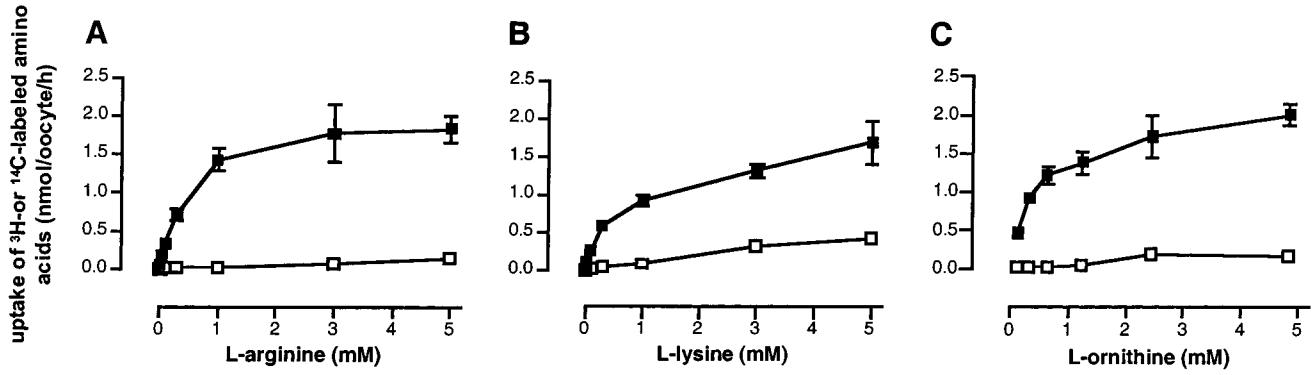


FIGURE 2: Concentration-dependent uptake of cationic amino acids by hCAT-3. *X. laevis* oocytes were injected with 36 ng of hCAT-3 cRNA (in 36 nL of water, closed squares) or with 36 nL of water alone (open squares). Two days later, uptake of the indicated concentrations of L-[^{3}H]arginine (A), L-[^{3}H]lysine (B), or L-[^{14}C]ornithine (C) was measured (15 min, 20 °C). Data points represent means ± SEM from one typical experiment with 5−10 replicates.

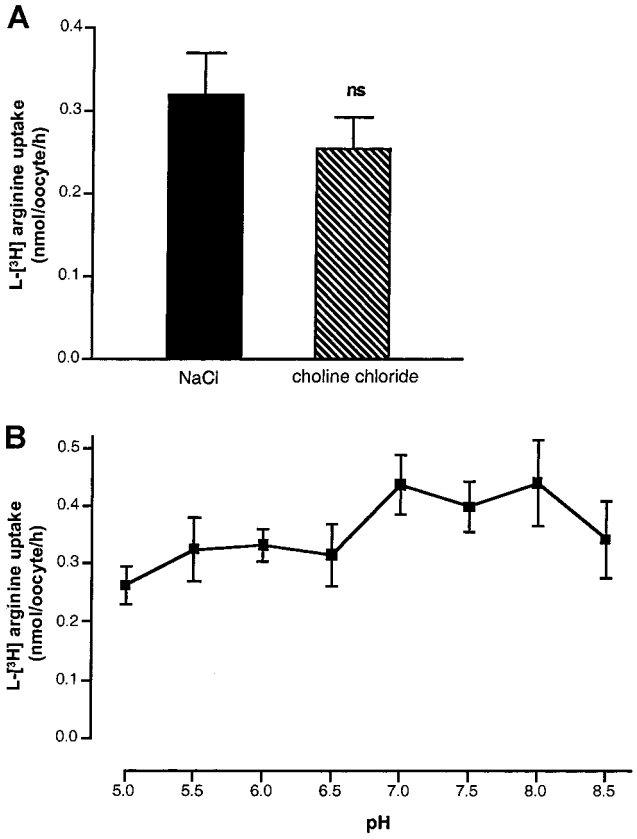


FIGURE 3: Na^{+}- and pH independence of hCAT-3. *X. laevis* oocytes were injected with 36 ng hCAT-3 cRNA (in 36 nL of water). Two days later, the uptake of 100 μM L-[^{3}H]arginine (15 min, 20 °C) was measured (A) in buffers containing either 100 mM NaCl (black bars) or 100 mM choline-chloride (hatched bars) or (B) in buffers with different pH as indicated. The values obtained with water-injected oocytes were subtracted from the respective value obtained with hCAT-3-injected oocytes. Data points represent means ± SEM, $n = 3$ with 5−10 replicates each.

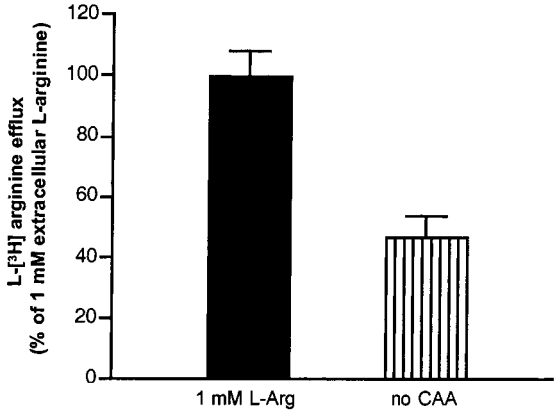
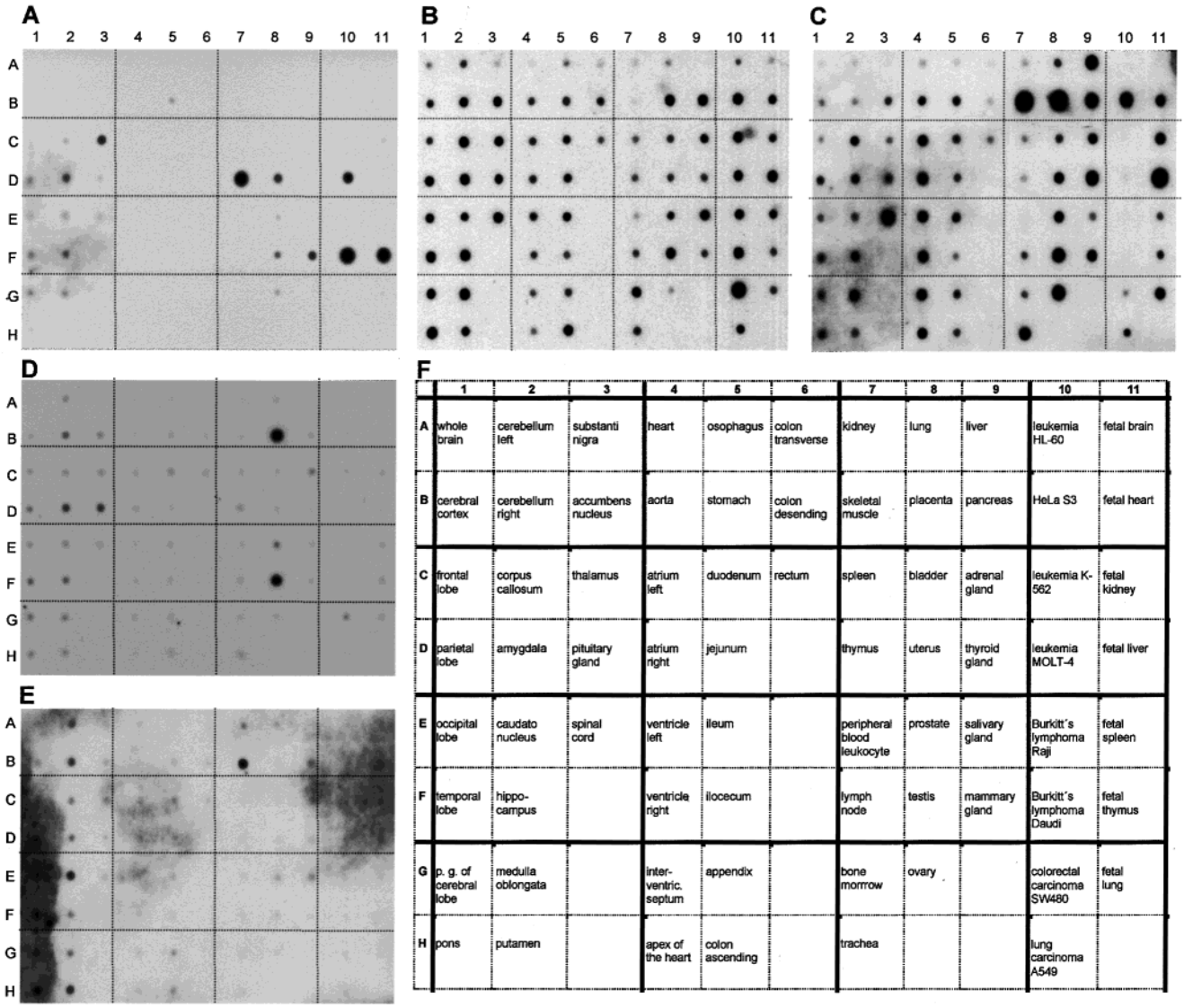


FIGURE 4: Trans-stimulation of hCAT-3. *X. laevis* oocytes were injected with 36 ng of hCAT-3 cRNA (in 36 nL of water). Two days later, the oocytes were injected a second time with 3.6 nmol of L-[^{3}H]arginine (in 36 nL of water) and immediately transferred to buffer containing either 1 mM L-arginine or no cationic amino acids. The efflux of L-[^{3}H]arginine was measured for 30 min at 20 °C. The values obtained with water-injected oocytes were subtracted from the respective value obtained with hCAT-3-injected oocytes. The resulting values were expressed as % of the mean efflux in 1 mM extracellular L-arginine. Data points represent means ± SEM, $n = 6$ with 5−10 replicates each.

was detected in mamma, probably due to the heterogeneity of this tissue. To compare the expression pattern of hCAT-3 with other CAT isoforms and with nNOS, the filter with multiple human RNAs was also hybridized with specific probes for hCAT-1, -2, and -4 and for human nNOS (Figure 5, panels B−E). In all areas of the central nervous system, a weak signal for hCAT-4 and a strong signal for hCAT-1 could be detected. Surprisingly, also a rather strong hCAT-2 expression was found in most tissues of the central nervous system. The tissues with the highest CAT-3 expression in the central nervous system (thalamus and amygdala) showed only a moderate nNOS expression. On the other hand, the tissues with the highest nNOS expression in the central nervous system showed either a weak hCAT-3 expression (caudato nucleus) or no signal at all for hCAT-3 (cerebellum, pons, putamen). In the periphery, hCAT-1 and -2 were also detected in most tissues. However, in thymus, hCAT-3 seemed to be the prominent CAT isoform. In uterus and mammary gland hCAT-3 was coexpressed with hCAT-1 and -2 and in testis with all known CAT isoforms. In addition to testis, a strong signal for hCAT-4 could only be detected in placenta. There was no overlap between nNOS and hCAT-3 expression in peripheral tissues. The strongest nNOS expression was found in skeletal muscle, but weaker signals could also be detected in kidney, pancreas, prostate, salivary gland and intestine.

Glycosylation and Membrane Localization of hCAT-3. EGFP Fusion Proteins Expressed in X. laevis Oocytes and Human Glioblastoma Cells. The low activity of mCAT-3 expressed in oocytes from *X. laevis* (*7*) had suggested to us



	1	2	3	4	5	6	7	8	9	10	11
A	whole brain	cerebellum left	substanti nigra	heart	osophagus	colon transverse	kidney	lung	liver	leukemia HL-60	fetal brain
B	cerebral cortex	cerebellum right	accumbens nucleus	aorta	stomach	colon desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart
C	frontal lobe	corpus callosum	thalamus	atrium left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia K-562	fetal kidney
D	parietal lobe	amygdala	pituitary gland	atrium right	jejunum		thymus	uterus	thyroid gland	leukemia MOLT-4	fetal liver
E	occipital lobe	caudato nucleus	spinal cord	ventricle left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt´s lymphoma Raji	fetal spleen
F	temporal lobe	hippo-campus		ventricle right	ilocecum		lymph node	testis	mammary gland	Burkitt´s lymphoma Daudi	fetal thymus
G	p. g. of cerebral lobe	medulla oblongata		inter-ventric. septum	appendix		bone morrow	ovary		colorectal carcinoma SW480	fetal lung
H	pons	putamen		apex of the heart	colon ascending		trachea			lung carcinoma A549	

FIGURE 5: Expression of hCAT-3 in different human tissues and cell lines (Dot blot analyses). A human multiple tissue expression array (Clontech, Heidelberg, Germany) containing poly(A)$^+$ RNA from human tissues and cell lines [as indicated in the table (panel F)] was hybridized consecutively with ^{32}P-labeled cDNA probes for hCAT-3 (A), hCAT-1 (B), hCAT-2 (C), hCAT-4 (D), and human nNOS (E). X-ray film was exposed to the membrane for 1−2 days.

that part of the overexpressed CAT-3 might stay in intracellular membranes. To study the subcellular localization and glycosylation of hCAT-3, we expressed fusion proteins between the enhanced green fluorescent protein (EGFP) and the C-terminus of hCAT-3 in *X. laevis* oocytes and U373 MG glioblastoma cells. In both cell types, hCAT-3 protein was mainly localized to the plasma membrane (Figure 7, panels A and B). Western blot analysis with lysates from U373 MG glioblastoma cells expressing hCAT-3.EGFP demonstrated that hCAT-3 was strongly glycosylated (Figure 7C).

DISCUSSION

Our study demonstrates that hCAT-3 is glycosylated and predominantly targeted to the plasma membrane in human cells and *X. laevis* oocytes. hCAT-3 exhibited a similar maximal transport activity as other CAT proteins. This is in contrast to the data obtained with mCAT-3 whose activity was reported to be about 100-fold lower when expressed in *X. laevis* oocytes compared to other CAT family members reported in the literature [V_{max} values, 0.02−0.06 versus 1−8 nmol/oocyte/h (*7*)]. Also Hosokawa and co-workers found only a 2-fold increase in the transport rate of cationic amino acids when they expressed rCAT-3 transiently in COS-7 cells (*6*). However, this relatively low activity might have been due to a low transfection rate. Although V_{max} values varied between different experiments in our hands, the higher transport activity for L-ornithine compared to L-arginine or L-lysine seems to be an intrinsic property of hCAT-3, as it was observed regularly also in parallel experiments where the transport of the different amino acids was measured with the same batch of oocytes. Like for hCAT-1, -2A, and -2B, hCAT-3-mediated transport was selective for cationic L-amino acids and Na^+-independent. In mouse and rat, CAT-3 mediated L-arginine transport has been reported to be competitively inhibited not only by other cationic amino acids, but also by L-citrulline, D-arginine (*6*), L-methionine, L-cysteine, and even L-aspartate and L-glutamate (*7*). In our experiments, hCAT-3 exhibited no transport activity for

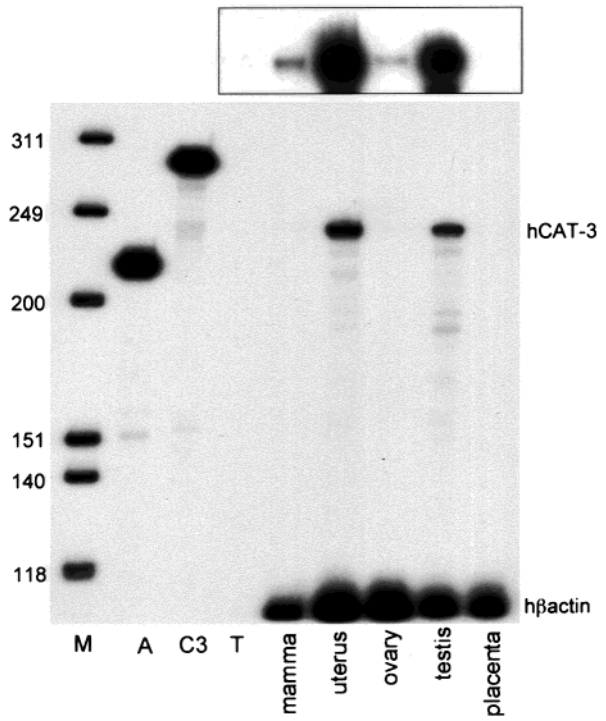


FIGURE 6: Expression of hCAT-3 in different human tissues (RNase protection analyses). Total mRNA was prepared from human mamma, uterus, ovar, testis, and placenta. The RNAs were hybridized with antisense cRNA probes specific for hCAT-3, and for hβactin (as an internal control). After RNase treatment, the protected RNA fragments (hCAT-3, 243 nt; hβactin, 108 nt) were separated on a 6% denaturing polyacrylamide gel. M, DNA size marker [pGl2-Basic (Promega), restricted with *Hin*fI]; A and C3, undigested probes for hβactin (228 nt) and hCAT-3 (292 nt), respectively; T, t-RNA used as a negative control. The inset on top shows a longer exposure of the gel, where a hCAT-3 signal could also be detected in mamma and ovary.

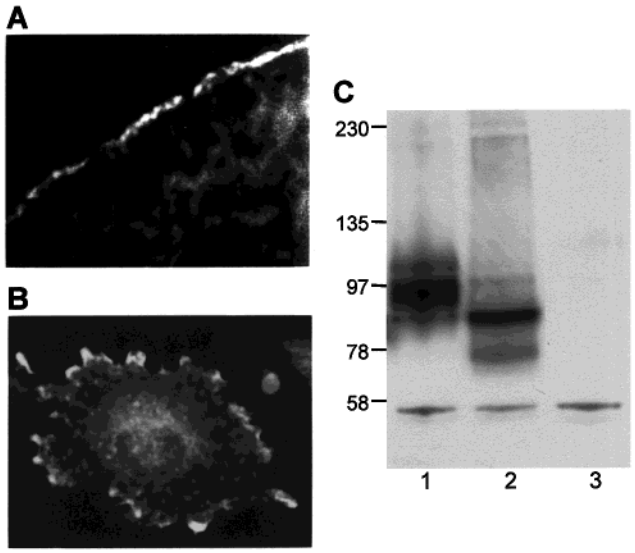


FIGURE 7: Protein expression of hCAT-3.EGFP fusion proteins. (A) Fluorescent micrograph of a Cryosection of a *X. laevis* oocyte injected with hCAT-3.EGFP cRNA. (B) Fluorescent micrograph of human U373 MG glioblastoma cells stably transfected with hCAT-3-pEGFP.N1. (C) Western blot with lysates from U373 MG glioblastoma cells, stably transfected with hCAT-3-pEGFP.N1 (lanes 1 and 2) or non transfected controls (lane 3). A total of 20 μg of protein/lane was separated by 8% SDS−PAGE, blotted and the membrane incubated with anti-GFP antibody. The lysate in lane 2 was treated with N-glycosidase F.

L-citrulline, L-methionine, L-cysteine and L-glutamate. In addition, D-arginine at concentrations up to 10 mM caused no significant inhibition of the transport of 50 μM L-arginine. These data suggest that the substrate recognition of hCAT-3 is very similar to that of the other CAT-isoforms. Yet, in contrast to hCAT-1, hCAT-3 did not transport L-histidine even at pH 5.5, where it is mostly protonated.

The apparent affinity of hCAT-3 for L-arginine was similar to that of hCAT-2B and consequently 4−5-fold higher than those reported for mouse or rat CAT-3. Also like hCAT-2B, hCAT-3 was only moderately trans-stimulated confirming results by Ito et al. (*7*) that showed an about 2-fold trans-stimulation of mCAT-3. The moderate trans-stimulation effect in our efflux experiments, e.g., the fact that considerable transport could be observed at zero substrate at the trans side, demonstrates that hCAT-3 is not an obligate exchanger, but can function as an unidirectional transporter. It is likely that this is also true for hCAT-3-mediated uptake of cationic amino acids, as in previous experiments with mCAT-1 and -2A, we could demonstrate that the dependence of the CAT proteins on trans-substrate is similar in influx and efflux experiments (*15*). hCAT-3 was largely independent of the extracellular pH, resembling more hCAT-1 in this respect (*5*). As reported for mCAT-2B (*16*, *17*), the apparent affinity of hCAT-3 for L-ornithine was about 2-fold lower than for L-arginine. The apparent affinity for L-lysine was intermediate. In contrast, Ito et al., have found an 2−3-fold higher affinity of mCAT-3 for L-arginine compared to L-lysine (*7*).

Despite the described differences in the transport properties, the sequence similarities strongly suggest that the cDNAs isolated in our study code for the human homologue of rat and mouse CAT-3. At present, no other CAT-related sequence can be found in the sequenced human genome. However, surprisingly, also no genomic hCAT-3 sequence is available to date in Genbank. The sequences of our independent cDNA fragments from NT2 cells are 100% identical, where overlapping also with the sequences obtained from fetal brain. In the functional domain, hCAT-3 exhibits a significant higher sequence identity with the high affinity isoforms hCAT-1 and -2B than with the low affinity hCAT-2A or with hCAT-4. However, the CAT-3 sequences show a relative high interspecies divergence in this domain. They differ in 11, 5, and 7 of 43 residues between human and mouse, human and rat, and mouse and rat, respectively. This might explain the species differences in the transport properties of CAT-3.

Our expression studies show clearly that the expression of CAT-3 is not confined to human brain. Indeed, in dot blot analyses, by far the highest hCAT-3 expression was found in thymus. However, also in uterus, testis and mammary gland, hCAT-3 expression was equal or higher than in most brain regions and could easily be detected by RNase protection analyses. The low abundance of hCAT-3 in human brain is also reflected in the low yield of positive clones in brain-derived cDNA libraries. The relative high expression of hCAT-3 in thalamus and hippocampus is consistent with earlier findings in mouse and rat brain (*7*, *18*). However in amygdala, that also showed a relative strong CAT-3 signal in our analyses, CAT-3 expression was barely detectable in the rat (*18*).

System y^+-like transport activity has been described in a variety of neural cells (*19*−*22*). Our dot blot analyses suggest that hCAT-1 and -2 (A or B) are the predominant CAT isoforms in the human brain. For CAT-1, an ubiquitous neuronal and glial expression has been shown in the rat brain (*18*). Also, in neuronal cultures derived from rat hypothalamus/brainstem basal rates of L-arginine transport were principally attributed to CAT-1 activity (*23*). The relative strong expression of CAT-2 (A or B) in human brain was rather surprising. The hybridization probe used does not distinguish the two splice variants, and further analyses are needed to determine the cell types that express each CAT isoform in the human brain.

Within the central nervous system, there was no correlation between the expression of hCAT-3 and nNOS, suggesting that hCAT-3 is not directly involved in supplying substrate for nNOS. However, there is increasing evidence that in the brain, L-arginine metabolizing and synthesizing or recycling enzymes are localized in distinct cell types requiring the flux of L-arginine and intermediates between cells (*18*; for review, see ref *24*). hCAT-3 might be expressed in cells that provide L-arginine or other cationic amino acids to other cells. The weak dependence of the hCAT-3 activity on substrate on the trans-side of the membrane makes it a good candidate to export cationic amino acids, especially when extracellular cationic amino acid concentrations are low.

The strong expression of hCAT-3 in peripheral tissues, e.g., in thymus, demonstrates that hCAT-3—unlike rat and murine CAT-3—is not neuron-specific. Also in the periphery, there was basically no overlap between tissues expressing hCAT-3 and nNOS, again excluding a direct role for hCAT-3 in the substrate supply of nNOS. The expression of mCAT-3 has been found in the mesoderm and in many developing tissues of mid streak mouse embryos (*7*). In addition, mCAT-3 has been shown to compensate for the loss of functional CAT-1 in cells derived from CAT-1 knockout mice (*25*). Yet, no data were available about CAT-3 expression in fetal tissues. In our dot blot analyses, hCAT-3 expression could only be detected in fetal thymus, but no other fetal tissue, indicating that a wide CAT-3 expression is confined to embryonic states. The specific role hCAT-3 might play in thymus and other peripheral tissues remains to be elucidated.

In conclusion, our data demonstrate that hCAT-3 is predominantly expressed in thymus and to a lesser extent in other peripheral tissues and in brain. They further suggest that hCAT-3 expression is not confined to neuronal cells. A direct role of hCAT-3 for supplying substrate for nNOS seems unlikely, as no correlation could be observed between the expression of the two proteins. The transport properties (K_M, V_{max}, trans-stimulation, substrate recognition) of hCAT-3 resembled most closely hCAT-2B, except for its pH independence.

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