# Expression of solute carrier 7A4 (SLC7A4) in the plasma membrane is not sufficient to mediate amino acid transport activity

Sabine WOLF*, Annette JANZEN*, Nicole VÉKONY*, Ursula MARTINÉ*, Dennis STRAND† and Ellen I. CLOSS*[1]

*Department of Pharmacology, Johannes Gutenberg University, Obere Zahlbacher Strasse 67, 55101 Mainz, Germany, and †Department of Internal Medicine, Johannes Gutenberg University, Obere Zahlbacher Strasse 63, 55101 Mainz, Germany

Member 4 of human solute carrier family 7 (SLC7A4) exhibits significant sequence homology with the SLC7 subfamily of human cationic amino acid transporters (hCATs) [Sperandeo, Borsani, Incerti, Zollo, Rossi, Zuffardi, Castaldo, Taglialatela, Andria and Sebastio (1998) Genomics **49**, 230–236]. It is therefore often referred to as hCAT-4 even though no convincing transport activity has been shown for this protein. We expressed SLC7A4 in *Xenopus laevis* oocytes, but could not detect any transport activity for cationic, neutral or anionic amino acids or for the polyamine putrescine. In addition, human glioblastoma cells stably overexpressing a fusion protein between SLC7A4 and the enhanced green fluorescent protein (EGFP) did not exhibit an increased transport activity for L-arginine. The lack of transport activity was not due to a lack of SLC7A4 protein expression in the plasma membrane, as in both cell types SLC7A4-EGFP exhibited a similar subcellular localization and level of protein expression as functional hCAT-EGFP proteins. The expression of SLC7A4 can be induced in NT2 teratocarcinoma cells by treatment with retinoic acid. However, also for this endogenously expressed SLC7A4, we could not detect any transport activity for L-arginine. Our data demonstrate that the expression of SLC7A4 in the plasma membrane is not sufficient to induce an amino acid transport activity in *X. laevis* oocytes or human cells. Therefore, SLC7A4 is either not an amino acid transporter or it needs additional (protein) factor(s) to be functional.

Key words: heteromeric amino acid transporter, human cationic amino acid transporter (hCAT), nitric oxide, polyamine.

## INTRODUCTION

The family of human cationic amino acid transporters (hCATs) consists of four members; hCAT-1, -2A, -2B and -3, with hCAT-2A and -2B being splice variants (for a review see [1]). The cationic amino acid transporter (CAT) proteins are distantly related to the so-called light subunits of heteromeric amino acid transporters (HATs; for reviews see [2,3]). Both gene families have therefore been classified in the same superfamily, solute carrier family 7 [SLC7; where hCAT-1 is SLC7A1, hCAT-2 (A + B) is SLC7A2, hCAT-3 is SLC7A3, and the HAT light subunits are SLC7A5–SLC7A11]. With optimal alignment, the sequence identity of the individual members of the same subfamily lies between 60 and 61 % for the CATs (74–75 % similarity) and between 39 and 54 % for the HAT light subunit family (61–72 % similarity). In contrast, the members of the two different subfamilies are only about 22–25 % identical (41–46 % similar).

The hCAT proteins seem to be the major entry route for cationic amino acids in most cell types. Owing to the involvement of cationic amino acids in important metabolic pathways, such as nitric oxide and polyamine synthesis, much interest has been attracted to the function and regulation of the hCAT proteins. When overexpressed in oocytes from *Xenopus laevis* or in mammalian cells, all hCAT proteins mediate the $Na^+$-independent transport of cationic amino acids (for reviews see [4–6]). hCAT-1 exhibits all the typical properties of system $y^+$. It has a relatively high affinity for cationic amino acids, is pH-independent and is stimulated strongly by substrate at the *trans* side of the membrane (*trans*-stimulation) [7]. The transport properties of hCAT-2B and -3 also resemble system $y^+$ [7,8]. However, both carrier proteins exhibit a slightly lower affinity for cationic amino acids than hCAT-1 and are less dependent on the presence of *trans*-substrate. hCAT-2A has an approx. 10-fold lower affinity for cationic amino acids and is largely insensitive to *trans*-stimulation [7]. In addition, both hCAT-2A and hCAT-2B are susceptible to pH changes. All hCAT proteins are glycosylated. They do not seem to need a second protein to form a functional transporter. In contrast, the HAT light subunits are not glycosylated and must associate with a glycoprotein (the so-called heavy subunit) to be targeted to the plasma membrane and exhibit transport activity (for reviews see [2,3,9,10]). Two such glycoproteins have been identified to date: rBAT (related to $b^{0,+}$ amino acid transporter, SLC3A1) and 4F2hc (also named CD98 or SLC3A2). The substrates transported by HATs range from cationic to neutral and anionic amino acids. All HATs work preferentially as exchangers.

Sperandeo et al. [11] have identified a cDNA in human placenta coding for a hydrophobic protein with 41–42 % sequence identity to members of the CAT family (59–61 % similarity) and 22–25 % identity to members of the family of HAT light subunits (41–44 % similarity). They have named the protein SLC7A4 and suggested that it is a new member of the hCAT family [11]. It is therefore often referred to as hCAT-4 in the literature. Here, we overexpressed SLC7A4 in *X. laevis* oocytes and mammalian cells and performed transport studies with a large number of different amino acids and polyamines. In addition, we investigated the protein expression and subcellular

Abbreviations used: CAT, cationic amino acid transporter; hCAT, human CAT; EGFP, enhanced green fluorescent protein; HAT, heteromeric amino acid transporter; SLC7, solute carrier family 7; rBAT, related to $b^{0,+}$ amino acid transporter.

[1] To whom correspondence should be addressed (e-mail Closs@mail.uni-mainz.de).

localization of SLC7A4 in these cells. We also identified a human cell line in which endogenous expression of SLC7A4 can be induced and measured L-arginine transport in these cells.

## EXPERIMENTAL

### 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 protocol given by Stratagene was used to differentiate NT2 cells into neurons (NT2N). The U373 MG glioblastoma cell line was obtained from the A.T.C.C. (Manassas, VA, U.S.A.). Cells were grown in Iscove's medium supplemented with 10 % fetal bovine serum. Cells were tested regularly for mycoplasma infection using 4,6-diamidino-2-phenylindole (DAPI; Roche Molecular Biochemicals, Mannheim, Germany). No contamination was detected.

### hCAT-4 cDNA in *X. laevis* oocyte expression vector

The insert of phCAT4-pBluescriptSK(−) [11] was amplified by PCR using the oligonucleotides GGAGGATCCTCTTCTCGGCCACCTGC (HC-4.start; sequence upstream of the start codon, with the *Bam*HI site underlined) and GGAAGATCTAGCTACTCCATATGGCCAGG (containing the stop codon and a *Bgl*II site, both underlined) as sense and antisense primers, respectively. The PCR product was subcloned into the *Bgl*II site of pSP64T [12]. Sequence analysis showed a single nucleotide mutation (G→A) at position 117 of the coding region that was also present in the original clone. The mutation was removed by insertion of a cDNA fragment encompassing the mutation, amplified by reverse transcription of testis mRNA and subsequent PCR. The resulting plasmid was named phCAT4-pSP64T. When PCR was used for further cloning steps, the absence of unwanted mutations was always verified by sequencing the PCR-derived portion of the respective cDNAs.

### Enhanced green fluorescent protein (EGFP) fusion constructs for mammalian expression

The insert of phCAT1-AB1C [7] was amplified by PCR using the oligonucleotides GGAGGATCCTGAACAGCAACATGGGGT (containing a *Bam*HI site and a start codon, both underlined) and GATCCCGGGCCTTGCACTGGTCCAAGT (removes the stop codon and introduces a *Xma*I site, which is underlined) as sense and antisense primers, respectively, and subcloned into the *Bgl*II/*Xma*I sites of pEGFP-N1 (Clontech, Heidelberg, Germany). The resulting plasmid was hCAT1.pEGFP-N1. The insert of phCAT-2B 181 [7] was amplified by PCR using the oligonucleotides CCGGAGCTCAGATCTGACGTCAGAATGATTCCT (containing a *Bgl*II site and a start codon, both underlined), and ACCGTCGACAAGGAATTCACTTGTCTTTTCAT (removes the stop codon and introduces an *Sal*I site, which is underlined) as sense and antisense primers, respectively, and subcloned into the *Bgl*II/*Sal*I sites of pEGFP-N1 (resulting plasmid hCAT2B-pEGFP-N1).

The insert of phCAT4-pSP64T was amplified by PCR using the oligonucleotides HC-4.start and ACCGTCGACTCCATATGGCCAGGGTCC (which removes the stop codon and introduces a *Sal*I site, which is underlined) as sense and antisense primers, respectively, and subcloned into the *Nhe*I/*Sal*I sites of pEGFP-N1. The resulting plasmid was named hCAT4-pEGFP-N1.

### EGFP fusion constructs for *X. laevis* oocyte expression

The *Nhe*I/*Not*I fragments of hCAT1-pEGFP-N1 and hCAT2B-pEGFP-N1 were inserted into the *Bgl*II site of pSP64T. Resulting plasmids were hCAT1.EGFP-pSP64T and hCAT2B.EGFP-pSP64T. The *Nde*I/*Bgl*II fragment of hCAT4-pSP64T was replaced by the *Nde*I/*Not*I fragment of hCAT4-pEGFP-N1, resulting in plasmid hCAT4.EGFP-pSP64T.

### Constructs for chimaeric proteins between SLC7A4 and hCAT-2A and hCAT-2B

In-frame *Bam*HI and *Sal*I sites were introduced into the coding region of SLC7A4 and hCAT-2A just upstream and downstream, respectively, of the sequence encoding the 'functional domain', using the QuikChange mutagenesis kit (Stratagene), the sense oligonucleotides GAACACCGTCCTGCTCGGATCCCTCTTCTCCCCTGC (*Bam*HI site for SLC7A4 underlined), GGACCTGGAGTCGCTGGTCGACTTCCTGTCCC (*Sal*I site for SLC7A4 underlined), GTCTTCTGGGATCCATGTTTCCTT (*Bam*HI site for hCAT-2A underlined) and CTGAAGGCGCTTGTCGACATGATGTCCATTG (*Sal*I site for hCAT-2A and -2B underlined), and the corresponding antisense oligonucleotides. A *Bam*HI site already exists at the corresponding site of hCAT-2B. Chimaeric cDNAs were obtained by exchanging the *Bam*HI and *Sal*I fragments between the SLC7A4 and hCAT-2A and -2B cDNAs (resulting plasmids were HC2/4-pSP64T, HC4/2A-pSP64T and HC4/2B-pSP64T). Constructs encoding fusion proteins between the chimaeras and EGFP were obtained by inserting the *Bst*XI/*Nde*I fragments of HC4/2A-pSP64T and HC4/2B-pSP64T into hCAT4.EGFP-pSP64T and the *Bst*XI/*Mun*I fragment of HC2/4-pSP64T into hCAT2B.EGFP-pSP64T (resulting plasmids were HC4/2A.EGFP-pSP64T, HC4/2B.EGFP-pSP64T and HC2/4.EGFP-pSP64T).

### Expression of cRNAs in *X. laevis* oocytes

The plasmids pSPhCAT1-AB1C, hCAT4-pSP64T, hCAT1.EGFP-pSP64T and hCAT4.EGFP-pSP64T were linearized with *Eco*RI, HC2/4-pSP64T with *Xma*I, HC4/2A-pSP64T and HC4/2B-pSP64T with *Sca*I, HC4/2A.GFP-pSP64T and HC4/2B.GFP-pSP64T with *Xba*I and HC2/4.GFP-pSP64T with *Afl*III. cRNA was prepared by *in vitro* transcription from the SP6 promoter (mMessage mMachine in vitro transcription kit; Ambion, AMS Biotechnology Europe, Wiesbaden, Germany). cRNA (36 ng in 36 nl of water) was injected into each *X. laevis* oocyte (Dumont stages V–VI). Oocytes injected with 36 nl of water were used as controls.

### Transport studies in *X. laevis* oocytes

Amino acid and polyamine uptake was determined 3 days after injection of cRNA as described previously [7]. 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 unlabelled L-amino acids or putrescine. The oocytes were then transferred to the same solution containing, in addition, 5 $\mu$Ci/ml putrescine or $^3$H- or $^{14}$C-labelled L-amino acids {39 Ci/mmol L-[$^3$H]arginine, 54 Ci/mmol L-[$^3$H]lysine, 63 Ci/mmol L-[$^3$H]serine, 63 Ci/mmol L-[$^3$H]leucine, 53 Ci/mmol L-[$^3$H]glutamine, 60 Ci/

mmol L-[$^3$H]proline, 15 Ci/mmol L-[$^3$H]phenylalanine, 46 Ci/mmol L-[$^3$H]glutamic acid, 55 mCi/mmol L-[$^{14}$C]ornithine (ICN, Eschwege, Germany), 56 mCi/mmol L-[$^{14}$C]citrulline (Dupont NEN, Bad Homburg, Germany), 80 Ci/mmol L-[$^3$H]methionine, 44 Ci/mmol L-[$^3$H]histidine and 110 mCi/mmol [$^{14}$C]putrescine (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% SDS. The incorporated radioactivity was determined in a liquid-scintillation counter.

For *trans*-stimulation experiments, three oocytes were injected with 3.6 nmol of L-[$^3$H]arginine or L-[$^{14}$C]ornithine (3.6 nCi), each in 36 nl of water. The oocytes were then transferred into uptake solution containing either no supplement, 1 mM L-arginine, a mixture of amino acids (1 mM each of L-arginine, L-lysine, L-ornithine, L-leucine, L-serine, L-proline, L-histidine, L-phenylalanine, L-methionine, L-glutamine, L-glutamic acid and L-citrulline) or a mixture of polyamines (1 mM each of spermine, spermidine and putrescine). After a 30 min incubation at 20 °C the radioactivity that had accumulated in the uptake solution was determined by liquid-scintillation counting.

### RNase protection analyses

Plasmids containing a 201 nt fragment of hCAT-1 (phCAT-1/riboII), a 243 nt fragment of hCAT-3 (pXcmHC3/4) and a 108 nt cDNA fragment of the human $\beta$-actin cDNA (pCR_$\beta$actin_hu_$\Delta$*Bst*EII-*Hin*dIII) were generated previously [8,13]. The 243 nt *Eco*47III fragment of SLC7A4 was subcloned into pCR-Script SK(+) (Stratagene; the resulting plasmid was named hCAT4-pCRScript). To generate radiolabelled antisense RNA probes, the plasmids were linearized: phCAT-1/riboII with *Xba*I, pXcmHC3/4 with *Eco*RI and hCAT4-pCRScript and pCR_$\beta$actin_hu_$\Delta$*Bst*EII_*Hin*dIII with *Asp*718. *In vitro* transcription was performed as described previously [14]. Total RNA was isolated from NT2 cells using the method of Chomczynski and Sacchi [15]. RNase protection analyses were performed with 20 $\mu$g of RNA/sample as described in [14].

### Transfection of U373 MG glioblastoma cells

Cells were seeded into 6-well plates ($2 \times 10^5$ cells/well) 1 day before transfection. Plasmid DNA (2 $\mu$g) was added to 100 $\mu$l of medium and 10 $\mu$l of Superfect (Qiagen, Hilden, Germany), mixed and incubated at room temperature for 5 min. Then, 600 $\mu$l of medium containing 10% serum was added. The cells were washed with PBS and incubated with the transfection reagent for 3 h at 37 °C. After transfection (2 days), cells were split into 10 cm plates. Stably transfected cell clones were selected in medium containing 200 $\mu$g/ml G418. For each construct, several independent clones were selected.

### Protein lysates and Western blots

U373 MG glioblastoma cells grown to confluence in culture plates (10 cm diameter) were washed three times with PBS, scraped from the plates and pelleted at 180 ***g***. Cells were then lysed with 2 vol. of Nonidet P-40 buffer (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$, 0.3% Nonidet P-40, 1 mM PMSF, 0.3 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin and 1 $\mu$g/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 1 h at 37 °C with peptide N-glycosidase F (Roche Molecular Biochemicals; 10 units/100 $\mu$g) and then an equal volume of the sample buffer containing 6 M urea was added.

Lysates (20 $\mu$g of protein) were separated by SDS/PAGE (8% gels) and then blotted on to nitrocellulose membranes (Protran83; Schleicher and Schuell, Dassel, Germany). Staining for EGFP fusion proteins and hCAT-1 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 and 5% non-fat dry milk] containing 10% goat serum for 2 h at room temperature, a dilution of the primary antibody {a 1:300 dilution of an anti-GFP polyclonal antibody (Clontech) or a 1:100 dilution of an hCAT-1 antibody [13]} in PBS containing 1% BSA and 0.1% Tween 20 overnight at 4 °C, and three changes of Blotto (each for 15 min at room temperature). This was followed by a 1:10000 dilution in Blotto of a peroxidase-conjugated secondary goat anti-rabbit IgG antibody (Calbiochem, Bad Soden, Germany) for 1 h at room temperature), three changes of TBST (10 mM Tris/HCl, pH 8, 150 mM NaCl and 0.05% Tween 20), one incubation in TBS (10 mM Tris/HCl, pH 8, and 150 mM NaCl) and finally a 1 min incubation with chemiluminescence reagent (Renaissance; Dupont NEN). An X-ray film (Agfa, Leverkusen, Germany) was then immediately exposed to the membranes.

### Confocal microscopy

Cells were grown in coverglass-bottomed chamber slides (Chambered Coverglass; Nalge Nunc, Naperville, IL, U.S.A.). After fixation for 5 min with 3% formaldehyde in PBS, the cells were stained with Hoechst 33342 (Molecular Probes, Eugene, OR, U.S.A.) and 1 mg/ml PBS for 30 min. After washing with PBS, the cells were analysed directly with a Zeiss 510 confocal laser scanning microscope equipped with a UV laser (Zeiss, Oberkochem, Germany). Images were collected with a 1.4 numerical aperture 63 × Zeiss Plan-aprochromat objective using identical scanning parameters.

### Transport studies in NT2 teratocarcinoma cells

Cells grown to confluence in 24-well plates were washed twice with Locke's solution (154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 3.6 mM $NaHCO_3$ and 5.6 mM glucose, pH 7.4) containing a defined concentration of L-arginine and then incubated at 37 °C for 1 h. After this pre-incubation, the Locke's solution was exchanged for the same solution containing L-[$^3$H]arginine (5 $\mu$Ci/ml), and the cells were incubated for 30 s at 37 °C. The cells were then transferred immediately to ice, washed three times with ice-cold Locke's solution and lysed in 0.5 M NaOH (250 $\mu$l/well for 30 min at 4 °C). After neutralization of the lysates with 250 $\mu$l of 0.5 M HCl and 500 $\mu$l of buffer A (50 mM Tris/HCl, pH 7.4, 0.5 mM EDTA and 0.5 mM EGTA) the protein content of each sample was determined using the Bradford reaction (Bio-Rad, Munich, Germany). The radioactivity in the samples was measured by liquid-scintillation counting. The background radioactivity derived from L-arginine bound to the cells (determined by addition of Locke's solution containing 5 $\mu$Ci/ml L-[$^3$H]arginine, followed by immediate washing steps) was subtracted from all values (usually less than 10% of experimental values).

## RESULTS

### Transport studies with SLC7A4 expressed in oocytes from *X. laevis*

To characterize the transport activity of SLC7A4, we first expressed the protein in oocytes from *X. laevis*. Assuming that SLC7A4 is a member of the hCAT family, we initially measured

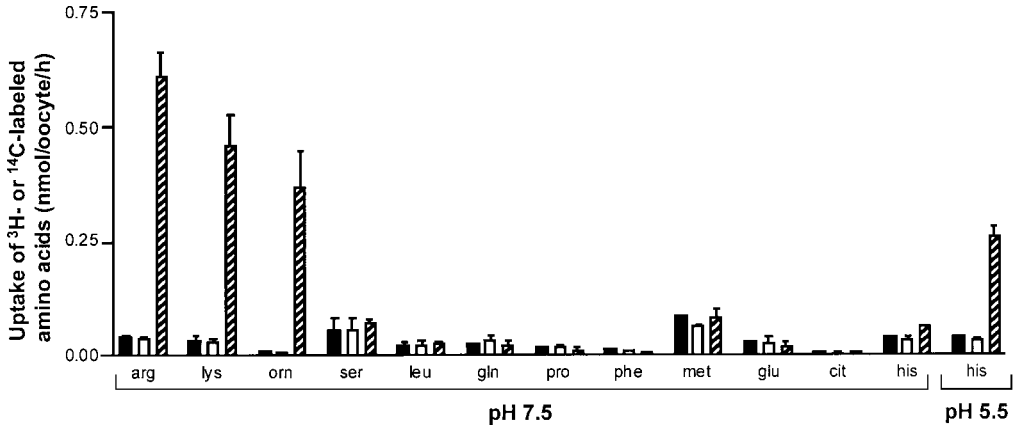


**Figure 1 Lack of transport activity of SLC7A4 expressed in oocytes from *X. laevis* for cationic, neutral or acidic amino acids**

*X. laevis* oocytes were injected with 36 ng of SLC7A4 cRNA (black bars) or hCAT-1 cRNA (hatched bars), each in 36 nl of water, or with 36 nl of water alone (white bars). Then 3 days later, uptake of the indicated $^{3}$H- or $^{14}$C-labelled amino acids (each 100 $\mu$M) was measured (15 min at 20 °C and pH 7.5). The uptake of L-[$^{3}$H]histidine was also measured at pH 5.5 as indicated. Data points represent means ± S.E.M., $n = 2$–4 with 6–10 replicates each. cit, citrulline.

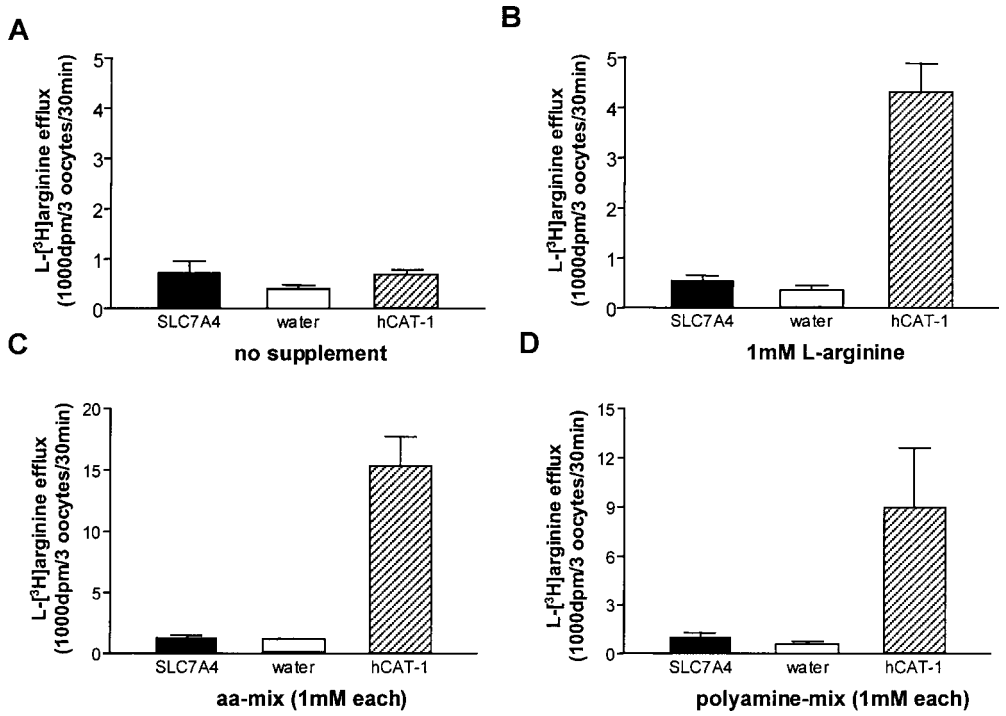


**Figure 2 *Trans*-stimulation experiments with SLC7A4 and hCAT-1 expressed in oocytes from *X. laevis***

*X. laevis* oocytes were injected with 36 ng of SLC7A4 cRNA (black bars) or hCAT-1 cRNA (hatched bars), each in 36 nl of water, or with 36 nl of water alone (white bars). Then 2 days later, the oocytes were injected for a second time with 3.6 nmol of L-[$^{3}$H]arginine (in 36 nl of water) and immediately transferred to buffer containing (**A**) no supplement, (**B**) 1 mM L-arginine, (**C**) a mixture of different amino acids (at 1 mM each, as listed in the Experimental section) or (**D**) a mixture of polyamines (1 mM each of spermine, spermidine and putrescine). The efflux of L-[$^{3}$H]arginine was measured for 30 min at 20 °C. Data points represent means ± S.E.M., $n = 2$–3 with 4–6 replicates each.

the uptake of the cationic amino acids L-arginine, L-lysine and L-ornithine (100 $\mu$M each) using radioactive tracers. However, we could not detect any transport mediated by SLC7A4 under conditions where other CAT isoforms exhibit significant transport activity (Figure 1). Varying the extracellular pH between 5.5 and 8.5 and increasing the temperature during the uptake assay from 20 to 30 °C did not result in SLC7A4-mediated L-arginine uptake (results not shown). We then tested a wide range of neutral and anionic amino acids (100 $\mu$M each), covering substrates of all known major mammalian amino acid transport systems, but could not find any transport activity of SLC7A4 (Figure 1). The transport of L-histidine was also assayed at pH 5.5, where it is protonated and becomes a substrate for hCAT-1 (Figure 1). When screening the Protein DataBank for proteins with sequence

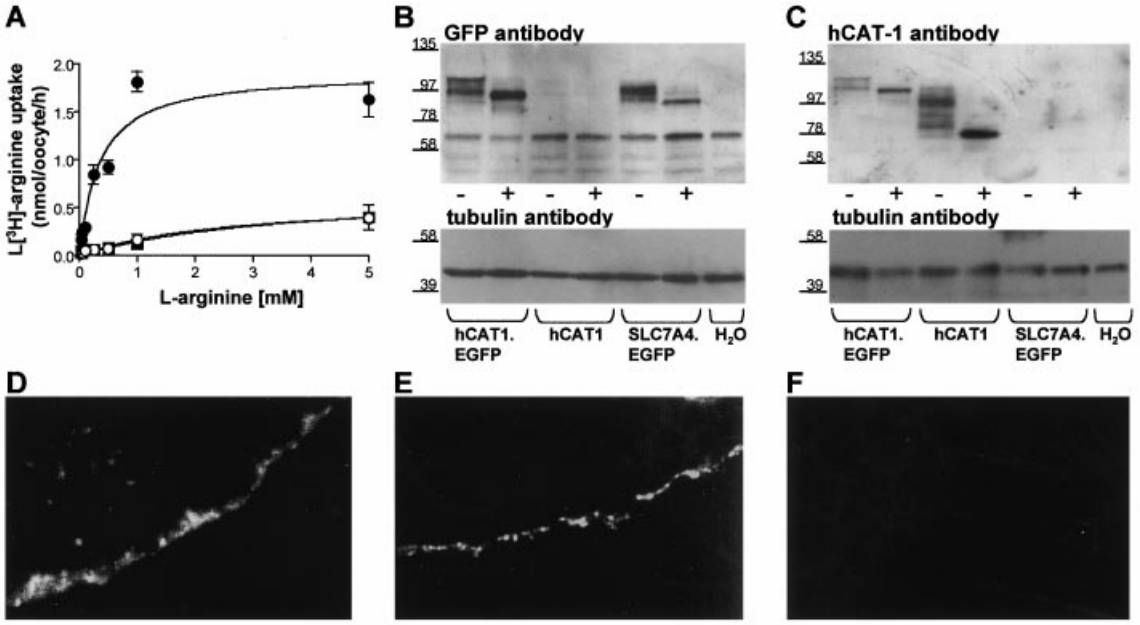


**Figure 3 Transport activity, expression level and subcellular localization of SLC7A4- and hCAT-1-EGFP fusion proteins expressed in oocytes from *X. laevis***

Oocytes were injected with 36 ng of SLC7A4-EGFP cRNA or hCAT-1-EGFP cRNA or with 36 nl of water alone, and 3 days later transport activity and protein expression were analysed. (**A**) Uptake studies with the indicated concentrations of L-[$^3$H]arginine for 15 min at 20 °C; ■, SLC7A4-EGFP; ●, hCAT-1-EGFP; ○, water. Data points represent means ± S.E.M. from one typical experiment with 5–10 replicates. Three different experiments with similar results have been performed. (**B**, **C**) Western blots with lysates from oocytes, injected with cRNA for hCAT-1-EGFP (lanes 1 and 2, reading from the left), hCAT-1 (lanes 3 and 4), SLC7A4-EGFP (lanes 5 and 6) or water (lane 7). SDS/PAGE (8% gels) was used to separate 20 $\mu$g of protein/lane, followed by blotting, and the membrane was incubated with an anti-GFP antibody (**B**) or an anti-hCAT-1 antibody (**C**). The lysates in lanes 2, 4 and 6 (+) were treated with peptide N-glycosidase F and the lysates in lanes 1, 3 and 5 (−) were untreated. The blots were then stripped and probed with an anti-tubulin antibody (**B** and **C**, lower panels). Three different experiments with similar results were performed. The multiple bands of hCAT-1 are most probably due to incomplete glycosylation and/or aberrant migration of this hydrophobic and glycosylated protein in SDS/PAGE. (**D**–**F**) Fluorescence micrographs of cryosections of *X. laevis* oocytes injected with cRNA for hCAT-1-EGFP (**D**) or SLC7A4-EGFP (**E**), or with water (**F**).

similarity to SLC7A4, transporters not only for amino acids but also for polyamines can be found. Therefore, we measured the uptake of 100 $\mu$M putrescine, but could not detect any SLC7A4-mediated uptake (results not shown). As some polyamine transporters of bacteria and yeast are antiporters, exchanging cationic amino acids with polyamines, we investigated whether SLC7A4 transports cationic amino acids in the presence of polyamines and vice versa.

Figure 2 shows one such experiment where we injected tritiated L-arginine into oocytes and then transferred the oocytes immediately to a buffer containing either no further supplement, 1 mM L-arginine, a mixture of different neutral, anionic and cationic amino acids, or a mixture of polyamines. No SLC7A4-mediated L-arginine efflux could be detected under these conditions. As expected, the presence of L-arginine or the amino acid mixture stimulated the hCAT-1-mediated efflux of L-arginine assayed in a parallel experiment. However, surprisingly, the polyamine mixture also had a strong *trans*-stimulation effect (Figure 2D), even though putrescine was not a substrate for hCAT-1 in influx experiments (results not shown). There was no SLC7A4-mediated L-ornithine efflux in the presence of the polyamine mixture in the extracellular buffer and no L-arginine or L-ornithine uptake after injection of the oocytes with the polyamine mixture (results not shown).

### SLC7A4-EGFP fusion proteins expressed in oocytes from *X. laevis*

To find out whether the lack of transport activity of SLC7A4 in our experiments was due to a lack of sufficient protein expression or the targeting of the protein to membranes other than the plasma membrane, we expressed fusion proteins between the EGFP and the C-terminus of hCAT-1 or SLC7A4 in *X. laevis* oocytes. Transport studies with hCAT-1-EGFP showed a concentration-dependent uptake of L-arginine with $K_m$ and $V_{max}$ values indistinguishable from the native hCAT-1, but no transport mediated by SLC7A4-EGFP (Figure 3A) or the native SLC7A4 (results not shown). Western-blot analysis with lysates from oocytes prepared in parallel demonstrated that SLC7A4 was glycosylated and expressed to a similar extent as hCAT-1 (Figure 3B). Equal loading of each gel was verified by staining the membrane with an antibody against $\beta$-tubulin (Figure 3B and 3C, lower panels). The hCAT-1-EGFP fusion protein was also recognized by an antibody raised against the C-terminus of hCAT-1 (Figure 3C). In cryosections of oocytes expressing hCAT-1 or SLC7A4, both proteins were mainly detected in the plasma membrane (Figures 3D and 3E).

### Chimaeric proteins between SLC7A4 and hCAT-2 expressed in oocytes from *X. laevis*

Our experiments with the SLC7A4-EGFP fusion protein demonstrated that SLC7A4 does not need a second protein to be targeted to the plasma membrane. It could not be ruled out, however, that it needs a second protein to form an active transporter. As the established hCAT isoforms are always active in *X. laevis* oocytes, they either do not need a second protein to be activated or interact with a protein that is present in the oocyte. We therefore hypothesized that chimaeric proteins be-

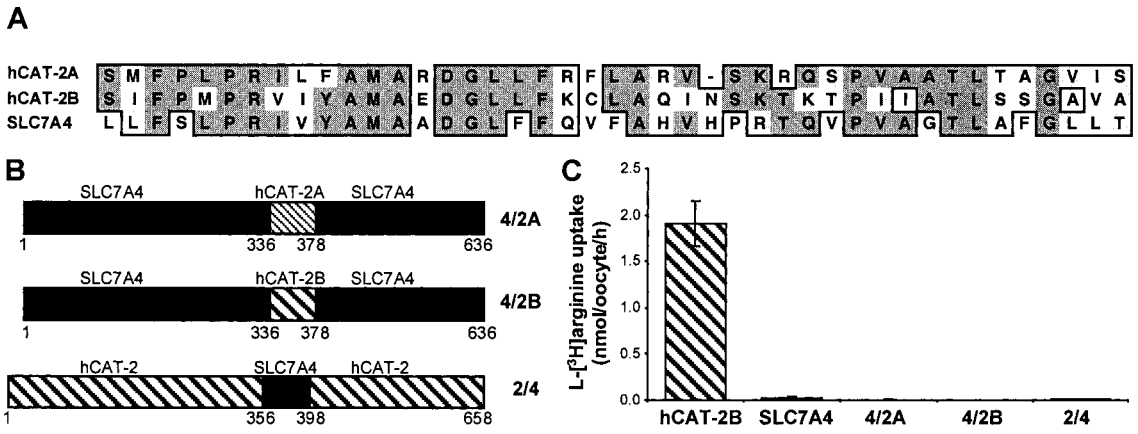


**Figure 4 Transport studies with chimaeras between SLC7A4 and hCAT-2 expressed in oocytes from *X. laevis***

(**A**) Comparison of the 43 amino acids of hCAT-2A and hCAT-2B that differ between the two proteins with the corresponding region of SLC7A4. This region was exchanged between SLC7A4 and hCAT-2A and hCAT-2B in the chimaeric proteins. (**B**) Scheme of the chimaeric proteins. The names of the chimaeras are listed on the right. Names above each bar indicate from which protein the different parts of the chimaeras derive. Numbers underneath correspond to amino acid residues in the chimaeras. (**C**) Uptake studies in *X. laevis* oocytes expressing hCAT-2B, SLC7A4 or the chimaeric proteins 4/2A, 4/2B or 2/4 using 1 mM L-[$^3$H]arginine (15 min for 20 °C and pH 7.5). The values obtained with water-injected oocytes were subtracted from the respective values obtained with cRNA-injected oocytes. Data points represent means ± S.E.M. from one typical experiment with 5–10 replicates.

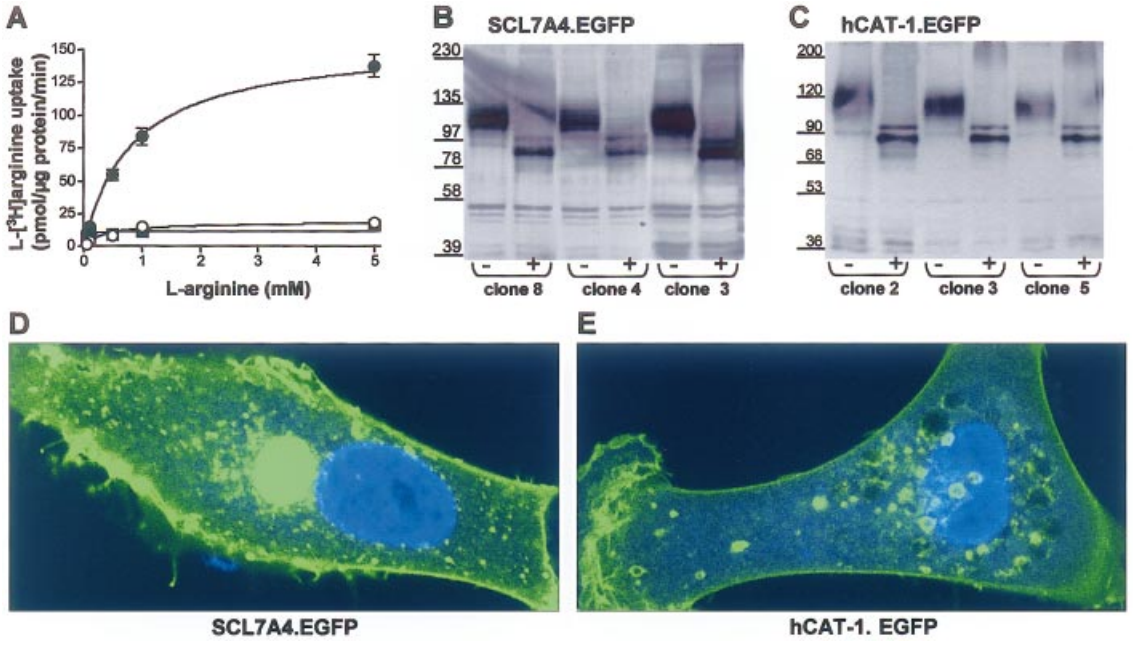


**Figure 5 Transport activity, expression level and subcellular localization of SLC7A4- and hCAT-1-EGFP fusion proteins expressed in human U373 MG glioblastoma cells**

Cells were stably transfected with expression vectors for SLC7A4-EGFP, hCAT-1-EGFP or EGFP alone. (**A**) Uptake studies with the indicated concentrations of L-[$^3$H]arginine (30 s at 37 °C; ■, SLC7A4-EGFP; ●, hCAT-1.EGFP; ○, EGFP). Data points represent means ± S.E.M. from one typical experiment with 5–10 replicates. (**B**, **C**) Western blots with lysates from cells expressing SLC7A4-EGFP (**B**) or hCAT-1-EGFP (**C**); 20 $\mu$g of protein/lane were separated by SDS/PAGE (8% gels), blotted and the membranes incubated with an anti-GFP antibody. The lysates in lanes 2, 4 and 6 of each blot were treated with peptide N-glycosidase F. (**D**, **E**) Confocal micrographs of cells expressing SLC7A4-EGFP (**D**) or hCAT-1-EGFP (**E**).

tween SLC7A4 and the established hCAT isoforms should be active when the part of SLC7A4 that needed the interaction with another protein was substituted for the respective part of one of the established hCAT isoforms. Chimaeric proteins were constructed between SLC7A4 and hCAT-2A and -2B by exchanging the area that is divergent between the two splice variants and the corresponding area of SLC7A4 (Figures 4A and 4B). This has been shown in the murine CAT proteins to determine the transport properties and is therefore referred to as the 'functional domain' [16]. Surprisingly, neither the chimaera with the hCAT-2 backbone and the functional domain of SLC7A4 (named 2/4), nor the reciprocal chimaeras with the backbone of SLC7A4 and the functional domain of hCAT-2A or -2B (named 4/2A and 4/2B, respectively) had any transport

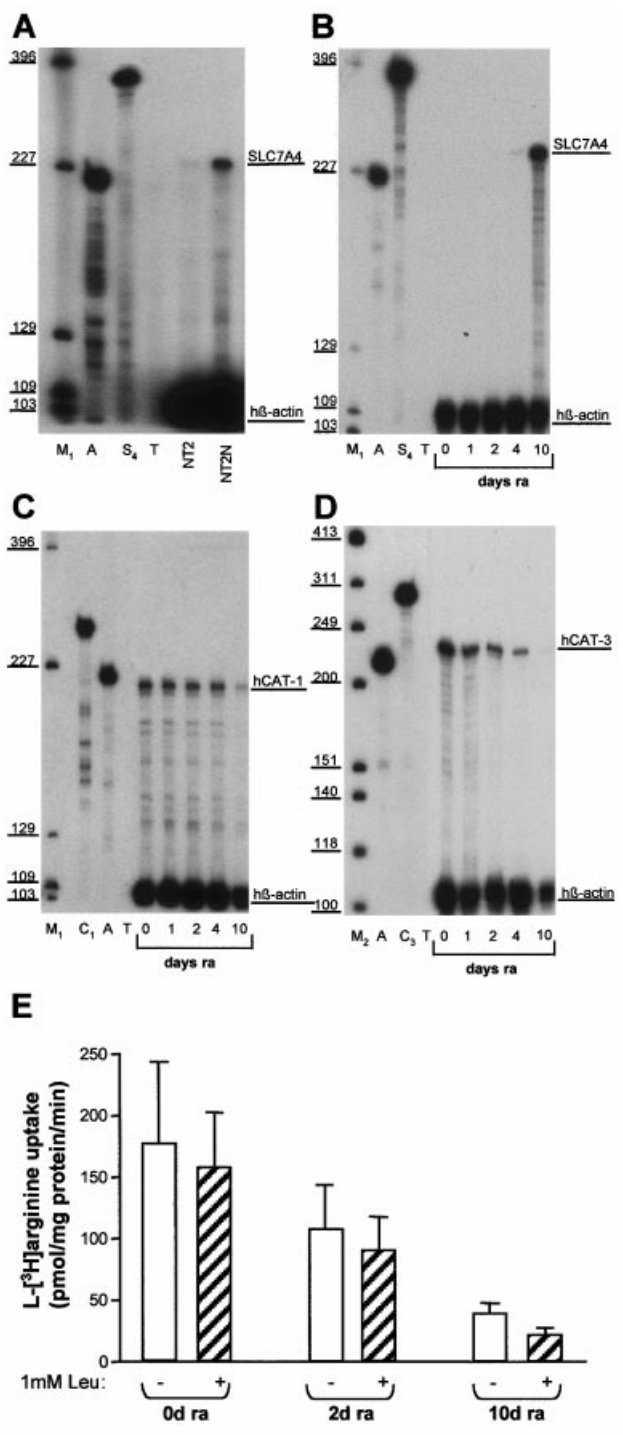


**Figure 6 Induction of SLC7A4 mRNA in NT2 teratocarcinoma cells by retinoic acid is not accompanied with an increase in L-arginine transport activity**

Cells were either fully differentiated into neurons (**A**; NT2, undifferentiated; NT2N, differentiated) or only treated with retinoic acid (ra) for 1–10 days (**B**–**D**) and the expression of SLC7A4 and the different hCAT isoforms as well as the transport rate for L-arginine were measured. (**A**–**D**) RNase protection analyses: total RNA prepared from NT2 cells was hybridized with antisense cRNA probes specific for human $\beta$-actin (as an internal control) and for SLC7A4 (**A** and **B**), hCAT-1 (**C**) or hCAT-3 (**D**). After RNase treatment, the protected RNA fragments (human $\beta$-actin, 108 nt; SLC7A4, 242 nt; hCAT-1, 201 nt; hCAT-3, 243 nt) were separated on a 6% denaturing polyacrylamide gel. $M_1$ and $M_2$, DNA size markers ($M_1$, pGl2-Basic; $M_2$, $\Phi$X174; both from Promega, Heidelberg, Germany; restricted with *Hin*fI); A, $S_4$, $C_1$ and $C_3$, undigested probes for human $\beta$-actin (228 nt), SLC7A4 (361 nt), hCAT-1 (252 nt) and hCAT-3 (292 nt), respectively; T, tRNA used as a negative control. (**E**) Uptake studies with 30 $\mu$M L-[$^3$H]arginine in the absence (white bars) or presence (hatched bars) of 1 mM L-leucine (30 s at 37 °C) and 0–10 days of exposure to retinoic acid. Data points represent means ± S.E.M. from one typical experiment with four replicates.

activity for L-arginine (Figure 4C). Experiments with fusion proteins between the chimaeras and EGFP demonstrated that the lack of transport activity was not due to a lack of chimaeric proteins in the plasma membrane (results not shown).

### SLC7A4-EGFP fusion proteins expressed in human U373 MG glioblastoma cells

As SLC7A4 did not show any transport activity in oocytes from *X. laevis*, we wondered if it had to be expressed in mammalian cells to be functional. Therefore, we transfected U373 MG glioblastoma cells with expression constructs encoding hCAT-1.EGFP and SLC7A4.EGFP. For each construct, several independent cell clones were isolated that stably express the fusion protein. Transport studies showed a 4–8-fold higher transport rate for L-arginine (100 $\mu$M) in each cell clone expressing hCAT-1-EGFP compared with cells expressing EGFP alone or untransfected cells (results not shown). The increase in the transport rate was concentration-dependent (Figure 5A). In contrast, at L-arginine concentrations ranging from 50 $\mu$M to 5 mM, none of the eight cell clones expressing SLC7A4-EGFP showed an increase in L-arginine transport. Also, no SLC7A4-mediated L-arginine transport could be detected when using PBS instead of Locke's solution for the uptake studies (results not shown). Western-blot analyses demonstrated that SLC7A4-EGFP was glycosylated in U373 MG cells and expressed to at least the same extent as hCAT-1-EGFP (Figures 5B and 5C). Confocal microscopy of the transfected cells revealed that both fusion proteins were localized to the plasma membrane, but also showed a significant expression in intracellular membranes (Figures 5D and 5E). The fluorescence was consistently higher in cells expressing SLC7A4-EGFP compared with hCAT-1-EGFP.

### SLC7A4 expressed endogenously in human NT2 teratocarcinoma cells

As the overexpression of SLC7A4 in *X. laevis* oocytes or mammalian cells had not resulted in any transport activity, we wondered whether SLC7A4 expressed endogenously would mediate transport of L-arginine. As SLC7A4 expression has been found predominantly in brain, testis and placenta [8,11,17], we screened a number of human cell lines derived from brain or testis for SLC7A4 expression. By far the highest expression was found in NT2 teratocarcinoma cells differentiated to neurons (NT2N) by treatment with retinoic acid (10 $\mu$M; 6 weeks) and afterwards with proliferation inhibitors (2 weeks; Figure 6A). Subsequently, we found that a 10 day treatment with retinoic acid (10 $\mu$M) was sufficient to induce SLC7A4 expression in NT2 cells (Figure 6B). The predominant hCAT isoform expressed in undifferentiated NT2 cells is hCAT-3 [8], but hCAT-1 was also found to be expressed in these cells. The expression of both hCAT isoforms was markedly down-regulated by the 10 day treatment with retinoic acid (Figures 6C and 6D). Transport studies demonstrated that cationic amino acids are transported predominantly via system $y^+$(CAT)-like transporters, as the presence of 1 mM L-leucine did not reduce the transport rate for L-arginine significantly (Figure 6E). Following treatment with retinoic acid the transport rate for L-arginine was reduced markedly, mirroring the down-regulation of hCAT-1 and -3 and

indicating that SLC7A4 does not function as a CAT in these cells.

## DISCUSSION

Owing to its sequence homology with hCAT proteins, SLC7A4 has been considered as a new member of the hCAT family [11]. It has therefore been named hCAT-4 and has been assumed to play an important role in the transport of cationic amino acids in certain tissues, e.g. the human placenta, where it is expressed throughout gestation [17]. However, there are no unambiguous data demonstrating SLC7A4-mediated transport of cationic amino acids. In the present study, we thus aimed to characterize the transport properties of SLC7A4. However, we could not detect any transport activity for L-arginine when overexpressing SLC7A4 in *X. laevis* oocytes or in mammalian cells. The lack of transport activity was not due to a lack of protein expression, as in both cell types a fusion protein of SLC7A4 and EGFP was expressed at least as highly as the corresponding fusion protein with hCAT-1. The function of the latter was indistinguishable from the native transporter, suggesting that the addition of the EGFP moiety to the C-terminus of the CAT proteins does not influence their transport properties. Similar results have been obtained with other hCAT isoforms (results not shown). These results are also consistent with an earlier report demonstrating that a fusion protein between murine CAT-1 and GFP retained its function as receptor for murine ecotropic leukaemia viruses [18]. In addition, SLC7A4-EGFP exhibited a similar subcellular localization to hCAT-1-EGFP. In *X. laevis* oocytes, both proteins were targeted predominantly to the plasma membrane, whereas in human U373MG glioblastoma cells a considerable portion of the fusion proteins was also detected in intracellular membranes. Yet also in U373 MG glioblastoma cells, the amount of SLC7A4-EGFP expressed in the plasma membrane seemed to be at least as high as the amount of hCAT-1-EGFP. So far, we have no explanation for the differential distribution of the fusion proteins in *X. laevis* oocytes and mammalian cells. Further studies are underway to determine whether the subcellular localization of the SLC7/CAT isoforms also differs between distinct mammalian cell types.

We also considered the possibilities that SLC7A4 might mediate efflux rather than influx of cationic amino acids, be strongly dependent on the presence of *trans*-substrate, work at a different pH optimum or transport only a specific cationic amino acid. However, we could not detect any transport activity for the cationic amino acids tested (L-arginine, L-lysine and L-ornithine), either in influx or in efflux experiments, by providing either cationic amino acids or a mixture of cationic, neutral and anionic amino acids as *trans*-substrates, or by varying the pH in the extracellular buffer. These data lead us to the conclusion that SLC7A4 does not work as a cationic amino acid transporter.

The members of the SLC7A4 superfamily transport a large variety of substrates, including neutral and anionic amino acids as well as cystine. Even some of the CAT proteins have been reported to recognize (with low affinity) some neutral and even anionic amino acids, e.g. murine CAT-1 recognizes L-histidine and L-cysteine [19], murine CAT-2B recognizes L-histidine [20], murine CAT-3 recognizes L-methionine, L-cysteine, L-aspartate and L-glutamate [21] and rat CAT-3 recognizes L-citrulline and D-arginine [22]. In addition, the degree of sequence identity between SLC7A4 and the hCAT proteins (about 40 %) is much less than amongst the different hCAT isoforms (about 60 %). It therefore seems plausible that SLC7A4 could transport substrates other than cationic amino acids. We tested nine additional neutral and anionic amino acids as possible influx substrates for SLC7A4 expressed in *X. laevis* oocytes. In addition, we examined the influx and efflux of the polyamine putrescine in the absence or presence of potential *trans*-substrates, such as L-arginine, a mixture of different amino acids and a mixture of polyamines. These experiments led to the discovery that polyamines *trans*-stimulate the hCAT-1-mediated efflux of L-arginine. However, we could not detect any transport activity of SLC7A4. These data suggest to us that SLC7A4 is not an amino acid transporter.

One might wonder if the protein we expressed in *X. laevis* oocytes or mammalian cells had the authentic amino acid sequence of SLC7A4. We have sequenced all of the constructs used and found the SLC7A4 sequence to be identical with sequences of two genomic clones in GenBank (AC002472 and AB002059). Alignment of the cDNA and genomic sequences indicates the coding region of SLC7A4 to span only 3.1 kb of genomic sequence and to be organized in four exons. This gene structure is completely different from that of hCAT-1 and -2(A + B), the coding sequences of which span more than 20 kb and are organized in 11 exons each {see [23] for hCAT-1; the gene structure of hCAT-2(A + B) has been deduced from the genomic sequence AB020863; a genomic sequence for hCAT-3 is not yet available}. Using the program GENSCAN [24], an additional intron of 33 bp is recognized in the genomic sequence of SLC7A4, predicting a protein that is 11 amino acids shorter than SLC7A4. The 11 amino acids are located immediately downstream of the functional domain. In the corresponding position, hCAT-3 also lacks 11 amino acids, whereas hCAT-1 and -2(A + B) are three amino acids shorter than SLC7A4. We wondered therefore if the cDNA sequence we used was derived from an incompletely spliced SLC4A7 mRNA. However, there are several expressed sequence tags in GenBank, derived from human colon carcinoma cells, cerebellum and cervix, that all contain the 33 bp sequence. We also recloned cDNA fragments from human placenta, using reverse transcriptase PCR, that all contained the 33 bp fragment. The sequence of the cDNA we used was 100 % identical with the GenBank sequence XM_009855 that has recently been compiled by the National Center for Biotechnology Information annotation project from all entries for SLC7A4. These analyses suggest strongly that we have expressed the authentic SLC7A4.

One explanation for the lack of transport function of SLC7A4 when overexpressed in *X. laevis* oocytes or mammalian cells would be a requirement for a second protein to form a functional transporter. Our data demonstrate that, unlike the members of the HAT light subunit family, SLC7A4 does not need a second protein to be targeted to the plasma membrane. However, it is possible that a protein may be necessary to activate the transport function of SLC7A4. In fact, rBAT is not only necessary for targeting the light subunit $b^{0,+}$AT to the plasma membrane, but also modulates the transport function of the holotransporter [10]. We hoped to overcome the need for a second protein by exchanging the functional domains of SLC7A4 and hCAT isoforms that are functional in *X. laevis* oocytes without the expression of additional proteins. This functional domain can be exchanged between different CAT isoforms, conferring the transport properties of the donor of that domain on the backbone of the recipient CAT isoform ([16] and A. Habermeier, I. Burck, S. Wolf, U. Martiné and E. I. Closs, unpublished work). However, the chimaeras between SLC7A4 and hCAT-2A and -2B were all non-functional, indicating that there are profound differences between SLC7A4 and the hCATs that must lead to a disruption of the interaction between the functional domain and the transporter backbone. These differences can be used in future studies to find important functional areas in the backbone of the CAT proteins. We also speculated that in cells expressing

SLC7A4 endogenously, all putative proteins necessary for the function of SLC7A4 should be present. However, in NT2N cells, where we found pronounced SLC7A4 expression concomitant with the down-regulation of hCAT-1 and -3 expression, we could not detect any SLC7A4-mediated transport activity for L-arginine. This makes it very unlikely that SLC7A4 is a transporter for cationic amino acids, although one has to take into account the fact that tumour cells often exhibit an aberrant expression of proteins.

Could SLC7A4 be a pseudoprotein? There are several points against this hypothesis. First, SLC7A4 is expressed in several human tissues and constitutes a stable membrane protein. Second, the divergence in the primary sequence and gene structure of SLC7A4 compared with the hCAT proteins indicates that SLC7A4 must have segregated from the hCAT family a long time ago. If the protein is not functional, its gene would be expected to have acquired a number of mutations, including frame-shift mutations and stop codons, leading to destruction of the open reading frame [25]. The intact open reading frame of SLC7A4 therefore suggests strongly that the protein has a cellular function. When screening the protein database using the BLASTP [26] program, all the proteins showing sequence homology with SLC7A4 are transport proteins. This makes it very likely that SLC7A4 is also a transporter. Besides the CAT proteins, which show by far the highest homology, the proteins found include the aforementioned HAT light subunits, as well as a number of amino acid and polyamine transporters (putrescine/ornithine exchanger) from bacteria and yeast. Most of the substrates of these transport proteins have been tested in our study and have proved negative as substrates for SLC7A4. Before searching for other substrates, it has to be elucidated whether SLC7A4 associates with other proteins that might be necessary for its function as a transporter. To this end, we are currently developing antibodies against SLC7A4 to immunoprecipitate SLC7A4 from human tissues.

Taken together, our data demonstrate that the expression of SLC7A4 in the plasma membrane is not sufficient to confer transport activity for amino acids. We therefore suggest that the name hCAT-4 should not be used until a transport function can be shown for this protein.

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## REFERENCES

1 Closs, E. I. and Mann, G. E. (2000) Membrane transport of L-arginine and cationic amino acids analogs. In Nitric Oxide: Biology and Pathobiology (Ignarro, L. J., ed.), pp. 225–241, Academic Press, San Diego
2 Palacin, M., Bertran, J. and Zorzano, A. (2000) Heteromeric amino acid transporters explain inherited aminoacidurias. Curr. Opin. Nephrol. Hypertens. **9**, 547–553
3 Verrey, F., Meier, C., Rossier, G. and Kuhn, L. C. (2000) Glycoprotein-associated amino acid exchangers: broadening the range of transport specificity. Pflugers Arch. **440**, 503–512
4 MacLeod, C. L. and Kakuda, D. K. (1996) Regulation of CAT: cationic amino acid transporter gene expression. Amino Acids **11**, 171–191
5 Deves, R. and Boyd, C. A. R. (1998) Transporters for cationic amino acids in animal cells: discovery, structure, and function. Physiol. Rev. **78**, 487–545
6 Closs, E. I. (2002) Expression and regulation of carrier proteins for cationic amino acids. Curr. Opin. Nephrol. Hypertens. **11**, 99–107
7 Closs, E. I., Gräf, P., Habermeier, A., Cunningham, J. M. and Förstermann, U. (1997) The human cationic amino acid transporters hCAT-1, hCAT-2A and hCAT-2B: three related carriers with distinct transport properties. Biochemistry **36**, 6462–6468
8 Vékony, N., Wolf, S., Boissel, J. P., Gnauert, K. and Closs, E. I. (2001) Human cationic amino acid transporter hCAT-3 is preferentially expressed in peripheral tissues. Biochemistry **40**, 12387–12394
9 Chillaron, J., Roca, R., Valencia, A., Zorzano, A. and Palacin, M. (2001) Heteromeric amino acid transporters: biochemistry, genetics, and physiology. Am. J. Physiol. Renal Physiol. **281**, F995–F1018
10 Wagner, C. A., Lang, F. and Broer, S. (2001) Function and structure of heterodimeric amino acid transporters. Am. J. Physiol. Cell Physiol. **281**, C1077–C1093
11 Sperandeo, M. P., Borsani, G., Incerti, B., Zollo, M., Rossi, E., Zuffardi, O., Castaldo, P., Taglialatela, M., Andria, G. and Sebastio, G. (1998) The gene encoding a cationic amino acid transporter (SLC7A4) maps to the region deleted in the velocardiofacial syndrome. Genomics **49**, 230–236
12 Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniati, R., Zinn, K. and Green, M. R. (1984) Nucleic Acids Res. **12**, 7035–7075
13 Gräf, P., Förstermann, U. and Closs, E. I. (2001) The transport activity of the human cationic amino acid transporter hCAT-1 is downregulated by activation of protein kinase C. Br. J. Pharmacol. **132**, 1193–1200
14 Closs, E. I. and Mann, G. E. (1999) Identification of carrier systems in plasma membranes of mammalian cells involved in transport of L-arginine. In Nitric Oxide, part C (Packer, L., ed.), pp. 78–91, Academic Press, San Diego
15 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156–159
16 Closs, E. I., Lyons, C. R., Kelly, C. and Cunningham, J. M. (1993) Characterization of the third member of the mCAT family of cationic amino acid transporters – identification of a domain that determines the transport properties of the mCAT proteins. J. Biol. Chem. **268**, 20796–20800
17 Ayuk, P. T., Sibley, C. P., Donnai, P., D'Souza, S. and Glazier, J. D. (2000) Development and polarization of cationic amino acid transporters and regulators in the human placenta. Am. J. Physiol. Cell Physiol. **278**, C1162–C1171
18 Masuda, M., Kakushima, N., Wilt, S. G., Ruscetti, S. K., Hoffman, P. M. and Iwamoto, A. (1999) Analysis of receptor usage by ecotropic murine retroviruses, using green fluorescent protein-tagged cationic amino acid transporters. J. Virol. **73**, 8623–8629
19 Wang, H., Kavanaugh, M. P., North, R. A. and Kabat, D. (1991) Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. Nature (London) **352**, 729–731
20 Kakuda, D. K., Finley, K. D., Dionne, V. E. and MacLeod, C. L. (1993) Two distinct gene products mediate $y^+$ type cationic amino acid transport in *Xenopus* oocytes and show different tissue expression patterns. Transgene **1**, 91–101
21 Ito, K. and Groudine, M. (1997) A new member of the cationic amino acid transporter family is preferentially expressed in adult mouse brain. J. Biol. Chem. **272**, 26780–26786
22 Hosokawa, H., Ninomiya, H., Sawamura, T., Sugimoto, Y., Ichikawa, A., Fujiwara, K. and Masaki, T. (1999) Neuron-specific expression of cationic amino acid transporter 3 in the adult rat brain. Brain Res. **838**, 158–165
23 Yoshimoto, T., Yoshimoto, E. and Meruelo, D. (1991) Molecular cloning and characterization of a novel human gene homologous to the murine ecotropic retroviral receptor. Virology **185**, 10–15
24 Burge, C. and Karlin, S. (1997) Prediction of complete gene structures in human genomic DNA. J. Mol. Biol. **268**, 78–94
25 Mighell, A. J., Smith, N. R., Robinson, P. A. and Markham, A. F. (2000) Vertebrate pseudogenes. FEBS Lett. **468**, 109–114
26 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389–3402

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