A chimera carrying the functional domain of the orphan protein SLC7A14 in the backbone of SLC7A2 mediates trans-stimulated arginine transport*

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*Running Title: Arginine transport by the hCAT-2/SLC7A14_BK chimera

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Background: The molecular identity of the lysosomal transporter for cationic amino acids, system c, remains unknown.

Result: SLC7A14 is a lysosomal localized protein with a functional domain that mediates arginine transport.

Conclusion: SLC7A14 may mediate cationic amino acid transport across lysosomal membranes.

Significance: As system c represents a salvage pathway in the therapy of cystinosis, characterization of SLC7A14 might help to develop better drugs.

Summary

In human skin fibroblasts, a lysosomal transport system specific for cationic amino acids has been described and named system c. We asked if solute carrier family 7 member A14 (SLC7A14), an orphan protein assigned to the SLC7-subfamily of cationic amino acid transporters (CATs) due to sequence homology, may represent system c. Fusion proteins between SLC7A14 and EGFP localized to intracellular vesicles, co-staining with the lysosomal marker LysoTracker®. To perform transport studies, we first tried to redirect SLC7A14 to the plasma membrane (by mutating putative lysosomal targeting motives), however without success. We then created a chimera carrying the backbone of hCAT-2 and the protein domain of SLC7A14 corresponding to the so-called “functional domain” of the hCAT proteins, a protein stretch of 81 amino acids that determines the apparent substrate affinity, sensitivity to trans-stimulation and (as revealed in this study) pH-dependence. The chimera mediated arginine transport and exhibited characteristics similar, but not identical to hCAT-2A (the low affinity hCAT-2 isoform). Western blot and microscopic analyses confirmed localization of the chimera in the plasma membrane of Xenopus laevis oocytes. Noticeably, arginine transport by the hCAT-2/SLC7A14 chimera was pH-dependent, trans-stimulated and inhibited by epsilon-Trimethyl-L-lysine, properties assigned to the lysosomal transport system c in human skin fibroblasts. Expression analysis showed strong expression of SLC7A14 mRNA in these cells. Taken together, these data strongly suggest that SLC7A14 is a lysosomal transporter for cationic amino acids.

Introduction

After degradation of proteins in lysosomes, amino acids have to be carried to the cytoplasm by specialized transport proteins. A lysosomal transport system for cationic amino acids has been described in human skin fibroblasts by Pisoni and co-workers and designated system c (1, 2). However, its molecular identity has not been identified so far. System c has therapeutic interest because it provides a salvage pathway in the therapy of patients with cystinosis (3). In these individuals, the loss of function of the lysosomal cystine transporter cystinosin leads to accumulation of the disulfide cystine, resulting in crystal deposition and cell damage (4). Patients are treated with oral application of the aminothiol cysteamine (5, 6), that enters the cells.
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and the lysosomes and forms a mixed disulfide with one cysteine molecule. This mixed disulfide resembles the cationic amino acid L-lysine and is transported out of the lysosome by a system c transporter. Administration is problematic, because cysteamine has an offensive taste and smell, and has to be taken according to a strict timetable (every 6 hours) (7). Therefore better therapeutic substances are needed. The knowledge of the salvage transporter would facilitate screening for such compounds. Due to their substrate specificity for cationic amino acids and their partial localization to lysosomes (at least in overexpressing cells), members of the solute carrier family 7 (SLC7) subfamily of cationic amino acid transporters (CAT) are potential candidates for system c (8, 9).

Lately, an orphan protein has been assigned to the SLC7 family as member A14 due to sequence homology. The SLC7 family is divided into two subgroups (9). The light chains of the heteromeric amino acid transporters (lcHATs) are predicted to comprise 12 transmembrane domains. They have to associate with a glycoprotein of the SLC3 family (heavy chain) to localize to the plasma membrane. The substrate selectivity and ion-coupling of those glycoprotein-associated amino acid transporters is diversified. In contrast, the CATs are predicted to comprise 14 transmembrane domains, are glycosylated and localize to the plasma membrane without co-expression of a second protein. They mediate exclusively Na⁺-independent transport of cationic L-amino acids. SLC7A14 exhibits a higher sequence identity to the hCAT compared to the lcHAT subfamily (Suppl. Fig.1). It is also predicted to have 14 putative transmembrane domains, which relates it even more closely to the hCAT subfamily (10). Our initial experiments expressing a SLC7A14-EGFP fusion protein in Xenopus laevis oocytes and U373MG glioblastoma cells exhibited an almost exclusive intracellular staining that coincided with the lysosomal marker LysoTracker® in the latter. This suggests that SLC7A14 may represent a lysosomal transport protein. In addition, SLC7A14 was expressed in human skin fibroblasts where system c has initially been described. We thus hypothesized that SLC7A14 may represent system c. To measure transport activity of the protein we first attempted to direct SLC7A14 to the plasma membrane by mutating putative lysosomal targeting sequences, a strategy that has successfully been used in the case of cystinosin (11). However, here we were without success. Therefore, we created a chimera carrying the functional domain of human SLC7A14 in the backbone of human CAT-2. This domain is defined by a stretch of 81 amino acids that resemble the forth intracellular and the fifth extracellular loop and the transmembrane domains (TM) nine and ten of all CAT proteins, according to the 14-TM model. For this domain it has already been described that it determines the apparent substrate affinity and sensitivity to trans-stimulation (12). The latter refers to a transport property typical for carrier proteins and distinguishing them from channels: Carrier often work better or even exclusively in an exchange mode as opposed to unidirectional transport. This can be seen by acceleration of transport by substrate at the trans-side of the membrane (the side where substrate is transported to) and is referred to as trans-stimulation. The hCAT-2/A14_BK chimera mediated arginine transport and was characterized regarding its localization, substrate affinity, pH-dependence, trans-stimulation properties and substrate specificity. The results from these experiments support the notion that SLC7A14 may indeed represent a system c transporter

Experimental procedures

Cell culture

The U373MG glioblastoma cell line was obtained from ATCC, Bethesda, MD. Stable transfected cells were grown in Iscove’s Modified Dulbecco’s Medium (Gibco/Invitrogen, Carlsbad) supplemented with GlutaMAX, 10% fetal bovine serum (FBS) and 0.2 mg/ml G418. Human skin fibroblasts were a kind gift from Martijn Wilmer (Department of Pharmacology/Toxicology 149; Nijmegen Centre for Molecular Life Sciences (M850-7.069); Nijmegen; NL) and were cultured in M199 medium + 10% FCS + penicillin/ streptomycin (100 U/ml).

Quantitative RT-PCR

Total RNA from human cells was isolated using the RNasy Mini Kit (Qiagen, Hilden). The expression of amino acid transporters and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as reference) was determined using the QuantiTect RT-PCR Kit (Qiagen, Hilden) and specific TaqMan hybridization probes as described (13). The following oligonucleotides and hybridization probe were used to detect SLC7A14 (5’-3’): ss: CTGGTGAACATCTATCTCATGC; as:
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CTGTTCCAGATGCCATATCC; TaqMan probe: [6-FAM]AAGCTCTCCACCATCATGAGATCC[Tamra-Q]

Site directed mutagenesis and generation of chimeric cDNAs

Site directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, Heidelberg). Restriction sites for BamHI and KpnI were introduced or deleted in the SLC7A14 coding sequence as silent mutations. The sequence of each oligonucleotide pair is presented in the supplemental table I. The introduction of a BamHI site into the hCAT-2A and hCAT-1 sequences and other chimeras have previously been described (4). This and a conserved KpnI sites were used to create chimeric cDNAs between SLC7A14 and hCAT-2.

Fluorescent protein fusion constructs and expression in U373MG cells

Full length cDNA clone from human KIAA1613/SLC7A14 (IRAKp961A1742Q2) was obtained from imaGenes (Source BioScience LifeScience, Berlin, Germany). The fluorescent protein fusion construct for mammalian expression were realized by amplification of the cDNA insert of hSLC7A14 by PCR (HotStar HiFidelity Polymerase, Qiagen, Germany) using the oligonucleotides TCAGATCTCTCAAGATGAGTGGCTTCTTACCTC (containing a BglII site, underlined; and a start codon, in italic characters) and CGACCGGTCGCAACTCTGGAGAGTATCTAACTC (removes the stop codon and introduces an AgeI site, which is underlined) as sense and antisense primers, respectively, and subcloned into the BglII/AgeI sites of pEGFP-N1. The resulting plasmid was named pSLC7A14-EGFP. Constructs for EGFP fusion proteins with hCAT-2A (3) and hCAT-1 (15) have previously been described. Cells were seeded into 6-well plates (2x10^8 cells/well) one day before transfection. 8 μl of Fugene HD (Promega, Madison) was added to 160 μl of plasmid DNA (2 μg) in water, mixed and incubated at room temperature for 10 min. Then, the reaction was added drop-wise to the cells in 1 ml medium containing FBS. The cells were incubated with the transfection reagent for 48 h at 37 °C. Stably transfected cell clones were selected in medium containing 0.2 mg/ml G418. For each construct, several independent clones were selected.

Fluorescent microscopy

U373MG cells expressing SLC7A14.EGFP were seeded in glass bottom dishes, stained with 50 nM Lysotracker® DND99 Red, 1μM MitoTracker® Orange CM-H2TMRos or 1μM ER-Tracker™ Red Dye (all Molecular Probes, Invitrogen, Eugene) and analyzed by confocal laser scanning microscopy.

Expression of cRNA in Xenopus laevis oocytes

All cDNAs were inserted between the SacII and ClaI site of pSGEM (14). The plasmids were linearized and cRNA was prepared by in vitro transcription from the T7 promoter (mMessage mMachine in vitro transcription kit, Ambion, AMS Biotechnology Europe, Cambridge). 10 ng cRNA in 20 nl water were injected in each X. laevis oocyte (Dumont stages V and VI). Non-injected oocytes were used as controls. All experiments were performed 3 days after cRNA injection.

Transport studies in X. laevis oocytes

To measure arginine uptake, oocytes were washed three times in ice cold uptake solution (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, and 5 mM Tris, pH 7.5). The oocytes were then transferred to the same solution supplemented with the indicated concentrations of unlabeled L-amino acids (0.01 – 10 mM) and [3H]-L-arginine (10 μCi/ml; ICN, Eschwege). After 15 min incubation at 20 °C, oocytes were washed 5 times with ice-cold uptake solution and solubilized individually in 2% SDS. The radioactivity of the lysates was determined in a liquid scintillation counter. For trans-stimulation and efflux experiments, cRNA-injected oocytes were each injected a second time with 3.6 nmol of [3H]-L-arginine (3.6 nCi) in 36 nl of water. The oocytes were then immediately transferred into uptake solution (pH and amino acid concentration as indicated). After 30 minutes incubation at 20°C, the [3H]-L-arginine that had accumulated in the extracellular buffer was determined by liquid scintillation counting.

Cell Lysates & Biotinylation of Cell Surface Proteins

All steps were performed at 4°C. Oocytes were rinsed 3 times in PBS (0.1 M NaCl, 2 mM KCl, 1.76 mM KH₂PO₄, 10.1 mM Na₂HPO₄), containing 0.1 mM CaCl₂ and 1mM MgCl₂ (PBS/CM) and then incubated in PBS/CM containing 1 mg/ml sulfosuccinimidobiotin (EZ-LinkTM: sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate,
Thermo Fisher Scientific Inc., Rockford) for 30 min. The biotinylation reaction was stopped by incubating oocytes in PBS/CM containing 50 mM NH₄Cl for 10 minutes, and rinsing 4 times in PBS/CM. After lysis in radioimmune precipitation assay buffer (RIPA: 0.15 mM NaCl, 1 mM EDTA pH 8, 0.1 mM Tris pH 7.2, 1% Triton X100, 1% deoxycholate, 0.1% SDS) containing protease inhibitors (Complete Mini EDTA-free protease inhibitor tablets, Roche, Basel). An aliquot of each whole oocyte lysate was mixed directly with an equal volume of 2x washing buffer II (50 mM Tris pH 7.4, 1 mM EDTA, 0.5% Triton X100, 0.1% SDS) and once in washing buffer I (50 mM Tris pH 8, 0.5% Triton X100, 0.1% SDS). All washing solutions contained 0.2 mM PMSF. Biotinylated proteins were released from the beads by incubation in 1x sample buffer for 10 min at 37 °C and analyzed by Western blotting using avidin-coated sepharose beads (immobilized NeutrAvidin™, Thermo Fisher Scientific Inc., Rockford). The beads were then washed three times with washing buffer I (50 mM Tris pH 8, 0.5 mM NaCl, 1 mM EDTA pH 8, 0.5% Triton X100, 0.1 % SDS) and once in washing buffer II (50 mM Tris pH 7.4, 1 mM EDTA, 0.5% Triton X100, 0.1% SDS). All washing solutions contained 0.2 mM PMSF. Biotinylated proteins were released from the beads by incubation in 1x sample buffer for 10 min at 37 °C and analyzed by Western blotting.

**Western Blot**

Lysates were separated by 10% SDS-PAGE and then blotted onto nitrocellulose transfer membranes (Protran 83, Whatman, Maidstone). Staining for EGFP proteins was achieved by sequential incubations in Blotto (50 mM Tris pH 8, 2 mM CaCl₂, 0.01% antifoam A (Sigma-Aldrich, St. Louis), 0.05% Tween 20, and 5% nonfat dry milk) for 2 h at room temperature to block unspecific binding sites; a 1:3000 dilution of rabbit anti-EGFP (living colors® full length A.v.polyclonal antibody, BD Biosciences Clontech, Mountain View) in Blotto overnight at 4 °C; three times in Blotto for 10 min at room temperature; a 1:15,000 dilution of peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Calbiochem, Darmstadt) in Blotto for 1 h at room temperature; three times in TBS-T (10 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 20); once in TBS (10 mM Tris-HCl, pH 8, and 150 mM NaCl); and finally in chemiluminescence reagent (Western Lightning® ECL-Plus, Perkin Elmer, Waltham) for 1 min. Chemiluminescence films (Amersham Hyperfilm™ ECL, GE Healthcare Limited, Buckinghamshire) were then immediately exposed to the membranes. For standardization, membranes were stained with anti-ß-tubulin monoclonal antibody (1:3000; Sigma-Aldrich, St. Louis) and peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000; Sigma-Aldrich, St. Louis).

**Results**

Our initial experiments where we expressed the orphan protein SLC7A14 in *Xenopus laevis* oocytes revealed no increase in arginine transport compared with control oocytes (data not shown). To prove protein expression and investigate the subcellular localization of the orphan, we fused the enhanced green fluorescent protein (EGFP) to the C-terminus of SLC7A14. Expression of this fusion protein revealed no plasma membrane staining in either *X. laevis* oocytes or human U373 MG glioblastoma cells. In the latter, staining of intracellular vesicles that also lit up with the lysosomal marker LysoTracker® was observed (Fig.1A). In contrast, overexpressed hCAT-1.EGFP (and all other hCATs) was clearly localized to the plasma membrane in these cells, in addition to intracellular membranes (not shown). EGFP alone was equally distributed in the cytoplasm (not shown). SLC7A14 and hCAT-1 coincided in intracellular membranes, but not at the plasma membrane (not shown). For SLC7A14.EGFP localization in mitochondria and endoplasmatic reticulum could be excluded by staining of the cells with MitoTracker® and ER-Tracker™. Western blot analyses revealed that like hCAT proteins (and unlike lCHATs) SLC7A14 is glycosylated (Fig.1B). As in *Xenopus laevis* oocytes, overexpression of SLC7A14.EGFP in U373MG cells did not lead to enhanced arginine transport in comparison to control cells (not shown). We thus hypothesized that SLC7A14 may represent a lysosomal transporter for cationic amino acids as described in human skin fibroblasts and designated system c. In fact, SLC7A14 mRNA was expressed in primary human skin fibroblasts from 6 different donors (Fig.2). Related to GAPDH (and assuming equal efficiency of the RT/PCR reaction), SLC7A14 mRNA expression was about half of hCAT-1. hCAT-2B and hCAT-3 mRNA expression was marginal and hCAT-2A mRNA could not be detected at all. There was no difference in SLC7A14 expression between cystinotic and wild type cells (not shown).

To measure transport activity, we first tried to redirect SLC7A14 to the plasma membrane by...
mutating putative lysosomal targeting sequences, however without success. Therefore, a chimera carrying the so-called functional domain of SLC7A14 in the backbone of hCAT-2 was created. This chimera was named hCAT-2/A14_BK, as the exchanged fragment in the respective cDNAs is framed by a BamHI and a KpnI restriction site (Fig.3A). According to the 14 transmembrane domain (TM) model, the SLC7A14 fragment introduced into hCAT-2 comprised the fourth intracellular and the fifth extracellular loop, part of the eighth TM and TM nine and ten (Suppl. Fig.2). Like hCAT-2A, an EGFP fusion protein of the chimera hCAT-2/A14_BK localized to the plasma membrane of Xenopus laevis oocytes, as revealed by fluorescence microscopy (Fig.3B). In contrast EGFP alone and SLC7A14.EGFP remained intracellular. Western blot analyses of whole cell lysates and cell surface-biotinylated protein fractions confirmed plasma membrane localization of the chimera. In contrast to fluorescent microscopy, this method revealed a very small portion of SLC7A14.EGFP to be localized to the plasma membrane (Fig.3C).

Transport measurements using 1mM [3H]L-arginine and an incubation time of 15 minutes (to stay in the linear part of the uptake, Suppl. Fig.3) demonstrated that the chimera hCAT-2/A14_BK mediates arginine transport (0.61 ± 0.06 compared to 1.38 ± 0.16 nmol arginine/oocyte/h mediated by hCAT-2A at pH 7.5).

The apparent substrate affinity of the chimera was determined at arginine concentrations between 0.01 and 10 mM (Tab.I). The arginine concentrations where half maximal transport rates were reached (apparent $K_M$) were calculated by fitting the data according to the Eadie-Hofstee equation after subtraction of the values obtained with non-injected oocytes. The apparent $K_M$ of the hCAT-2/A14_BK chimera (1.93 mM ± 0.19) was low, similar to the apparent $K_M$ of hCAT-2A (1.45 mM ± 0.02). As reported before, hCAT-1 exhibited a significantly higher apparent affinity for arginine ($K_M$; 0.6 mM ± 0.06).

Next, we determined in efflux experiments where labeled substrate was injected into the oocytes whether the chimera is trans-stimulated (Fig.4) e.g. if it exhibits higher transport rates, when substrate is present at the opposite side of the membrane. This is characteristic for hCAT-1. In the absence of trans-substrate, efflux mediated by the chimera was reduced by 47 ± 4.5% (compared to efflux into buffer containing 1 mM arginine). Although trans-stimulation was not as pronounced as for hCAT-1 (87 ± 0.7% reduced transport without trans-substrate), it was clearly different from hCAT-2A that exhibited no trans-stimulation.

We then asked if pH-differences influenced the transport activity of the chimera by varying the extracellular pH from 5 to 8.5 in influx experiments. Indeed, the transport activity of the chimera was continually increased from pH 5 to 8.5: transport rates at pH 5 were only 24.7% ± 3.2 of the rates measured at pH 7.5 (Fig.5A). The pH-dependence of the chimera was thus similar to hCAT-2(A or B) and significantly different from hCAT-1 that is largely pH-independent (Fig.5A and (15)). Until now it was not clear if the functional domain also defines the pH-dependence of the hCAT proteins. To answer this question, a chimera, carrying the functional domain of hCAT-1 in the backbone of hCAT-2 (hCAT-2/1_BK) was investigated. Like hCAT-1, hCAT-2/1_BK was pH-independent, suggesting that pH dependence is indeed determined by the functional domain (Fig.5A).

Arginine efflux mediated by hCAT-2/A14_BK and hCAT-2A was also reduced at extracellular pH 5 compared to pH 7.5, while hCAT-1-mediated efflux was again pH-independent (Fig.5B).

Pisoni et al. identified cationic amino acid derivatives that interfere with transport by lysosomal system c, but not system y$^+$ (2). In accordance with these observations, transport of the chimera, but not of hCAT-1, hCAT-2B and hCAT-3 (all system y$^+$ transporters) was inhibited by ε-trimethyl-L-lysine (Fig.6). However, the inhibition of the chimera was less pronounced than reported for system c in human skin fibroblasts (34% versus 50%). Transport by hCAT-2A (which is not expressed in skin fibroblasts and not a y$^+$ transporter) was inhibited to a similar extent as the chimera. In contrast, hCAT-2/1_BK remained unaffected. This suggests the functional domain to be involved in substrate recognition.

To further elucidate this question, we investigated whether histidine recognition is determined by the functional domain. We have previously established that histidine is a substrate for hCAT-1 at low pH (when it carries a positive charge). Here we found, that in contrast, hCAT-2A transported only very little histidine under this condition (Fig. 7A). The hCAT-1/2A_BK chimera was very similar to hCAT-2A, supporting the notion that the functional domain is involved in substrate recognition. Histidine
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Discussion

The aim of our study was to elucidate the physiological function of SLC7A14, an orphan protein assigned to the amino acid transporter family SLC7 due to sequence homology. Within this family SLC7A14 is most closely related to the subfamily of cationic amino acid transporters (42-45% identity in amino acid sequence in comparison to 16-20% identity to the subfamily of light chains of heterodimeric amino acid transporters, lchATs) (Suppl. Fig.1). In addition like all hCATs, SLC7A14 is predicted to exhibit 14 TM by most analysis software. This is in contrast to lCHATS with only 12 putative TMs. In mammalian cells, SLC7A14 localized almost exclusively to intracellular vesicles that were not stained with MitoTracker® or ER-Tracker™ but with the lysosomal marker LysoTracker®. This is in contrast to all other known hCAT proteins that are clearly residents of the plasma membrane (Fig.1 and (8)). However, hCAT proteins are also found in lysosomes where they coincide with lysosomal markers (unpublished). If they function as amino acid transporters in these compartments remains to be determined. The intracellular localization of SLC7A14 is different from the intracellular localization of lCHATs that are trapped in the ER when their respective glycoprotein partner is missing (16). In contrast, hCAT proteins do not seem to need a partner protein to traffic to the plasma membrane. Using fluorescence microscopy the SLC7A14.EGFP fusion protein was solely detected in lysosomes also in all other cell types tested (U373 glioblastoma, Huh7 hepatoma, A673 neuroepithelioma, NT2 teratocarcinoma and TGW neuroblastoma cells, data not shown), including two that exhibited endogenous expression of SLC7A14. This was also true when the fluorescent protein DsRed (dimeric or monomeric) was fused to the C- or N-terminus of SLC7A14, indicating that the subcellular localization was independent of the position or type of fluorescent protein in the fusion protein (not shown). We found a very small portion of SLC7A14 to be biotinylated at the cell surface of Xenopus laevis oocytes, suggesting that the transporter may first be incorporated into the plasma membrane before it traffics to lysosomes. This has also been observed for other lysosomal proteins (17). Taken together, these results indicate that SLC7A14 is truly a lysosomal resident. However, antibodies against SLCA14 are necessary to examine the subcellular localization of the native protein in primary cells and tissues.

In addition to its expression in human skin fibroblasts, high levels of SLC7A14 mRNA were detected in the CNS (brain, cerebellum, spinal cord) using a human multi tissue RNA panel (Suppl. Fig.4A). This is consistent with findings in other bilateral species (18). Marginal mRNA levels could be detected in trachea, kidney, prostate, salivary gland, testis and whole skin, while no A14 mRNA was detectable in heart, fetal liver, placenta, thymus and thyroid gland. Amongst various human cell lines tested, human umbilical vein endothelial cells (HUVEC), NB-OK-1 and TGW-1 neuroblastoma cells exhibited considerable SLC7A14 expression (Suppl. Fig.4B). SLC7A14 thus seems to be expressed primarily in skin fibroblasts, neuronal cells and primary endothelial cells. If SLC7A14 represents indeed system c in these cells, a different transporter has to mediate lysosomal transport of cationic amino acids in tissues lacking SLC7A14 expression.

Our mRNA analyses show that hCAT-1 and SLC7A14 are the only members of the hCAT subfamily that are expressed at an appreciable level in human skin fibroblasts. As Pisoni et al. found clear differences in the transport activities of systems c and y⁺ (hCAT-1) it seems unlikely for hCAT-1 to be responsible for the system c activity described in fibroblast lysosomes. On the other hand, the chimera carrying the functional domain of SLC7A14 had transport properties very similar to system c (and different from system y⁺/ hCAT-1): apparent low substrate affinity, pH dependence, moderate trans-stimulation, inhibition by ε-trimethyl-L-lysine.

A crucial question is thus if the transport properties of the chimera reflect those of SLC7A14. Several findings indicate that this is indeed the fact: (i) first of all, the observation that the chimera mediates arginine transport indicates that its functional domain is derived
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from a transporter that recognizes this amino acid as substrate. In addition, the cationic amino acid lysine is recognized by the chimeric protein equally well as arginine (Suppl. Fig.5). (ii) Our previous work has shown that the functional domain defines the apparent substrate affinity and sensitivity to trans-stimulation (12). Here we further show that pH-dependence of the transport activity is also determined in this domain: Like the donor of the functional domain in the chimera hCAT-2/1_BK (hCAT-1), the chimera itself was pH independent. As the chimera hCAT-2/A14_BK is pH-dependent, it seems very likely that the donor of its functional domain, SLC7A14, is a pH-dependent transporter as described for system c. (iii) In contrast to hCAT-1, the chimera hCAT-1/2A_BK recognized histidine very poorly, demonstrating that the functional domain is necessary for substrate recognition. However, other protein regions seem also to be involved in this process as the introduction of the functional domain of hCAT-1 into the hCAT-2 backbone was not sufficient to fully confer histidine recognition. However, the importance of the functional domain in substrate recognition together with the observed arginine transport activity for hCAT-2/A14_BK highly suggests that SLC7A14 is also an arginine transporter. In addition, this indicates that epsilon-three methyl-L-lysine is most likely recognized by SLC7A14.

(iv) The transport properties of hCAT-2/A14_BK are similar but not identical to hCAT-2A: the chimera exported a 2-fold higher amount of arginine into buffer containing 1mM arginine than into buffer containing no cationic amino acid, while hCAT-2A was not trans-stimulated. Therefore, hCAT-2/A14_BK is clearly different from its backbone donor, hCAT-2A, and also from the system y* transporter hCAT-1 that exhibited a more pronounced (7.6-fold) trans-stimulation than the chimera. The trans-stimulation of the chimera resembles in fact system-c-mediated lysine efflux from lysosomes that is 2-fold higher into lysine containing buffer compared to buffer without any cationic amino acid (1).

Taken together, our data suggest that the system-c-like transport properties of the chimera hCAT-2/A14_BK reflect the transport properties of SLC7A14. The “wrong” pH dependence of system c has already been discussed by Pisoni et al. (2). For a lysosomal exporter, one would rather expect that the transport activity increases at low luminal pH (which is equivalent to extracellular pH in our transport assay). Instead the activity of system c and the functional domain of SLC7A14 decrease at low pH, suggesting that they mediate substrate uptake into rather than efflux out of lysosomes. In our experiments, we varied only the pH at the extracellular space that corresponds to the intr lysosomal space when considering the orientation of the transporter. In contrast, measuring uptake in isolated lysosomes, Pisoni et al. varied the pH at the extra lysosomal side (corresponding to the cytoplasmic side). Efflux out of oocytes (that corresponds to uptake into lysosomes) was just as poor at a low pH as influx into oocytes (that corresponds to export out of lysosomes). In addition the pH-dependence was identical under trans-stimulated and not trans-stimulated conditions (not shown). This indicates that in general SLC7A14 has only a weak transport activity at low pH.

hCAT-2/A14_BK showed an apparent low substrate affinity, similar to the low affinity hCAT-2A and significantly different from the high affinity hCAT-1. In comparison to system y* low substrate affinity has also been described by Pisoni et al. for system c (2).

Until now it was not clear whether the functional domain also determines pH-dependence. The pH independence of hCAT-2/1 (a chimeric protein with the functional domain of the pH-independent hCAT-1 in the backbone of the pH-dependent hCAT-2) supports this notion. It seems thus likely that the pH-dependent arginine transport of the hCAT-2/A14 chimera is a genuine characteristic of SLC7A14.

In summary the data presented here suggest that SLC7A14 may represent a lysosomal transporter for cationic amino acids resembling system c because (i) it localizes almost exclusively to lysosomes in mammalian cells, (ii) it is expressed in human skin fibroblasts where system c has been first described and most important, (iii) its functional domain confers arginine transport activity similar to system c. However, final proof has to await establishment of an assay where transport of the native protein can be measured directly. In addition, other lysosomal transporters for cationic amino acids must exist in cells not expressing SLC7A14.
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References


Footnotes

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The abbreviations used are: hCAT, human cationic amino acid transporter; SLC, solute carrier; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; lchAT, light chain of heteromeric amino acid transporter.

Figure Legends

Figure 1: Localization of SLC7A14 in human U373 MG glioblastoma cells
A) U373MG cells overexpressing the SLC7A14.EGFP-fusion protein (stable transfection, green) were stained with LysoTracker®, MitoTracker® or ER-TrackerTM (all Invitrogen, red). Cells were analyzed by confocal laser scanning microscopy. Scale bars represent 10 µm. B) U373MG overexpressing SLC7A14.EGFP were lysed and analyzed by Western blotting. Proteins with (+) or without (-) glycosidase treatment were separated by 7.5 % SDS-PAGE, blotted and probed with an anti-GFP antibody (upper panel) and an anti-tubulin antibody as loading control (lower panel).

Figure 2: mRNA Expression of SLC7A14 and CATs in human skin fibroblasts
Total RNA was isolated from skin fibroblasts of six donors. Messenger RNAs of SLC7 members were measured by quantitative RT-PCR. GAPDH was chosen as housekeeping gene for relative determinations and was set 1. Columns represent means ± SEM (n=6).

Figure 3: Localization of SLC7A14, hCAT-2/A14_BK, and hCAT-2A in Xenopus oocytes
A) Scheme of the chimeric protein hCAT-2/A14_BK. The functional domain of hCAT-2 (aa 357 - 431) was replaced by the corresponding fragment of SLC7A14 (aa 352-433, dark grey), which resembles the forth intracellular and the fifth extracellular loop and the transmembrane domains (TM) nine and ten of both proteins, according to the 14-TM model. B) Xenopus oocytes were frozen in tissue freezing medium 3 days after cRNA injection. Cryosections (12µm) were analyzed by fluorescence microscopy. C) Proteins of Xenopus oocytes expressing the EGFP fusion protein or EGFP alone as indicated were biotinylated at the cell surface. Non-injected oocytes served as controls. Subsequently oocytes were lysed for Western blot analysis. Total lysates (left side) or surface proteins (separated from non-biotinylated intracellular proteins by avidin-coated beads, right side) were separated by 10 % SDS-PAGE, blotted and probed with an anti-GFP antibody (upper panels) and subsequently with an anti-tubulin antibody as control for loading (lower panel, left side) and successful withdrawal of intracellular proteins (lower panel, right side).

Figure 4: Trans-stimulation of hCAT-2/A14_BK in comparison to hCAT-1 and -2A
Xenopus oocytes expressing the indicated transporters were incubated in amino acid free medium for 4-6 h and then each injected with 36 nl 100 mM [3H]-L-arginine [100 µCi/ml]. Three oocytes each were then incubated for 30 minutes in buffer (pH 7.5) containing either no amino acids or 1 mM arginine. Subsequently, the radioactivity in the supernatants was determined. Values obtained with non-injected oocytes were subtracted and the radioactivity in buffer containing no amino acids was expressed as percent of the respective radioactivity detected in extracellular buffer containing 1mM L-arginine (= 100%: dotted line). Columns represent means ± S.E.M (n=9-15), with at least three different batches of oocytes. Statistical analysis was performed using analysis of variance with Bonferroni’s post-hoc test. Three stars correspond to p ≤ 0.001, n.s. stands for not significant.

Figure 5: pH dependence of L-arginine-transport mediated by hCAT-2/A14_BK in comparison to hCAT-1 and -2A
Xenopus oocytes were injected with cRNA encoding individual transporters. A) Uptake of 1 mM [3H]-L-arginine into oocytes expressing: -••- hCAT-2/A14_BK, -◆- hCAT-2A, •- hCAT-1, or -Φ- hCAT-2/1_BK was measured in dependence of various pH values in the extracellular buffer. B) 36 nl 100 mM [3H]-L-arginine [100 µCi/ml] were injected into the oocytes. Efflux measured in extracellular buffer containing 1mM arginine at pH 5 is expressed as percent of efflux at pH 7.5 (dotted line). Data points and bars represent means ± S.E.M (n=10-20 for influx and 9-15 for efflux experiments), with at least two different batches of oocytes. Statistical analysis was performed using analysis of variance with Bonferroni’s post-hoc test. Three and two stars correspond to p ≤ 0.001 and 0.01, respectively; n.s. stands for not significant.
Figure 6: Transport activity in the presence of the system c inhibitor ε-trimethyl-L-lysine

*Xenopus* oocytes were injected with cRNA encoding the indicated transporter, respectively. Uptake of 30 µM [³H]-L-arginine in the absence or presence of 3 mM ε-trimethyl-L-lysine was determined. Values obtained in the absence of the putative inhibitor were set as 100% (not shown). Columns represent means ± S.E.M (n=10-20), with at least two different batches of oocytes. Statistical analysis was performed using analysis of variance with Bonferroni’s post-hoc test. Three and two stars correspond to p ≤ 0.001 and 0.01, respectively, when compared to untreated controls.

Figure 7: Substrate recognition of several chimeric proteins in comparison to the wild type transporters

*Xenopus* oocytes were injected with cRNA encoding the indicated transporters. A) Uptake of 1 mM [³H]-L-arginine (black bars) or 1 mM [³H]-histidine (grey bars) was measured at pH 5.5. Data points represent means ± S.E.M (n=13-28), with at least two different batches of oocytes. B) The same data as shown in A) but here, histidine transport is expressed as percent of the arginine transport measured in parallel by the same transporter, respectively.

### Table I

Table I

*Apparent Kₘ of the chimera hCAT-2/A14_BK in comparison to hCAT-1 and -2A*

For expression of hCAT-2/A14_BK, hCAT-2A and hCAT-1 cRNA was injected in *Xenopus laevis* oocytes. Not injected oocytes served as controls. [³H]-L-arginine uptake in dependence on the extracellular amino acid concentration (0.01 mM, 0.03 mM, 0.1 mM, 0.3 mM, 1 mM, 3 mM, and 10 mM) was measured. The results represent means ± SEM (n= 3-4)

<table>
<thead>
<tr>
<th></th>
<th>Kᵢₘ [µM L-arginine]</th>
<th>Vᵢₘ [nmol L-arginine/oocyte/h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT-1</td>
<td>0.567 ± 0.055</td>
<td>3.083 ± 0.466</td>
</tr>
<tr>
<td>CAT-2A</td>
<td>1.445 ± 0.018</td>
<td>4.208 ± 0.615</td>
</tr>
<tr>
<td>CAT-2/A14_BK</td>
<td>1.929 ± 0.194</td>
<td>2.511 ± 0.38</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3

A

CAT-2  SLC7A14  CAT-2

\[ \text{BamHI} \quad \text{KpnI} \]

B

CAT-2/A14 BK  CAT-2A

EGFP  A14

C

\begin{tabular}{c|c|c|c|c|c|c}
 & EGFP & A14 & CAT-2 & CAT-2/A14_BK & Control & EGFP & A14 & CAT-2 & CAT-2/A14_BK & Control \\
\hline
kDa & & & & & & & & & & \\
170 & & & & & & & & & \\
130 & & & & & & & & & \\
95 & & & & & & & & & \\
72 & & & & & & & & & \\
55 & & & & & & & & & \\
43 & & & & & & & & & \\
55 & & & & & & & & & \\
\end{tabular}

whole cell lysates  surface proteins
Figure 4

![Bar chart showing transport activity for CAT-2/A14_BK, CAT-2A, and CAT-1.](chart.png)
Figure 5
Figure 6

![Graph showing transport activity as a percentage of uninhibited for different conditions: CAT-1/A14_BK, CAT-2A, CAT-1, CAT-2B, CAT-3, and CAT-2/1_BK. The graph includes error bars indicating variability.](http://www.jbc.org/)
A chimera carrying the functional domain of the orphan protein SLC7A14 in the
backbone of SLC7A2 mediates trans-stimulated arginine transport*
Isabel Jaenecke, Jean-Paul Boissel, Matthias Lemke, Johanna Rupp, Bruno Gasnier and
Ellen I. Closs

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