CRF grant 'Identification and characterization of the lysosomal transporter involved in cysteamine-mediated cysteine efflux'

6-month Progress Report, Feb 6th 2008

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Cysteamine depletes cystine from cystinotic lysosomes through formation of a mixed cysteamine-cysteine disulfide which exits lysosomes through the lysosomal cationic amino acid transporter, system c.
The molecular identity of system c is unknown. In preliminary experiments, the Mainz and Paris groups showed that several members of the SLC7 family of cationic amino acid transporters (CATs) localize to lysosomes and late endosomes, thus representing candidate system c transporters. In particular, two characterized CAT proteins, CAT2A or B, and an orphan SLC7 family member, SLC7A14, showed preferential lysosomal localization after transient expression in HeLa cells.

The aim of the project is to examine whether these SLC7 proteins are involved in lysosomal cysteamine-cysteine efflux, with the long-term goal to improve small-molecule treatment of cystinosis. Since these hypotheses on the identity of system c are not exclusive, they were examined in parallel.

The Paris and Mainz groups regularly met over the past 6 months to concert their efforts, share data and exchange tools (plasmids, etc).

Progress Report Paris group (Christine Anne, Samira Boubekeur, Bruno Gasnier)

The Paris group focused on two objectives:

- characterize the interaction of cysteamine-cysteine with characterized CAT proteins
- search potential sorting motifs of SLC7A14 to induce plasma membrane expression as a preliminary step to functional studies

Recognition of the mixed disulfide cysteamine-cysteine by CAT transporters

A custom synthesis of 500 mg cysteamine-cysteine disulfide was purchased from the company Idealp-Pharma (http://www.idealp-pharma.com/).

The ability of cysteamine-cysteine to interact with human CAT1, CAT2A or CAT2B was examined by performing arginine transport assays in transiently transfected HEK cells using plasmids and protocols provided by the Mainz group. CAT1 was included as a control and because previous data from Mainz and Paris showed that it partially localizes to lysosomes and late endosomes in addition to the plasma membrane.

Surprizingly, hCAT1 turned out to be the most sensitive to cysteamine-cysteine, with ~80% inhibition at 10 mM concentration as compared to ~10% for CAT2B. The sensitivity of CAT2A is intermediate. These experiments thus suggest that CAT1 and/or CAT2A might be involved in cysteamine-mediated cystine depletion since they both share lysosomal localization and recognition of the mixed disulfide. The potential role of CAT1 is interesting since it is ubiquitously expressed.

Because of the high level of endogenous arginine uptake in mammalian cells, IC50 values of CATs for cysteamine-cysteine could not be determined in HEK cells. These experiments are thus now pursued in Xenopus oocytes, an expression system which provides a much higher signal-to-noise ratio, using plasmids provided by Mainz. Measurements will be repeated at acidic extracellular pH to mimic conditions of the lysosomal lumen.

Next step: The ability of CAT1, CAT2A and CAT2B to translocate cysteamine-cysteine across membranes using a custom-synthesized 3H-labeled molecule.

Development of an *in situ* cystine depletion assay in whole cells

The above transport experiments will tell whether CATs *can* export cysteamine-cysteine from lysosomes –and help focusing on particular isoforms– but not whether they actually do so. It is thus necessary to develop a lysosomal cystine depletion assay to provide a final conclusion on the identity of system c. This assay can be performed on isolated lysosomes or in whole cells, using an ester derivative to artificially load lysosomes with cystine.

To this purpose, we ordered a custom synthesis of [3H]L-cystine dimethyl ester labeled on the cystine residue to the company Hartmann Analytic (http://www.hartmann-analytic.de/). However, because of purification problems the compound ordered in July was received only in late December.

Experiments are now ongoing to find optimal conditions for loading [3H]cystine into lysosomes of whole cells and perform cysteamine depletion experiments.

Screening of putative sorting motifs of the orphan transporter SLC7A14

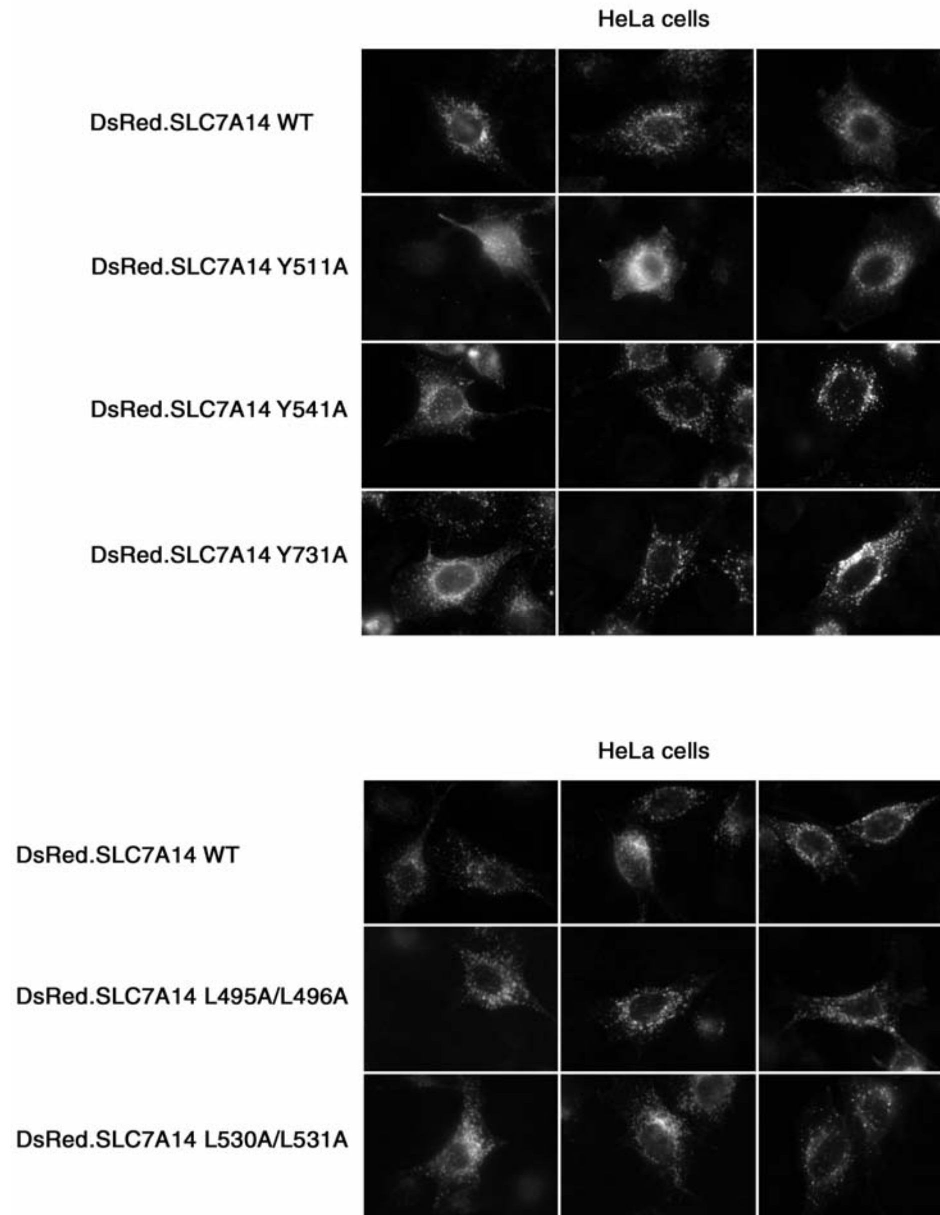
As shown in previous studies from the Paris group (Kalatzis et al EMBO J 2001; Morin et al EMBO J 2004), the functional characterization of lysosomal transporters can be facilitated by redirecting them to the plasma membrane for transport measurement in whole cells. Paris and Mainz thus tried different approaches in order to redirect SLC7A14 to the cell surface.

Attempts made in Paris were based on the mutation of potential lysosomal sorting motifs (see Bonifacino and Straub 2003 Ann. Rev. Biochem. 72:395-447). Several tyrosine-based and dileucine-based candidate motifs were identified in cytosolic loops or in the C-terminal end of SLC7A14.

Their critical residues were mutated to alanine as follows:
L495A/I496A
Y511A
L530A/I531A
Y541A

Y731A

The mutations were introduced into a DsRed-SLC7A14 construct provided by Mainz. However, as illustrated below, none of these mutants was redirected to the plasma membrane after transient expression in HeLa cells.



Two hypotheses may explain these results: either neither of the above motifs is involved (the lysosomal localization of SLC7A14 resulting from other signals) or several of them are involved. The latter hypothesis (motif redundancy) will be examined by combining several of the above mutations in a single construct. In parallel, we will explore a novel strategy bypassing knowledge of the sorting mechanism by inserting a *dominant* motif conferring surface expression into wild-type SLC7A14 (see Shikano et al 2005 Nat. Cell Biol. 7:985-992).

Other attempts by the Mainz group are described below.

Progress Report Mainz group (Ellen Closs and Jean-Paul Boissel)

Our focus in the first 6 months of the grant period lay on SLC7A14, the orphan member of the SLC7 family that exhibited an exclusive lysosomal localization in our preliminary studies. The following points were addressed:

- Investigation of the expression pattern of SLC7A14 in human tissues and cell lines
- Subcellular localization of SLC7A14 in different human cell lines
- Attempts to induce plasmalemmal localization of SLC7A14
- Creation of stable cells lines expressing SLC7A14.EGFP fusion proteins

Expression pattern of SLC7A14 in human tissues and cell lines

To find out which tissues and cell lines express SLC7A14, its mRNA expression was assessed by qRT- real time PCR. High levels of message were detected in the CNS (brain, cerebellum, hippocampus, spinal cord), and in the bladder (Figure 1). Amongst various human cell lines tested, human umbilical vein endothelial cells (HUVEC), NB-OK-1 and TGW- 1 neuroblastoma cells exhibited considerable SLC7A14 expression (Figure 2A). Only little SLC7A14 mRNA was detected in NT2 neuron-committed teratocarcinoma cells under basal condition. However, when neuronal differentiation was induced in these cells by treatment with retinoic acid, a time-dependant upregulation of SLC7A14 was observed (Figure 2B). SLC7A14 thus seems to be expressed primarily in neuronal cells, endothelial cells and in the bladder.

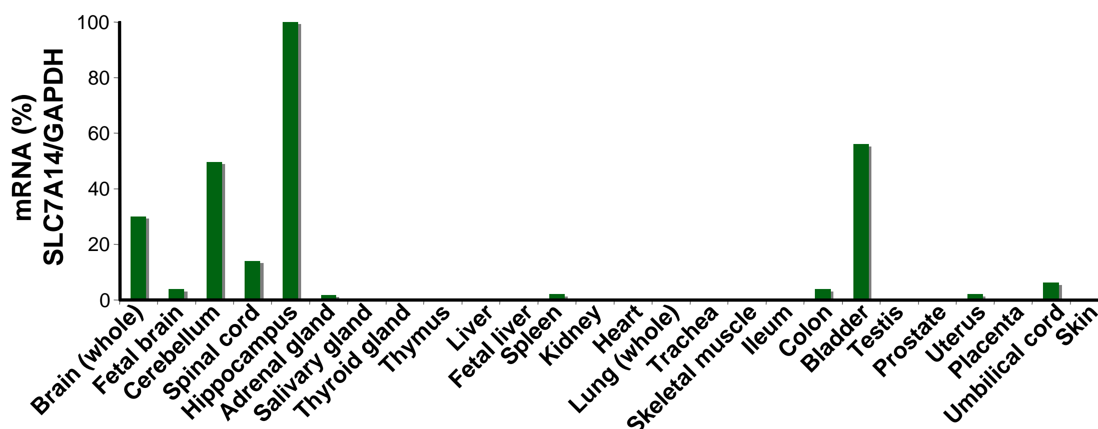


Figure 1
Expression of SLC7A14 in various human tissues was assessed by quantitative RT/PCR using a primer pair and a taqman hybridization probe specific for SLC7A14.

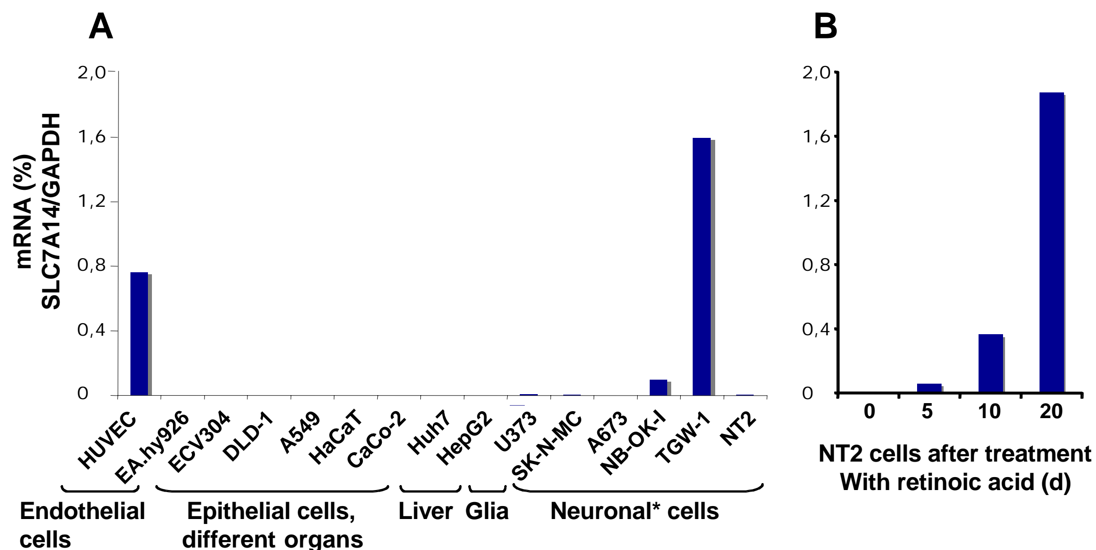


Figure 2
Expression of SLC7A14 was assessed as described in Fig.1: A) in various human cell lines, B) in human NT2 teratocarcinoma cells after treatment with retinoic acid for the time periods indicated. Retinoic acid induces neuronal differentietion in NT2 cells.

Localization of SLC7A14 in different human cell lines

Our preliminary studies indicated an exclusive lysosomal localization of SLC7A14 in human HeLa cells. To find out if this subcellular distribution is also seen in other cell types, a fusion construct between the SLC7A14 cDNA and enhanced green fluorescent protein (EGFP) was transfected in various human cell lines. In addition, the fusion protein was also expressed in oocytes from *Xenopus laevis*. In all cell lines tested (U373 glioblastoma, A673 neuroepithelioma, NT2 teratocarcinoma, Huh7 hepatoma and TGW neuroblastoma cells), SLC7A14 exhibited an exclusive intracellular staining. 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. An example is shown for TGW neuroblastoma cells and NT2 teratocarcinoma cells (that exhibit endogenous expression of SLC7A14) in Figure 3. In all cases, the localization of the fusion proteins overlapped with the lysosomal markers Lamp1 (a type 1 transmembrane protein that is primarily localized in lysosomes and late endosomes) and the fluorescent dye “lysotracker” (that accumulates specifically in compartments with low pH) (Invitrogen). 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.

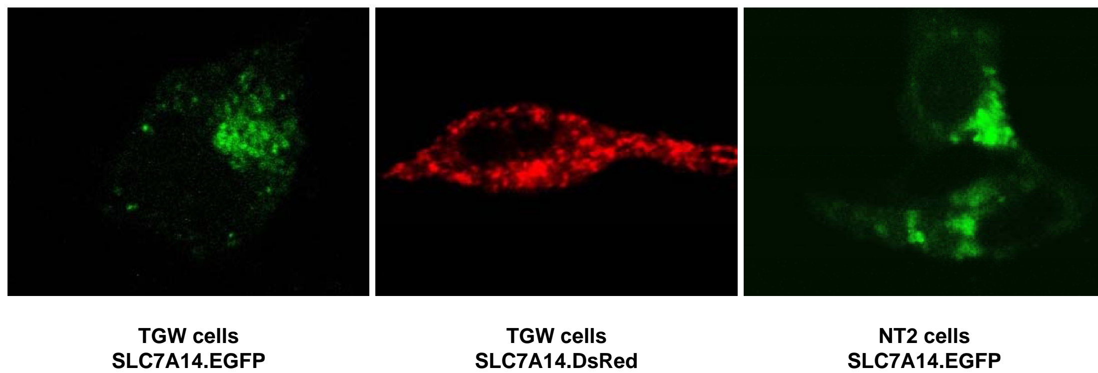


Figure 3
TGW neuroblastoma and NT2 teratocarcinoma cells were transiently transfected with expression constructs coding for fusion proteins between SLC7A14 and EGFP or DsRed as indicated.

Attempts to induce plasmalemmal localization of SLC7A14

The subcellular localization of SLC7A14 is clearly different from that of the related CAT proteins. Although the latter exhibit also intracellular staining compatible with a lysosomal localization, all CAT isoforms localize to the plasma membrane, where they mediate Na^+-independent transport of cationic amino acids. Co-expression of SLC7A14 with CAT-1 revealed nearly 100% co-localization of the two proteins in intracellular compartments. However, only CAT-1 was expressed in the plasma membrane (Figure 4).
SLC7A14 exhibits extended C- and N-termini as compared to CAT-1 (Figure 5). To find out if either extension was responsible for the lack of plasma membrane localization, deletion mutants were constructed. However, DsRedmonoC1-SLC7A14 fusion proteins lacking the 20 C-terminal, 74 N-terminal or both, C- and N-terminal amino acids of SLC7A14 remained completely intracellular. Finally SLC7A14 was fused to rBAT, a glycoprotein necessary to pull SLC7A9 to the plasma membrane. This approach has recently been used to force a new SLC7 member to the plasma membrane and to subsequently show transport activity of the fusion protein (Chairoungdua et al. J Biol Chem 2001, 276: 49390-9). However, cells expressing SLC7A14/rBAT fusion proteins underwent apoptosis. We thus plan to create chimeras between SLC7A14 and CAT-1 in order to localize the protein region responsible for the exclusive intracellular localization.

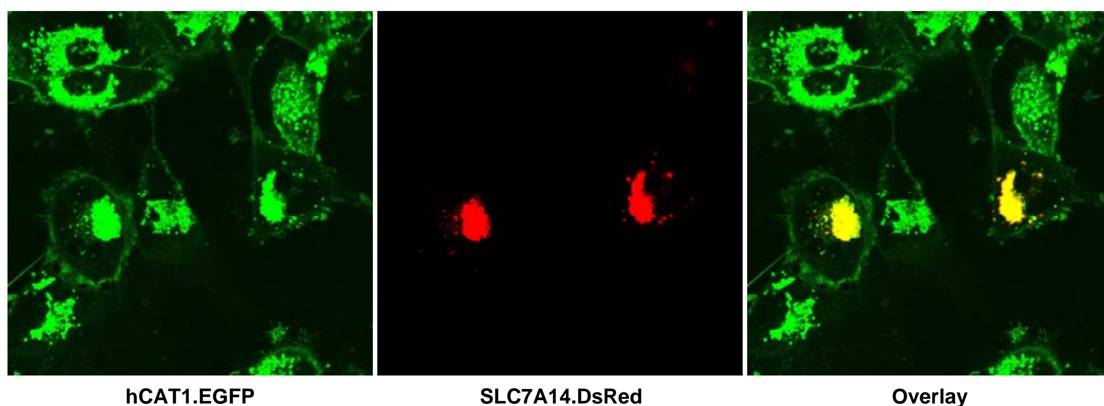


Figure 4
U373MG glioblastoma cells stably expressing a fusion protein between human CAT-1 and EGFP were transiently transfected with an expression construct for a fusion protein between SLC7A14 and DsRed. The overlay shows co-expression of the two proteins in intracellular compartments. However, only CAT-1 is also expressed in the plasma membrane.

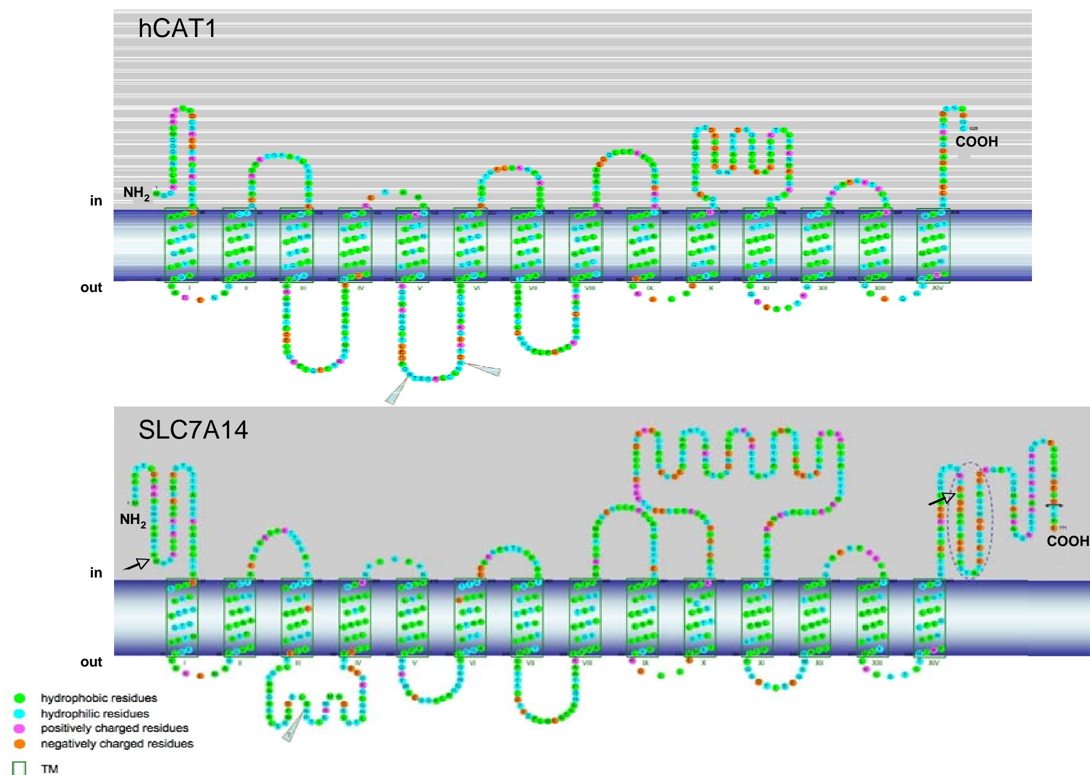


Figure 5
Comparison of the amino acid sequences of human CAT-1 and SLC7A14. Both proteins are predicted to span the plasma membrane 14 times. SLC7A14 exhibits extended N- and C-termini as compared with CAT-1. The arrows indicate the N- and C-terminal deletions.

Creation of stable cells lines expressing SLC7A14.EGFP fusion proteins

Our results so far indicate that SLC7A14 is a lysosomal resident and may be difficult to divert to the plasma membrane. It is thus important to try an alternative approach to measure transport by this protein directly in the lysosomal compartment. We have thus generated a U373MG cell line with stable overexpression of the fusion protein EGFP-N1-SLC7A14 (Figure 6). The subcellular localization of the fusion protein in this cell line is identical with the localization of the transiently expressed protein. We are now planning to prepare lysosomes from this cell line as well as from untransfectd cells and cells stably expressing EGFP-CAT-1 fusion proteins to perform transport studies. The CAT-1 lysosomes are expected to exhibit increased transport of cationic amino acids and can thus be used as positive controls.

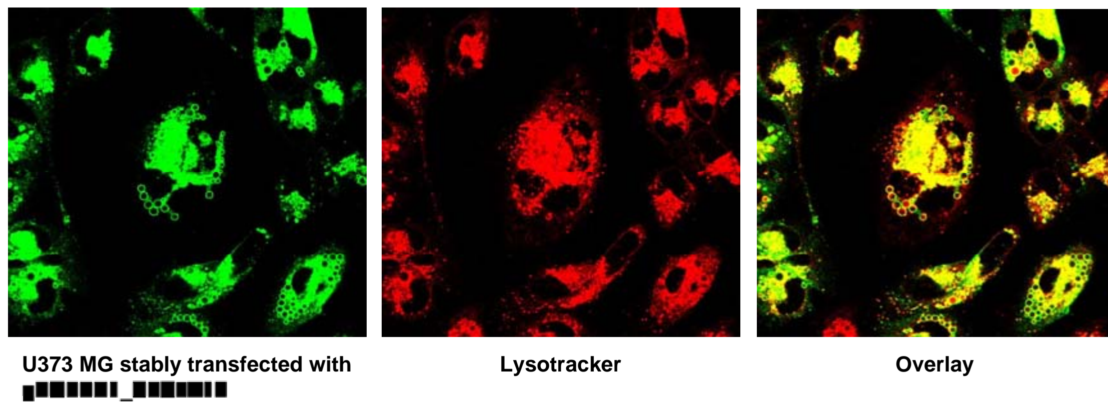


Figure 6
EGFP-N1-SLC7A14 stably expressed in U373MG glioblastoma co-localizes with lysotracker. Its localization is thus similar to the transiently expressed protein.

Ongoing projects

Antibodies against SLC7A14 and hCAT-1/ hCAT-2A

Previously, we have generated antibodies against the CAT proteins using fusion proteins between the CAT C-termini and the bacterial protein TrpE. However, the immune sera obtained against these fusion proteins have relatively low titer. In an attempt to increase the immune response, we are now planning to use fusion proteins between the CAT C-termini and glutathione-S-transferase (GST) in the first immunization and the TrpE fusion proteins for the boosts. If this strategy is more successful, GST and TrpE fusion proteins containing different regions of SLC7A14 will be generated for subsequent immunizations.

Subcellular localization of SLC7A14 in primary cells with endogenous expression

The localization of SLC7A14-EGFP or -DsRed fusion proteins will be examined in primary cells that express SLC7A14, e.g. HUVEC and primary neurons.

Chimeras between SLC7A14 and hCAT-1

We are currently subcloning the different SLC7A14 deletion and fusion constructs into an vector for *Xenopus laevis* oocyte expression. Especially for the rBAT fusion protein, we hope that the oocytes will support overexpression better then the mammalian cells. We will also generate chimeras between SLC7A14 and hCAT-1 to localize the protein domains important for transport and subcellular targeting. These experiments will also be carried out in oocytes and represent an alternative approach to find the transport substrates for SLC7A14.

Measurements of lysosomal transport

We are also planning to establish a protocol for lysosome preparation (from cells with stable overexpression of SLC7A14 and hCAT-1) and subsequent transport assays.