CRF grant "Molecular study of lysosomal transporters involved in the cystine-depleting effect of cysteamine"

Progress Report- January 2013

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The long-term objective of this grant is to identify the membrane transporters underlying the cystine-depleting effect of cysteamine as this would provide a rationale to improve its efficiency or discover more efficient, better tolerated drugs.

Paris Group

(Adrien Jézégou, Christine Anne, Seana O'Regan, Christopher Ribes and Bruno Gasnier)

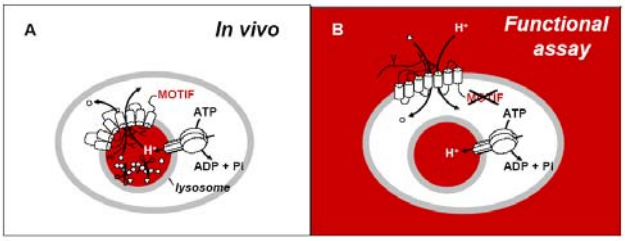
We recently identified with collaborators a cystinosin homologue termed PQLC2 as a novel lysosomal cationic amino acid (CAA) exporter and showed that this transporter plays a major role in the cystine-depleting effect of cysteamine.

Our interest for PQLC2 arose from two initially independent research lines. Firstly, we identified PQLC2 as a novel lysosomal membrane protein in a proteomic study conducted by Drs. Agnès Journet and J. Garin, in Grenoble (submitted). Secondly, our expertise on lysosomal transporters had attracted yeast geneticists led by Pr. Bruno André at the Université Libre de Bruxelles. The Brussels group had identified homologues of PQLC2, termed Ypq1, 2 and 3, at the yeast vacuole membrane and obtained genetic evidence for a role in the homeostasis of CAAs. Ypq1 and ypq2 mutants are resistant to canavanine, a toxic analogue of arginine, and the ypq3 gene is transcriptionally repressed by excess lysine. Therefore, the geneticists postulated that Ypq1-3 proteins export CAAs from the vacuole. In joint experiments, heterologous expression of mammalian PQLC2 at the yeast vacuole restored canavanine sensitivity in *ypq2* mutant yeasts, confirming that the mammalian and yeast proteins represent functional orthologues.

We thus tested whether PQLC2 transports CAAs. In preliminary experiments, we redirected PQLC2 to the plasma membrane by site-directed mutagenesis as this would facilitate transport measurements by replacing the poorly tractable lysosomal assay by a whole-cell assay equivalent to lysosomal efflux (*Figure 1*). Recombinant PQLC2 expressed in cultured mammalian cells localizes to lysosomes. We screened the amino acid sequence for candidate lysosomal sorting motifs and identified a dileucine-type motif in the C-terminal tail. After mutating the critical leucine pair in this motif, PQLC2 was dramatically misrouted to the cell surface, thus confirming that it represents resident protein of the lysosomal membrane.

We next used the dileucine mutant to develop a whole-cell assay of PQLC2-mediated transport. Although encouraging data were initially obtained in transfected mammalian cells, we rapidly shifted to Xenopus oocytes because they lack the strong endogenous CAA transport found in mammalian cells. Signal-to-noise ratios are thus much higher in Xenopus oocytes. A series of radiotracer flux and electrophysiological measurements carried out in this expression system showed that PQLC2 catalyzes a robust, electrogenic transport which is selective for cationic amino acids and strongly activated at low extracytosolic pH, in agreement with the hypothesis derived from the yeast genetic data. PQLC2 is thus a novel, evolutionarily conserved CAA exporter from the lysosomal membrane.

Figure 1: Approach used to facilitate the study of lysosomal transporters. These proteins export hydrolysis products from lysosomes through a process driven by the V-type H+-ATPase. They can be redirected to the plasma membrane by mutating their lysosomal sorting motifs. In this experimental design, the mutated transporter takes up substrates from the extracellular medium by a process topologically equivalent to lysosomal efflux. Acidification of the medium (red color) mimics the lysosomal lumen environment and stimulates uptake. This whole-cell design requires much less cells and time than assays on purified lysosomes and it is open to diverse biochemical and biophysical techniques.



According to the early biochemical model of cysteamine therapy, cysteamine induces a 'salvage' export pathway for cystine molecules accumulating in lysosomes from cystinosis patients by reacting with cystine and forming a mixed cysteine-cysteamine disulfide which resembles lysine. The lysine-like compounds subsequently exits through the hitherto unknown lysosomal CAA exporter. Therefore, our identification of PQLC2 as a novel CAA transporter prompted us to examine its potential role in this cystine-depletion mechanism.

To address this question, we first examined whether the mixed disulfide interacts with PQLC2. Electrophysiological measurements of the transport current carried by PQLC2 showed that, indeed, this compound is recognized and translocated by the novel transporter, with efficiency similar to the natural CAA substrates. We thus next tested whether PQLC2 is required for the effect of cysteamine, using human skin fibroblast cultures kindly provided by Pr. Corinne Antignac as a cellular model of cystinosis. The PQLC2 gene was silenced, or not, with small interfering RNAs in healthy and cystinotic fibroblasts. Cells were then treated, or not, with cysteamine and the levels of cystine and mixed disulfide were measured by mass spectrometry by Dr. Bernadette Chadefaux-Vekemans at Necker Children's Hospital in Paris. Three independent experiments showed that PQLC2 silencing dramatically increases (up to 15-fold) the level of mixed disulfide in cysteamine-treated patient fibroblasts, thus implying that the early biochemical model is correct and that PQLC2 is a major salvage route for lysosomal cystine depletion during cysteamine therapy.

This study has been published in PNAS:
Heptahelical protein PQLC2 is a lysosomal cationic amino acid exporter underlying the action of cysteamine in cystinosis therapy.
Jézégou A, Llinares E, Anne C, Kieffer-Jaquinod S, O'Regan S, Aupetit J, Chabli A, Sagné C, Debacker C, Chadefaux-Vekemans B, Journet A, André B and Gasnier B. (2012) *Proc Natl Acad Sci U S A.* 109(50), pp. E3434- E3443.

It is worth mentioning that although our studied established a major role for PQLC2 in cysteamine therapy, we cannot exclude an additional contribution from the SLC7 transporter candidates studied in

this grant (see below). The gene silencing technique used in our study cannot address easily this question as the presence of residual PQLC2 activity could mask a partial contribution from SLC7 transporters.

Progress Report Mainz Group – January 2013

(Ellen Closs, Jean-Paul Boissel and Isabel Jaenecke)

The goal of our project is to find out if members of the SLC7 sub-family of human cationic amino acid transporters (hCAT) mediate not only plasma but also lysosomal membrane transport of these amino acids and thus are candidates to provide the salvage efflux pathway for cystine after cysteamine treatment. The rational of this approach is based on the observation that all SLC7 members investigated so far (as EGFP fusion proteins), localize to lysosomes either in addition to the plasma membrane (hCAT-1, -2A+B and -3) or exclusively (SLC7A14). In this project, we furthermore established that a chimera carrying the functional domain of SLC7A14 in the backbone of hCAT-2 mediates transport of cationic amino acids (CAA), suggesting that SLC7A14 is a lysosomal CAA transporter (Jaenecke et al. 2012). Using the method of solid supported membrane (SSM)-based electrophysiology, we demonstrated in the last grant period, that lysosomal membrane preparations of hCAT-2B-overexpressing cells exhibit increased transport activity for CAA. However, no signal was detected in preparations from SLC7A14-overexpressing cells. With this approach we could not exclude if:

1) the positive signal detected for hCAT-2B was due to contaminations of the lysosomal preparations with plasma membranes and
2) the negative result for SLC7A14 was based on a possible lack of electrogenic transport by this isoform.

Although we have previously shown time-dependent [^{3}H]arginine uptake into fraction C lysosomes, our attempts to demonstrate increased uptake rates in lysosomes derived from transporter overexpressing cells had failed. In this grant period, we therefore improved the isolation protocol for intact lysosomes and for uptake of radiolabeled amino acids based on the protocol established by Harms et al. and Pisoni et al. (Harms, Kern, and Schneider 1980; Pisoni et al. 1987) to isolate the so called fraction C from cultured cells that contains intact lysosomes. We improved our protocol at several steps (e.g. incubation of cell in MST directly, less strokes with the potter, pre-incubation of all solutions at 20°C) and performed more experiments with SLC7A14.EGFP, hCAT-1.EGFP and also hCAT-2B.EGFP overexpressing cells in comparison to cells overexpressing EGFP alone.

Isolation of fraction C

Cells of one confluent T150 flask were distributed to ten plates with a diameter of 145 mm and cultivated for eight days. Under these conditions the cells were confluent for at least two to three days. Cells were harvested in 4 ml ice-cold MST per plate (MOPS-Sucrose-Tris buffer: 250 mM Sucrose; 50 mM MOPS; 1 mM EDTA; pH 7.6 with Tris) and combined. After washing twice in ice cold MST (600g; 10 min), cells were resuspended in 3 ml MST containing protease inhibitors (Complete Mini, Roche) and homogenized with 15-20 strokes in a potter connected to a drilling machine.

A small amount of the homogenized cells was kept as fraction A. The remaining homogenate was divided into two 1.5 ml reaction tubes, followed by a differential centrifugation. To get rid of whole cells and cell debris, the samples were centrifuged at 900g for 5 minutes. Then the supernatants were transferred to new reaction tubes and spun at 3000g for 15 minutes to get rid of nuclei and heavy mitochondria. This step was repeated once. Supernatants were then centrifuged for 15 minutes at 10,000g in new tubes, to enhance the removal of mitochondria. Finally the lysosomes were pelleted in new tubes at 20,000g for 15 minutes. The pellets were taken up in 500 µl MST each and combined, followed by four washing steps (each 15 minutes, 20,000g, 1 ml MST). The resulting pellet was taken up in 250 µl MST and named fraction C.

We then measured the protein concentration (BCA assay) and performed a latency assay (ß-hexosaminidase assay) to determine what percentage of lysosomes were still intact in each preparation.

[^{3}H]amino acid uptake

Fraction C, as well as a solution of 200 µM [^{3}H]arginine or [^{3}H]lysine [20 µCi/ml] in MST were pre-incubated in a water bath (20°C). Then fraction C was mixed with the equal volume of the amino acid solution, resulting in a final concentration of 100 µM [^{3}H] amino acid [10 µCi/ml]. After mixing once by pipetting up and down, an aliquot of the mixture was removed at each given time point and added to 4 ml ice cold MST to stop all processes. Lysosomes were washed thrice with 2 ml ice cold MST each, on a membrane filter (Cellulose-mix, pore size 0.45 µm) attached to a vacuum pump. After lysis of the lysosomes in 2% SDS, the amount of radioactivity was determined by liquid scintillation counting. A sample without fraction C served as negative control.

Results

Fraction C was isolated from U373 glioblastoma cells overexpressing EGFP fusion proteins of hCAT-1, hCAT-2B, and SLC7A14, respectively. The latency assay indicated that 70 - 75% of the lysosomes were still intact in all preparations. All EGFP fusion proteins enriched in fraction C compared to fraction A, together with the lysosomal protein LAMP1, confirming lysosomal localization of the SLC7 members. To begin with, we tested transport of the cationic amino acid arginine in fraction C. While arginine uptake was similar in all samples after just 5 seconds incubation, at later time points (60 – 120 sec) it was significantly higher in samples of cells overexpressing either hCAT-1.EGFP or hCAT-2B.EGFP in comparison to samples of cells overexpressing EGFP alone. However, arginine uptake into fraction C derived from cells overexpressing SLC7A14.EGFP, was not different from control. As the mixed cysteine-cysteamine disulfide (supposed to be a generated within lysosomes and to be a substrate of the lysosomal CAA transporter) is more similar to lysine than to arginine, we also tested [^{3}H]lysine uptake into fraction C. In these experiments we got similar results, with only hCAT-1.EGFP and hCAT-2B.EGFP exhibiting transport activity, but not SLC7A14.EGFP.

These results establish that hCAT-1 exhibits transport activity in the lysosome and confirm lysosmal transport activity for hCAT-2B also detected by SSM-based electrophysiology. Together with previous results obtained in Bruno Gasnier's laboratory, showing lysosomal localization of rat and mouse CAT-2A in the liver, this suggests that CATs function also as lysosomal CAA transporters. It needs to be elucidated what role hCATs and the newly discovered PQLC2 play for clearing the mixed cysteine/cysteamine disulfide from lysosomes and for total lysosmal CAA transport, respectively.

In contrast to the CATs, we could not detect a transport activity for the orphan protein SLC7A14 with the modified protocol. This was unexpected, because we have shown that the so called "functional domain" of SLC7A14 was sufficient to mediate CAA transport (Jaenecke et al. 2012). As inhibition of transport due to the fusion to EGFP cannot be excluded, studies with cells overexpressing SLC7A14 without EGFP have to be performed. With a newly generated antibody against native SLC7A14, we are currently working on generating such a cell line. This new antibody enabled us to show the enrichment of endogenous SLC7A14 in fraction C derived from human skin fibroblasts together with LAMP1, confirming lysosomal localization of this protein.

Literature

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