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

Progress report- June 2011

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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 and, hopefully, reduce its side effects.

More specifically, we are interested in the (still unknown) membrane transporter that exports the product formed in patients' lysosomes when cysteamine reacts with cystine, a mixed disulfide resembling the cationic amino acid lysine. This transporter lies at center stage of the therapeutical mechanism. However, other proteins involved in the 'periphery' of the process such as the entry of cysteamine into lysosomes or other steps may be important as well.

Paris group

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

In a previous CRF-funded project, we focused on the mechanism of cystine depletion in liver using biochemical, proteomic and genetic approaches. We showed with the Mainz group and with a partner in Grenoble that (i) the type-2 cationic amino acid transporter (CAT2) sits in the lysosomal membrane, in addition to its reported plasma membrane localization, and (ii) that it is the sole transporter known to recognize lysine among the lysosomal membrane proteins detected by mass spectrometry. CAT2 thus initially represented the best, and possibly sole, candidate for the core process of cysteamine therapy in liver (with presumably other CAT isoforms playing a similar role in tissue with low or undetectable CAT2 expression).

Subsequently, our studies on isolated lysosomes showed that, in vitro, cystine depletion is not affected by genetic ablation of CAT2. These data argued for a minor role of CAT2, despite its localization and transport properties, and for the existence of another unanticipated lysosomal transporter efficiently translocating lysine and the mixed disulfide.

However, as our in vitro assay might not recapitulate all aspects of the cystine-depleting process occurring in live cells, we decided to pursue our analysis of CAT2 and examine its role in vivo using mouse models (the search for the second lysine transporter will be described in future reports).

We crossed two mouse lines inactivated for the cystinosin (*ctns*) and the CAT2 genes provided by C. Antignac (Paris) and L. Ellies (San Diego), respectively, and generated a double knock-out. Then, we treated, or not, with Cystagon 4 groups of mice with distinct homozygous genotypes: wild-type, *ctns-/-*, *cat2-/-* and double KO. The drug was provided by Orphan Europe. We initially performed daily intraperitoneal injections of cysteamine hydrochloride in an attempt to obtain

faster effects and better control of the amount of drug administered to each mice. However, this approach proved practically difficult and poorly efficient. We thus repeated the experiment using oral application of Cystagon in drinking water, as originally reported by Cherqui et al. (Mol. Cell Biol. 2002). Mice were treated for two months and cystine measurements were performed in our University by B. Chadefaux and colleagues using liquid chromatography coupled to mass spectrometry.

Surprisingly, in marked contrast with our in vitro data, the in vivo cystine-depleting effect of cysteamine in liver turned out to be fully dependent on the presence of CAT2: whereas Cystagon decreased cystine levels by 75% (unpaired t-test: $P<0.02$), it had no effect in the double knock-out ($P=0.41$).

The apparent discrepancy between these in vitro and in vivo results could have several possible origins, including a poor physiological relevance of our in vitro assay (favoring the detection of the second, unknown transporter) or a role of CAT2 at other steps than lysosomal egress of the mixed disulfide. Current measurements of arginine and lysine levels in liver of the knock-out mice seem to favor the second group of hypotheses.

Measurements in other tissues than liver are under progress. We plan to establish ex vivo cellular models from the knock-out mice to dissect further the mechanism.

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

During the first 6 months of the grant period, we:

- established a protocol for the isolation of a granular, lysosome enriched fraction derived from human U373MG glioblastoma and EA.hy926 endothelial cells,
- established a protocol for uptake experiments with lysosome enriched fractions,
- investigated new ways to isolate a purer lysosome enriched fraction.

For the isolation of a lysosome-enriched fraction, the following protocol was applied:

Cells were seeded in 15cm dishes (10) and grown to confluency. The medium was then removed, and the cells washed once with PBS. After scraping the cells off the plates, samples were centrifuged at 800xg for 5min, then again washed in PBS, and resuspended in MST-buffer (50mM MOPS, 250mM sucrose, 1mM EDTA, pH7.6 with Tris). The cells were further disrupted with a potter (20-30 strokes) resulting in fraction A. This fraction was further purified by several centrifugation steps to remove whole cells and cell debris (10min, 100xg), nuclei and heavy mitochondria (15min, 3000xg) and lighter mitochondria (10min, 10000xg). After each step, the supernatant was kept and the pellet was discarded. Finally, the lysosomes were pelleted at 20.000xg (10min) and the pellet was washed 4 times with MST. In this lysosme-enriched fraction (LEF) endogenous CAT-1 in EA.hy926 cells (A) and also overexpressed CAT-1- and SLC7A14-EGFP fusion proteins in U373MG cells (B) were prominantly present, concomitant with the lysosomal markers cathepsin D and LAMP1 (Figure 1)

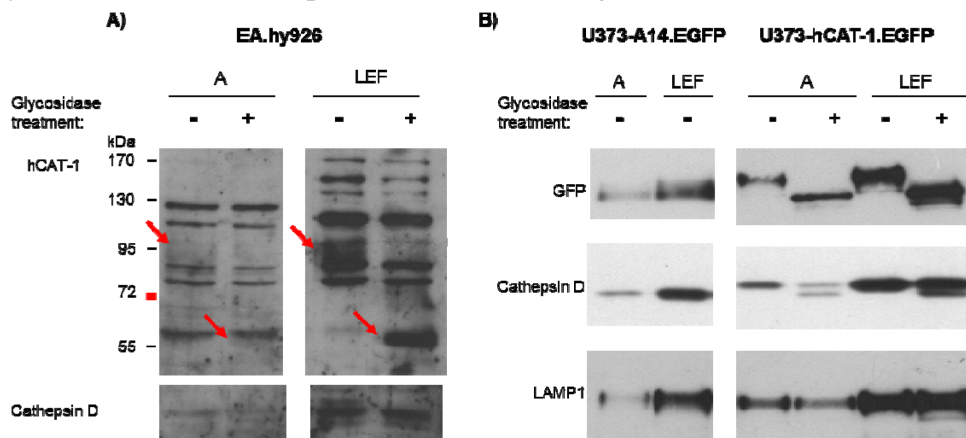

Figure 1: Western blot with total cell lysates (A) or lysosome-enriched fractions (LEF) from indicated cells, probed with the indicated antibodies.

Membrane integrity was conserved to the extent of 80% in most granular preparations as judged by the latency of hexosaminidase activity, determined as the difference in hexosaminidase activity in the presence and absence of 1% Triton.

Uptake of radioactive labeled [^{3}H]L-arginine (100µM) in the LEF fraction was measured by pipetting aliquots on filters at different time points after addition of the radioactive tracer. The

filters were washed 3 times with MST. The lysosomes were then lysed with 2% SDS and the radioactivity was measured. Control values of [^{3}H]-labeled arginine loaded directly on filter were subtracted from all other values. As expected, a time-dependent uptake of radioactivity was observed. However, the transport of arginine into the lysosome-enriched fraction derived from U373MG cells overexpressing either hCAT-1_EGFP or SLC7A14_EGFP was identical to the transport detected in the lysosome-enriched fraction derived from U373MG-EGFP control cells (Figure 2).

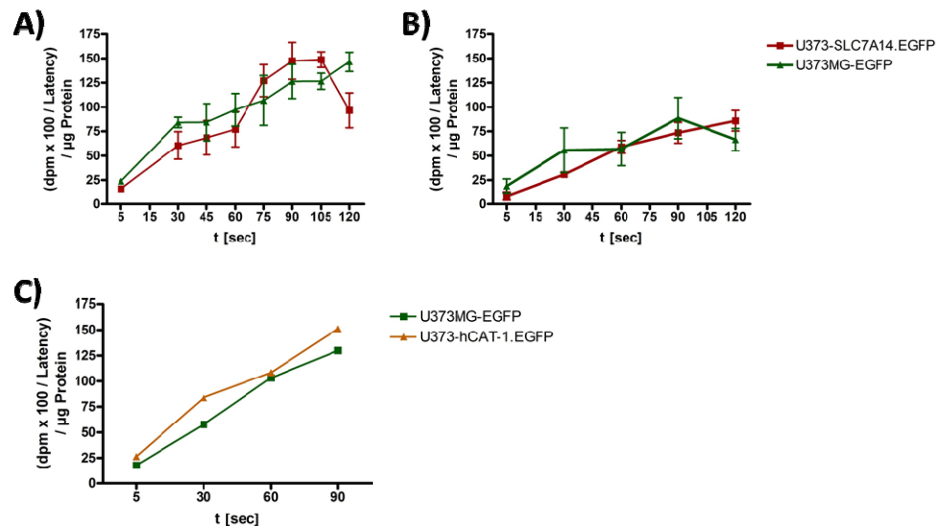

Figure 2: Time-dependent uptake of 100µM [^{3}H]L-arginine [10µCi/ml] into lysosome-enriched fractions derived from U373MG glioblastoma cells stably over-expressing SLC7A14 (panels A: 37°C and B: 20°C) or hCAT-1 (panel C: 37°C), in comparison to uptake into untransfected control cells.

To exclude that the negative result is based on contamination with other organelles, we aimed at increasing the purity of the lysosome fraction. Our first approach was to further purify the LEF fraction on a self-forming Percoll gradient. As described in the literature, after centrifugation a purer lysosome fraction should be found in the lower third of the gradient (green arrow in Figure 3A). However, even under several different centrifugation conditions (17.5 – 30% Percoll, 30.000 – 50.000xg) a single band in the upper third of the gradient (red arrow in Figure 3A) was always observed. All the same, the lower part of the gradient was extracted, named fraction P and analyzed by Western blotting. After applying the same amount of protein in each lane, there was a decrease instead of an increase of lysosomal markers in fraction P in comparison to LEF.

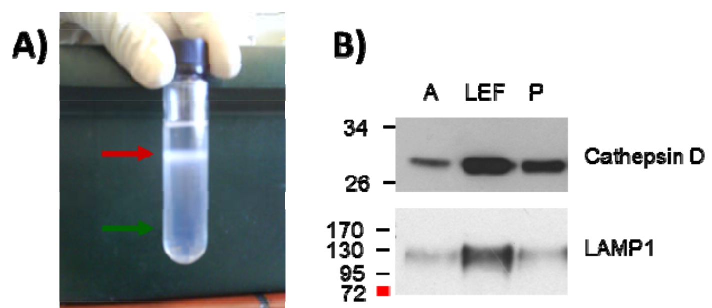

Figure 3: A) Representative picture of a Percoll gradient after centrifugation. The Green arrow indicates the region where lysosomes were expected, red arrow indicates the only band visible after centrifugation. B) Western blot of total cell lysate (A), lysosome-enriched fraction (LEF) and the further purified fraction (P) from U373MG_A14.EGFP cells, probed with the indicated antibodies. Each lane was loaded with 20 µg protein.

In a second approach, we tested the lysosome isolation kit from Sigma (LYSISO1). After cell disruption, only two centrifugations were performed, removing of cell debris at 1000xg and pelleting of lysosomes at 20.000xg. The resulting granular fraction was layered onto a OptiPrep®

gradient (Figure 4A, left picture). After 4 hours of centrifugation several bands were formed. Fractions 1 (top) to 10 (bottom) were collected. According to the manual, lysosomes should be enriched in fractions 4-6. However, Western blot analysis showed a decrease of the lysosomal marker Cathepsin D from fraction 1 to 10 (Figure 4B). An enzymatic assay of 10µl aliquots of each fraction showed a peak activity of the lysosomal enzyme hexosaminidase in fractions one and two, but also in fractions five to seven (Figure 4C, red curve). This peak activity correlated with a peak in protein content (Figure 4C, blue curve). We have thus not been successful in obtaining a purer lysosme fraction.

In the following months, we plan to focus on alternative ways to assess arginine export from lysosomes. As lined out in our proposal, we will measure the activity of endothelial NO synthase as a reporter for the transport of lysosome-derived arginine into the cytosol.

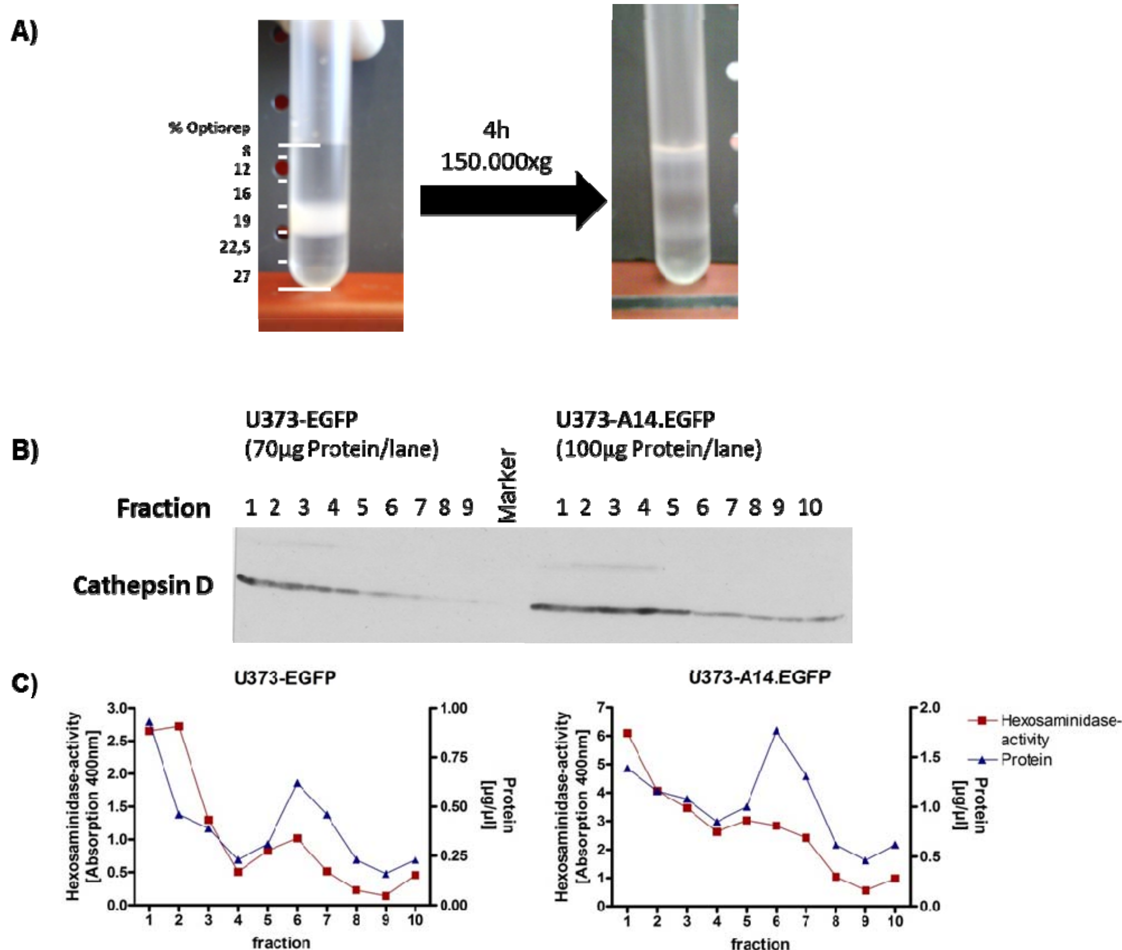

Figure 4: A) Representative pictures of the OptiPrep® gradient before (left side) and after centrifugation (right side). The white band in the left picture indicates the LEF. B) Western blot of fractions obtained from an OptiPrep® gradient and probed with an cathepsin D antibody. The fractions were obtained of the cells indicated. C) Red curve: The activity of the lysosomal enzyme hexosaminidase was measured by incubation of 10µl of the OptiPrep-fractions with Triton X100 and 1mM 4-Nitrophenyl-N-acetyl-ß-D-glucosaminide. Reaction was stopped after 30min by adding 200µl 0,8M Glycin/NaOH pH 10,4 and absorption at 400nm was measured. Blue curve: Protein concentration per fraction [µg/µl].