CRF grant "Molecular study of a cystinosin homologue and its impact on cystinosis and cysteamine therapy"

1st Progress Report

Principal investigators: Bruno Gasnier, Ph.D. (Paris Descartes/CNRS)
Bruno André, Ph.D. (ULB Gosselies)
CRF PhD fellow : Xavier Leray (Paris Descartes/CNRS)

Paris group: Xavier Leray; Christine Anne; Cécile Debacker; Bruno Gasnier
Gosselies group: Elisa Llinares, Abdoulaye Barry; Bruno André

In a recent study, we identified a cystinosin homologue termed PQLC2 as a lysosomal exporter of cationic amino acids (CAAs) using proteomic analysis, yeast genetics and functional analysis in Xenopus oocytes. We also showed that PQLC2 is critical for cysteamine therapy of cystinosis as it exports from the lysosome a lysine-like mixed disulfide formed by a chemical reaction between cysteamine and cystine (Jézégou et al, 2012 PNAS 109:E3434–E3443).

The aim of this grant is to characterize further the properties of PQLC2, explore its potential regulation and interactions with other proteins, and characterize further the molecular events associated with cysteamine therapy. This research may provide rationales to improve cysteamine therapy.

The two groups frequently interact and had a joint progress meeting in Paris on May 6. The following milestones were achieved during the first semester.

In a first research line, we pursued our in vitro analysis of the cystine-depleting effect of cysteamine. Cultured human cystinotic cells were treated or not with cysteamine and cellular levels of cystine and mixed disulfide were measured by liquid chromatography coupled with tandem mass spectrometry, using deuterated cystine as an internal standard. In these experiments, the PQLC2 gene silencing step of our previous study was omitted and we focused on the time course of cystine and mixed disulfide levels after application of cysteamine. Interestingly, we observed that the formation of mixed disulfide occurs at a faster rate than the clearance of mixed disulfide, suggesting that PQLC2, which is responsible for the clearance step, is rate-limiting. PQLC2 overexpression experiments are in progress to test this conclusion.

Secondly, we examined the ion-coupling mechanism of PQLC2 by measuring simultaneously CAA uptake and transport current activities in sets of Xenopus oocytes expressing the sorting mutant of PQLC2 at their surface. Oocytes were recorded under two-electrode voltage clamp and perfused with radiolabelled arginine. The area under the current trace evoked by [^{3}H]arginine gives the amount of electric charge accumulated during arginine transport. After recording, electrodes were disimpaled and oocytes were quickly washed and counted by liquid scintillation. This measurement gives the amount of accumulated arginine. Repeating this assay with several oocytes under diverse durations of arginine application, PQLC2 expression level, etc allows plotting the amount of charge as a function of accumulated arginine. Although the resulting plots showed some variability across oocyte preparations for an unknown reason, our data excluded the proton gradient of the lysosomal membrane as a driving force for CAA transport, in

contrast with the transport activity of cystinosin. We are currently analyzing the origin of the aforementioned variability and exploring the physiological consequences of the lack of proton coupling.

Finally, our experiments addressed the question of how does PQLC2 traffic to the lysosomal membrane. This aspect of PQLC2 function is important as improper targeting of proteins normally destined to the lysosome (including cystinosis) may perturb their biological role. Previous work showed that newly synthesized lysosomal transmembrane proteins initially reach the Golgi from where they are targeted to the lysosome via different pathways. In a simple eukaryote like yeast, where the Golgi and early endosomes are not readily distinguishable, two pathways have been described. In the so-called CPY (carboxypeptidase Y) pathway, proteins present in the Golgi are first sorted via AP-1 or GGA adaptors into clathrin-coated vesicles. These vesicles then fuse with endosomes from where the proteins migrate to the vacuole (the lysosome of yeast). In the ALP (alkaline phosphatase) pathway, proteins reaching the Golgi are first sorted in vesicles in a manner dependent on the AP-3 adaptor complex, and these vesicles then directly fuse with the vacuolar membrane, bypassing endosomes. In mammalian cells, the AP-1 and AP-3 adaptor complexes were also reported to promote budding from the Golgi of distinct types of clathrin-coated vesicles incorporating different cargo proteins. However, a role of these AP complexes at the level of early endosomes has also been reported and some transmembrane proteins exiting the Golgi may first reach the plasma membrane (and cycle between it and early endosomes) before being delivered to the lysosomal membrane.

To investigate the mechanisms involved in targeting PQLC2 to the lysosome, we first used yeast as a model system since mechanisms of basic cellular functions tend to be highly conserved from yeast to human cells. Furthermore, we previously showed that three yeast proteins named Ypq1, -2 and -3 are similar in sequence to PQLC2 and promote export of CAAs from the vacuole, the lysosome of yeast (Jézégou et al, 2012 PNAS 109:E3434–E3443). Our results show that the yeast Ypq proteins mainly use the ALP pathway to reach the vacuolar membrane. In the case of Ypq1, this sorting is dependent on an acidic di-leucine motif present in the second cytosolic loop of the protein. When components of the AP-3 complex are defective, or when substitutions are introduced into the acidic di-leucine motif, the Ypq proteins transit through endosomes (via the CPY pathway) to reach the vacuole. When components of both the CPY and ALP pathways are defective, localization of Ypq1 to the vacuolar membrane is strongly impaired. In HeLa cells, PQLC2 is localized to the lysosomes and substitutions in its acidic di-leucine motif (present in the C-terminal tail of the protein) missort the protein to the plasma membrane (Jézégou et al, 2012 PNAS 109:E3434–E3443). We treated HeLa cells with siRNAs targeting the mRNA encoding the μ3A subunit of the AP-3 complex and confirmed by immunoblotting that this leads to a severe reduction of the amount of μ3A. In these siRNA treated cells, PQLC2 failed to localize to the lysosomes. It rather accumulated in a perinuclear compartment likely corresponding to the Golgi whereas part of the protein was also deviated to the cell surface. In contrast, the localization at the cell surface of the mutant $PQLC2^{LL>AA}$ variant was unaltered after treatment of cells with the siRNAs targeting μ3, indicating that this mutant form of PQLC2 exits the Golgi in a manner independent of AP-3. These experiments suggest that, similar to what has been observed for the yeast Ypq proteins, the AP-3 adaptor complex plays a major role in targeting to the lysosomes of the PQLC2 transporter, likely via recognition at Golgi level of its acidic di-leucine motif.