CRF grant ¨Molecular anatomy and physiology of human cystinosin¨

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1. Topology study of cystinosin

For the topological study, cDNAs encoding the fusion proteins depicted in Figure 1 were constructed.

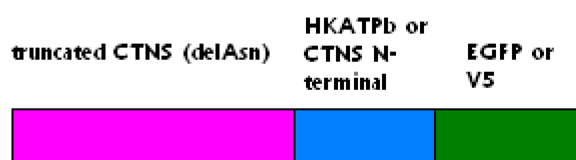

Figure 1. Scheme of cystinosin fusion proteins made for the topology study

As mentioned in the previous report, a series of C-terminally truncated fragments of human cystinosin (pink segment) lacking endogenous N-glycosylation sites was fused to a glycosylation-based topology reporter (in blue) followed by an expression reporter (in green). The glycosylation state of the topology reporter (glycosylated when it is translocated into the ER lumen) indicates the topology of the truncation site tested for each construct.

Most constructs fused with the glycosylated domain of H^+,K^+-ATPase β subunit and EGFP did not express well in Xenopus oocytes. The reason might be the excessive size of the reporters (> 400 aa together), which were larger than cystinosin itself. The resulting constructs are presumably degraded by the protein quality control system of Xenopus oocytes.

To circumvent this difficulty, we tried to express them in vitro by coupled transcription/ translation in the presence of microsomes. On SDS-PAGE, the size of the resulting translation products was in good accordance with those obtained in vivo, in Xenopus oocytes, after deglycosylation. However, we could not observe any glycosylation of the reporter in these in vitro products for a reason that remains unclear.

EGFP was thus replaced by a much smaller tag, the V5 epitope. The resulting fused genes were all well expressed in Xenopus oocytes. Nevertheless, the level of expression was largely time-dependent and construct-dependent. In SDS-PAGE experiments, some of the truncated constructs underwent a ~12-kDa decrease in apparent molecular mass after treatment with PNGase-F or other N-deglycosydases, thus bringing topological information.

With the information gathered from these experiments, we propose a novel topological model of human cystinosin which significantly differs from the current model predicted by topology softwares. The major difference lays in TM1 and TM6. This difference was supported by a similar experiment, where a N-terminal segment of cystinosin was used as topology reporter instead of H^+,K^+-ATPase β.

To verify this novel topological model, we are now performing cysteine-scanning experiments on full-length, active cystinosin constructs. A cystinosin mutant lacking endogenous cysteines was constructed and shown to be active for cystine transport. Ectopic cysteine residues will then be substituted for endogenous residues at chosen sites to test their topological orientation in the full-length context. After a functional assay aimed at confirming that the single cysteine mutants studied are properly folded, biotinylation with impermeant thiol reagents will be performed to get a *validated* topological map of cystinosin.

Our first priority is to check the relative location of the 2 PQ motifs. According to the current topology model, both motifs are on the same side of the membrane whereas, in our revised model, they should sit on opposite sides.

2. Functional analysis of cystinosin

In preliminary experiments of this project, we developed an electrophysiological assay of cystinosin-mediated cystine transport and proposed, based on voltage-dependence properties, that cystine binding is coupled to protonation from the luminal compartment of a residue buried in the membrane. Site-directed mutagenesis of several conserved residues revealed mutants with strongly altered voltage dependence properties that corroborated our working hypothesis. However, these experiments failed to unveil the identity of the proton binding site.

To address this issue, we used a different approach consisting in recording transient currents elicited by applying voltage jumps to cystinosin-expressing oocytes. The underlying rationale was that if cystine-laden cystinosin is protonated at a site buried in the membrane, and therefore located in the voltage gradient, voltage steps should challenge the protonation equilibrium and induce capacitive currents associated to proton transfer.

In good agreement with this model, we observed that, in the presence of cystine, voltage jumps induced transient currents in wild-type cystinosin, with time constants of 4 to 6 ms. 'Titration' of this electrogenic transition by applying a wide range of voltage values indicated a mean transient charge moved of ~8 nC per oocyte. This value, which is proportional to the number of cystinosin molecules, in turn allowed deriving a maximal transport rate of 28 cystine molecules per second per cystinosin molecule.

To identify the protonatable residue coupled to cystine binding, we next repeated this analysis on site-directed mutants. Interestingly, mutation of a single aspartate could abolish the cystine-dependent transient currents and thereby unveil the identity of the proton binding site. Because of its geometric properties (proximity to the cytosol but accessibility from the lysosomal lumen), we propose that this aspartate residue represents a relay site for the translocated H^+ that actively drives cystine out of the lysosome.

A manuscript describing this electrophysiological study is currently in progress.

We hope that these deep biochemical and biophysical analyses of human cystinosin may provide an accurate framework for a better understanding of the cellular roles of cystinosin.