

# CRF grant "Molecular anatomy and physiology of human cystinosin"

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### Topology study of cystinosin

As mentioned in the previous report, we recently started performing a scanning cysteine-accessibility mutagenesis (SCAM) in order to get a validated topological map of cystinosin.

A cystinosin mutant lacking all the 5 endogenous cysteines was constructed and shown to be active for cystine transport. Ectopic cysteine residues were then individually introduced to substitute endogenous ones at chosen sites to test their topological orientation in cystinosin. The corresponding RNA was *in vitro* transcribed and microinjected into *Xenopus* oocytes for expression.

To test the topological orientation of the ectopic cysteine, surface biotinylation was performed on oocytes. The biotinylation reagent EZ-link Biotin-HPDP (Pierce) is membrane-permeable and cleavable, and the SH-blocking reagent MTSET is membrane-impermeable. According to the methodology shown in Table 1, we expected to distinguish the different possible orientations of cysteine residues.

**Table 1: Criteria used to determine the topological orientation of tested cysteine residues based on surface biotinylation**

Cysteine location	No pre-blocking	Pre-blocking with MTSET
Extracellular	Biotinylation	No biotinylation
Intracellular	Biotinylation	Biotinylation
In the membrane	Little or no biotinylation, unaffected by pre-blocking	

Our results showed that the predicted extracellular residues behaved as expected, i.e., could be only biotinylated without pre-blocking. However, under our experimental conditions, no biotinylation was observed for presumed intracellular or membrane-buried cysteine residues. To solve this problem, we perforated oocytes with digitonin to facilitate entry of Biotin-HPDP into oocyte and to minimize the quenching effect of endogenous thiol-containing molecules present in the oocyte cytosol. After short-period permeabilization, biotinylation could be successfully observed for predicted intracellular cysteine residues, which could also be blocked by pre-treatment with MTSET. Furthermore, under these improved conditions, little or no biotinylation was observed for cysteine residues predicted to be localized in the membrane.

Until now, we have studied with this method more than 30 cystinosin mutants, covering all extramembrane loops and some membrane segments. Our results support the idea that cystinosin contains 7 transmembrane segments. However, some transmembrane segment borders are under correction and require further experimental analysis.

We also tested the transport activity of the new cystinosin mutants, using  $^{14}\text{C}$ -labelled cystine as the substrate. Several mutants showed significant decrease in cystine transport. Furthermore, pretreatment of oocyte with N-ethylmaleimide (NEM) could abolish the transport activity of some new mutants, which suggests that these mutated positions are either exposed to the transport pathway, or essential for the conformational changes involved in cystine translocation.

Therefore, as the next step, we will carry out TEVC electrophysiological recordings on these mutants to explore in depth the vital roles of these residues for the transport function of. We expect that a validated, more precise topological map will help direct our attention to functionally important residues in cystinosin; while the detailed study of transport mechanism will in turn increase our knowledge on the structure-function relationship of cystinosin. The novel topological and functional map should also be useful to understand the molecular effects of some pathogenic mutations and the interactions of cystinosin with other proteins.

Continuous efforts are still devoted to investigate the interplay between the two important PQ motifs present in the cystinosin structure, as suggested by previous electrophysiological and phylogenetic data. We tried to rescue the non-active mutant D305N (in the second PQ motif) with a 'mirror' mutation N166D or N166E in the first PQ motif. However, transport activity, monitored by labelled cystine uptake or TEVC recording, was not rescued in this double mutant, thus indicating that the two PQ motifs do not play equivalent roles.