CRF grant "Molecular anatomy and physiology of human cystinosin"

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1. Topology study of cystinosin

Cystinosin is predicted to comprise seven transmembrane domains but its actual topology remains unknown. As a first step to address this issue, we used a classical gene fusion approach in which a topology reporter is fused to various truncated fragments of the membrane protein of interest in order to map the topological orientation of a set of truncation sites along the polypeptide (see Manoil and Beckwith 1986, Science 233:1403-8).

A series of C-terminally truncated fragments of human cystinosin linked to a glycosylation-based topology reporter were constructed as depicted in Figure 1.

<table>
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<tr>
<th>truncated CTNS (delAsn)</th>
<th>HKATPb</th>
<th>EGFP</th>
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Figure 1. Scheme of cystinosin fusion constructs used for topology study.

To facilitate analysis of the topology reporter, the seven endogenous N-glycosylation sites of human cystinosin were inactivated by mutating the corresponding Asn residues to Ala. This unglycosylated cystinosin mutant was kindly provided by C. Antignac (Necker Hospital). A total of six C-terminal truncations were produced at the first amino acid residue of predicted transmembrane segments number 2 to 7. In addition, an almost full-length, unglycosylated construct lacking the C-terminal lysosomal targeting motif was also made. The topology model used to design the truncations was obtained using a Hidden Markov Model-based algorithm (http://www.cbs.dtu.dk/services/TMHMM/).

The truncated cystinosin fragments were linked at their C-terminus to the β subunit of H⁺,K⁺-ATPase, which harbours five N-glycosylation sites and may thus serve as a glycosylation reporter. EGFP was also fused downstream the β subunit domain to detect the fusion protein.

A commercially available cystinosin antibody was also tested as an alternative to the EGFP tag, but did not show specific activity. The fused coding sequences were cloned into the pOX vector which allows expression either in vivo in *Xenopus* oocytes or in vitro in mammalian microsomes.

All the aforementioned constructs were proved by DNA sequencing. In vitro transcription was performed (Ambion) from linearized plasmids to prepare mRNAs, which were eventually microinjected into *Xenopus laevis* oocytes. After several days of expression, oocytes are lysed. SDS-PAGE is performed for the cleared lysate, and the target protein is detected by immunoblotting with anti-GFP antibody. The size of the target protein and its sensitivity either in vivo to an N-glycosylation inhibitor (tunicamycin) or in vitro to the enzyme peptide:N-glycopeptidase F gives information about its glycosylation status. The presence of
N-glycans in the H\textsuperscript{+},K\textsuperscript{+}-ATPase domain reflects localization of the truncation site in the extracytoslic compartment. The approach has been validated on a construct lacking the last (7\textsuperscript{th}) putative transmembrane domain and the study is in progress for other constructs. If in vivo expression of these truncated constructs proves difficult, we will express them in vitro by coupled transcription/translation in the presence of endoplasmic reticulum microsomes.

2. Functional analysis of natural cystinosin mutants

The transport activity of cystinosin can be studied using a whole cell assay based on a functional sorting mutant expressed at the plasma membrane. At acidic extracellular pH, cells expressing this mutant take up cystine through a transport process equivalent to lysosomal efflux. The transport activity can be followed by measuring the cellular accumulation of radiolabelled cystine (Kalatzis et al. 2001 EMBO J. 20:5940-9) or by recording an electrical current carried by protons co-transported with cystine molecules (Ruivo et al, manuscript in preparation).

Natural mutants displaying a residual cystine transport activity (Kalatzis et al. 2004, Hum Mol Genet. 2004 13:1361-71) were selected from different clinical phenotypes (infantile, juvenile and ocular) and cloned into the pOX vector. Following the same procedure as above, the gene was transcribed in vitro and expressed in \textit{Xenopus laevis} oocytes. Two different techniques were employed to characterize the transport activity of these pathogenic mutants: the uptake of labelled cystine and the recording of cystine-evoked currents by Two Electrodes Voltage Clamp (TEVC) measurement. After a preliminary survey, those mutants which showed at least moderate signal in one technique were further studied.

The ocular mutant G197R was one of them. It showed only a small signal (about 30 nA) in TEVC recording compared to the control protein (‘DelAd’ construct lacking the lysosomal targeting signal, about 300 nA). However, the G197R mutant showed more obvious response in the tracer uptake measurement (with \textsuperscript{14C} cystine) as illustrated in Figure 2.

![Figure 2. \textsuperscript{14C}-cystine uptake measurement for G197R and control (‘DelAd’) cystinosin.](image)

The discrepancy between TEVC measurements (10-fold decrease at 1mM cystine) and biochemical measurements (2-fold decrease at saturation; Figure 2) suggests that the coupling
between $\text{H}^{+}$ and cystine transport may be altered by this ‘ocular’ G197R mutation. However, this interpretation requires combined electrophysiological and \([^{14}\text{C}]\)cystine uptake measurements on individual oocytes to be firmly established.

Another interesting mutant was P200L, which is associated to juvenile phenotype in clinics. It showed moderate signal (about 60 nA) in TEVC measurements, which could provide opportunity for a more detailed kinetic study. In addition, this mutant also showed significant response (similar to that of G197R) in cystine uptake measurement, thus offering chance to compare the saturation kinetics obtained from both techniques.

A deeper knowledge on the topology and on the transport mechanism of human cystinosin may help pinpointing molecular alterations associated with the existence of distinct clinical phenotypes.