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Identification and subcellular localization of a new cystinosin isoform

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[1]*Department of Nephrology and Urology, Division of Nephrology, and* [2]*Department of Laboratory Medicine, Bambino Gesù Children's Hospital and Research Institute, Rome, Italy; and* [3]*Department of Pediatrics, Pediatric Nephrology, Radboud University Medical Center, Nijmegen, The Netherlands*

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Taranta A, Petrini S, Palma A, Mannucci L, Wilmer MJ, De Luca V, Diomedi-Camassei F, Corallini S, Bellomo F, van den Heuvel LP, Levtchenko EN, Emma F. Identification and subcellular localization of a new cystinosin isoform. *Am J Physiol Renal Physiol* 294: F1101–F1108, 2008. First published March 12, 2008; doi:10.1152/ajprenal.00413.2007.—Nephropathic cystinosis is a lysosomal disorder caused by functional defects of cystinosin, which mediates cystine efflux into the cytosol. The protein sequence contains at least two signals that target the protein to the lysosomal compartment, one of which is located at the carboxy terminal tail (GYDQL). We have isolated from a human kidney cDNA library a cystinosin isoform, which is generated by an alternative splicing of exon 12 that removes the GYDQL motif. Based on its last three amino acids, we have termed this protein cystinosin-LKG. Contrary to the lysosomal cystinosin isoform, expression experiments performed by transient transfection of green fluorescent protein fusion plasmids in HK2 cells showed that cystinosin-LKG is expressed in the plasma membrane, in lysosomes, and in other cytosolic structures. This subcellular localization of the protein was confirmed by transmission electron microscopy. In addition, immunogold labeling was observed in the endoplasmic reticulum and in the Golgi apparatus. Expression of the protein in renal tubular structures was also directly demonstrated by immunostaining of normal human kidney sections. The plasma membrane localization of cystinosin-LKG was directly tested by [^{35}S]cystine flux experiments in COS-1 cells. In the presence of a proton gradient, a marked enhancement of intracellular cystine transport was observed in cells overexpressing this isoform. These data indicate that the expression of the gene products encoded by the CTNS gene is not restricted to the lysosomal compartment. These finding may help elucidate the mechanisms of cell dysfunction in this disorder.

genetic disease; nephropathic cystinosis; cystine transport

NEPHROPATHIC CYSTINOSIS (NC) is an autosomal recessive disorder characterized by severe renal Fanconi syndrome, leading to early onset chronic renal failure in the majority of the patients (8). With time, other complications develop, including photophobia, anterior chamber abnormalities, retinal degeneration, hypothyroidism, diabetes mellitus, exocrine pancreatic insufficiency, myopathy, neurological deterioration, and liver involvement (8, 11). Despite significant clinical improvements related to the use of the cystine-depleting agent cysteamine, most patients develop end-stage renal disease in their second or third decade of life (7, 9, 15).

NC is caused by mutations in the CTNS (cystinosis, nephropathic) gene, which encodes for the cystinosin protein that mediates cystine efflux from lysosomes (12, 27). The CTNS gene is composed of 12 exons, with the start and stop codons located in exon 3 and exon 12, respectively, and encodes for a 367-amino acid protein (27). Cystinosin is predicted to have seven transmembrane domains that form a proton-driven, ATP-dependent cystine transporter across lysosomal membranes (12, 27). To date, at least two lysosome-targeting motifs have been identified at the COOH terminal tail (GYDQL) and in the third predicted cytoplasmic loop (YFPQA) (amino acids 281–285) (5, 12). Experimental mutations or deletion of the last five amino acids has been shown to cause partial mislocation of the protein to the plasma membrane (5).

In the past years, several cystinosin isoforms have been registered in *Homo sapiens* online databases [isoform CRA_b (gi: EAW90495), cystinosis nephropathic (gi: AAH32850), cystinosis nephropathic isoform 1 (gi: NP_001026851), unnamed protein product (gi: BAF84708)]. Among these, partial expressed sequence tag sequences and complete sequences from human spleen and testis (gi: AAH32850 and BAF84708) indicate the existence of a longer transcript, resulting from an alternative splicing of the last exon. Similar isoforms have also been recovered from nonhuman DNA and protein databases (XP_001089495.1, XP_854520.1, XR_021668.1), although no functional data are available on these alternative cystinosin sequences.

Herein, we report the characterization of the cystinosin protein isoform resulting from an alternative splicing of exon 12, which changes the protein sequence at the level of the lysosome-targeting motif and directs part of the protein to other cell compartments, including the plasma membrane, the Golgi apparatus, the endoplasmic reticulum (ER), and cytosolic vesicles resembling endosomes.

MATERIALS AND METHODS

Isolation and sequencing of CTNS isoforms. cDNA synthesis was performed with first-strand cDNA synthesis kit (Roche Applied Science, Penzberg, Germany) from 3 μg of total RNA extracted with TRIzol reagent (Invitrogen, Paisley, UK) from human or mouse kidney specimens. PCR conditions and primer sequences are listed in Table 1. PCR fragments were extracted and purified from agarose gels with gel extraction kit (QIAGEN, Milan, Italy). Direct sequencing was performed with big dye terminator kit (Beckman Coulter, Brea, CA) and CEQ2000XL DNA Analysis System (Beckman Coulter).

Cloning of CTNS isoforms and mutated cDNAs into expression plasmids. CTNS isoforms and a mutated variant lacking the last five amino acids were cloned into pDsRed1-N1 plasmid, pEGFP-N1

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Table 1. *Primer sequences and PCR conditions*

Primer Label	Primer Sequence	Position, bp
hCTNS fw	5′-ATGATAAGGAATTGGCTG-3′	340–357
hCTNS bw	5′-TCAGCCCTTCAAGCTGC-3′	1792–1808
mCTNS fw	5′-TCAGTCTCACTGCTCCTCC-3′	193–211
mCTNS bw	5′-CTCCCAGTTGCAAGGTTAGC-3′	1679–1698
mCTNS fw1	5′-GCTTTAGCCTCCTCCAGATG-3′	1051–1070
	Primers used with the pDsRed1-N1 vector	
hCTNS xI fw	5′-GAGAAATCGCTCGAGATGATAAGGAAT-3′	340–351
hCTNS-bH1-S1 bw	5′-GGTCCCTGGATCCCGGTTCAGCTGGTC-3′	1429–1440
hCTNS-bH1-S2 bw	5′-CCGGCTTGGATCCCGGCCCTTCAAGCT-3′	1794–1805
	Primers used with the pcDNA3.1 vector	
hCTNS-bH1 fw	5′-GGTACCGAGCTCGGATCCATGATAAGGAATTGGCTGACTA-3′	340–361
hCTNS-xI S1 bw	5′-TCTAGATGCATGCTCGAGCTAGTTCAGCTGGTCATACCCC-3′	1422–1443
hCTNS-xI S2 bw	5′-TCTAGATGCATGCTCGAGTCAGCCCTTCAAGCTGCTTGC-3′	1788–1808
hCTNS-xI ΔGYDQL bw	5′-TCTAGATGCATGCTCGAGCTACGGTCTCTTTCTGTAC-3′	1407–1422

Reference sequences: NM_004937 (*Homo sapiens* CTNS) and NM_031251 (*Mus musculus* CTNS). PCR condition for hCTNS: 1 cycle of 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 63°C, and 90 s at 72°C. PCR condition for mCTNS: 1 cycle of 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 62°C, and 90 s at 72°C. CTNS, cystinosis, nephropathic; bp, base pairs; h, human; xI, *Xho*I; bHI, *BamH*I; m, mouse; fw, forward; bw, backward.

(Clontech Laboratories, Palo Alto, CA), or pcDNA3.1 (Invitrogen) after digestion with *Xho*I and *BamH*I restriction enzymes (Fermentas, Hanover, MD). Sequence integrity was tested by direct sequencing.

Cell transfection. HK2 cell lines were cultured in DMEM-F12 (Gibco, Grand Island, NY), supplemented with 5% FCS, 2 mM L-glutamine, 1× penicillin/streptomycin, and 1× insulin-transferrin-sodium selenite (Sigma-Aldrich, St. Louis, MO). COS-1 cell lines were cultured in DMEM high glucose (Gibco), supplemented with 10% FCS, 1× penicillin/streptomycin, and 2 mM L-glutamine. Cells were transfected at 80% confluence with lipofectamine-2000 (Invitrogen), according to the manufacturer's protocol.

Immunofluorescence microscopy. Transfected cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA.

For colocalization studies, rabbit anti-zonula occludens-1 (ZO-1) antibodies (Zymed, South San Francisco, CA) were used according to protocols suggested by the manufacturer and revealed with goat anti-rabbit Alexa Fluor 488 (Molecular Probes). Negative controls were performed using 1% BSA solutions lacking the primary antibody.

Transfected cells were also incubated with a rabbit affinity-purified anti-cystinosin-LKG antiserum using a 1:10 dilution in 1% BSA for 90 min. This antiserum is directed against a cystinosin-LKG-specific sequence spanning amino acids 366–389 (ARTGSGSRLRQDWAPSLQPKALPQ) and was produced by a commercial biotechnology company (PRIMM, Milan, Italy). Slides were then incubated with goat anti-rabbit Alexa Fluor 488 or 555 antibodies (Molecular Probes).

Transfected cells were also incubated with a 75 nM solution of lysotracker Red DND-96 (Invitrogen) for 2 h in growth medium.

Fluorescence microscopy was performed on a Nikon Eclipse E600 microscope equipped with epifluorescence optics. Images were processed with Metamorph software. Colocalization analysis was performed using an Olympus fluoview FV1000 confocal microscope equipped with FV10-ASW version 1.6 software, Multi Ar (458–488 and 514 nm) and 2× He/Ne (543 and 633 nm) laser with ×60 oil immersion objective. Images were processed with Adobe Photoshop 5.5.

Immunoelectron microscopy. HK2 cells overexpressing cystinosin-LKG were fixed in 4% formaldehyde-0.001% glutaraldehyde.

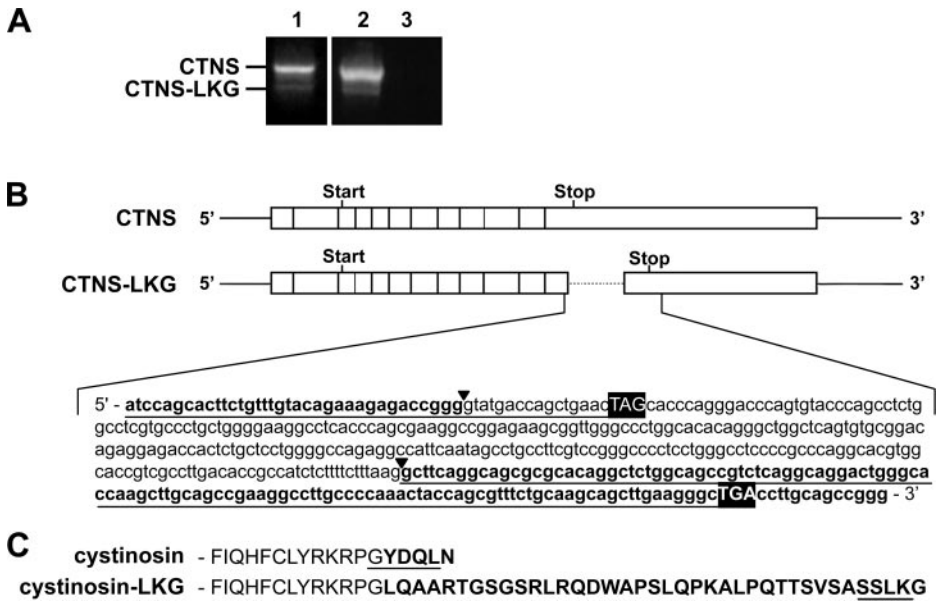

Fig. 1. Cystinosin isoforms. *A*: agarose gel of PCR products obtained from a cDNA human kidney library (*lane 1*) and from total RNA extracted from proximal tubule cell lines, derived from a healthy subject (*lane 2*) and a patient with nephropathic cystinosis (NC) (*lane 3*). PCR reactions were performed using primers "hCTNS fw" and "hCTNS bw" (see Table 1). The more abundant transcript corresponds to the lysosomal isoform. The smaller and less abundant transcript corresponds to cystinosin-LKG. *B*: CTNS (cystinosis, nephropathic) gene isoforms and nucleotide sequence of the exon 12 splicing portion. The cystinosin-LKG sequence is represented in bold type. Arrows indicate splice sites; stop codons are shown with a black background. Translated sequences are underlined. *C*: carboxy-terminal protein sequences of the two cystinosin isoforms. The GYDQL and SSLK sequences are known lysosome and endoplasmic reticulum (ER) targeting motifs, respectively (underlined).

Fig. 2. Comparison of cystinosin-LKG carboxy terminal tail sequences in different species. Specific residues of cystinosin-LKG are reported. Identical amino acids are indicated with a black background, residues with mild-to-high similarity are shown with a gray background, and residues with low similarity are reported with a white background.

A preembedding procedure was performed: cells were permeabilized with 0.1% saponin, preincubated with 0.15% glycine, blocked with 5% normal goat serum/1% BSA, and incubated overnight at 4°C with anti-cystinosin-LKG antiserum diluted 1:10. Samples were then incubated with 5-nm gold-conjugated goat anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK), fixed in 2.5% glutaraldehyde, and collected by centrifugation at 2,000 rpm for 15 min at 4°C. Pellet was enclosed in 2% gelatine, postfixed in OsO_4, dehydrated in ethanol, and embedded in EMbed-812 (Electron Microscopy Sciences, Hatfield, PA).

Ultrathin sections were obtained with a Leica Ultracut S ultramicrotome (Leica, Vienna, Austria) and analyzed with a Zeiss EM 10C transmission electron microscope (Zeiss, Cambridge, UK) after staining with lead citrate and uranyl acetate.

Control experiments were performed without primary antibodies.

Immunoperoxidase staining. Human kidney paraffin sections were treated with xylene, hydrated in alcohol and water, incubated in pH 8.3 EDTA buffer, and treated with 3% hydrogen peroxide for 20 min to quench endogenous peroxidase activity, according to standard protocols. Specimens were then incubated for 1 h at 37°C with affinity-purified anti-cystinosin-LKG antiserum and diluted 1:10 in 1% BSA/PBS. Antibodies were revealed with biotinylated goat anti-rabbit IgG, streptavidin-conjugated peroxidase, and 3,3′-diaminobenziodine (Dako, Carpinteria, CA). Nuclei were stained with Mayer's hematoxylin, and specimens were examined with a Nikon eclipse E600 light microscope. Peptide competition experiments were performed after overnight incubation of the antiserum with excess of peptide.

Cystine uptake experiments. COS-1 cells were transfected with cystinosin isoforms or mutant proteins in 24-well plates. After washing with Krebs-Henseleit-HEPES buffer (4 mM KCl, 135 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM monobasic NaH_2PO_4, 5 mM D-glucose, and 10 mM HEPES, pH 7.4), cells were incubated at 20°C with 1 μCi L-[^{35}S]cystine in 200 μl of Krebs-Henseleit-HEPES buffer, pH 7.4 or pH 5.6. After washing in ice-cold buffer, cells were lysed with 0.1 N NaOH and counted in a Beckman Coulter liquid scintillation counter (Fullerton, CA).

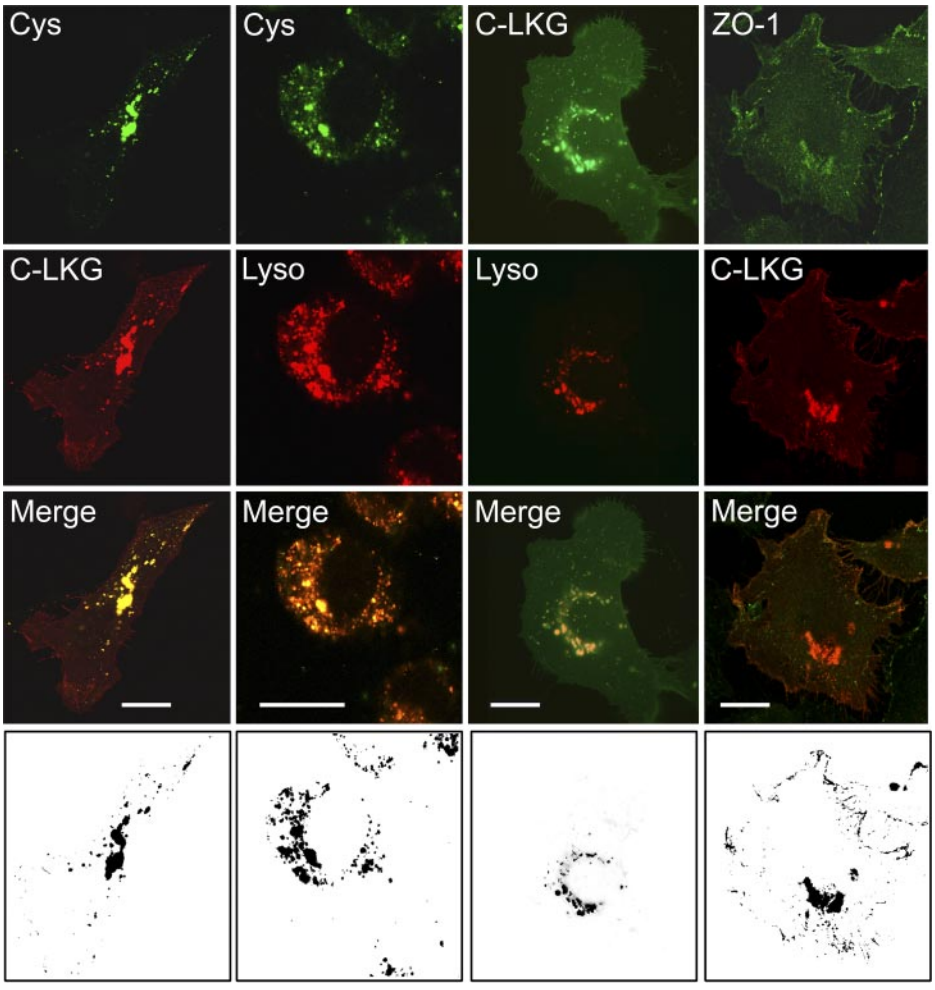

Fig. 3. Expression of green fluorescent protein (GFP) fusion proteins in HK2 cells. The figure shows colocalization experiments of GFP fusion proteins, lysotracker lysosomal marker, and anti-zonula occludens (ZO)-1 antibodies. Each colocalization experiment is shown in columns. *Row 3* shows the overlays between the first two rows. Colocalization masks are shown in *row 4*. HK2 cells were transiently transfected with cystinosin (Cys) or cystinosin-LKG (C-LKG) GFP fusion proteins. Lysosomes were stained with lysotracker Red (Lyso). The plasma membrane was stained with anti-ZO-1 monoclonal antibodies (ZO-1). Overlay between the two cystinosin isoforms is limited to vesicular structures that correspond to lysosomes, as indicated by the complete overlap of the GFP-cystinosin signal with the lysotracker die. In addition, a diffuse, fine granular cytosolic fluorescence was observed only in GFP-cystinosin-LKG-transfected cells. In these same cells, a plasma membrane signal was also observed, which partially colocalizes with ZO-1. Bars: 20 μm.

Differential centrifugation. HK2 cell lines overexpressing green fluorescent protein (GFP)-cystinosin fusion proteins were lysed by ultrasonification in the presence of a protease inhibitor cocktail (Sigma). Sequential centrifugations were performed at 4°C at different speeds, as indicated in RESULTS. Pellets were then resuspended in the homogenization buffer. The relative amount of GFP-cystinosin fusion proteins in each subcellular fraction was measured fluorometrically at 480 nm with a luminescence spectrometer (Perkin Elmer, Schelton, CT). Fractions were then run on 10% polyacrylamide gels, blotted on nitrocellulose membrane, and incubated with primary antibody directed against cathepsin D (Santa Cruz Biotechnology) protein. After incubation with horseradish peroxidase-conjugated secondary antibodies, signals were detected by chemiluminescence (Amersham) and measured by densitometry using Scion Image for Windows software (www.scioncorp.com).

RESULTS

Cystinosin isoforms. Cystinosin isoforms were searched by PCR amplification of a human kidney cDNA library. Two different PCR fragments were recovered, using primers encompassing the entire open-reading frame and extending into the 3′-UTR region (Fig. 1*A*). Both PCR products were not expressed in a proximal tubule cell line derived from a patient with NC carrying a 65-kb deletion of the CTNS genomic region (Fig. 1*A*). Sequence analysis confirmed that these amplicons correspond to splicing variants of the CTNS mRNA. The most abundant fragment matches the originally reported lysosomal CTNS isoform (NM_004937). The second fragment originates from an alternative splicing of exon 12, which removes 264 nucleotides, including the sequences encoding for the "GYQDL" lysosome-targeting motif and the stop codon (Fig. 1*B*). The open-reading frame continues downstream to the spliced region, resulting in a longer protein of 400 amino acids, with a different carboxy terminal tail (Fig. 1*C*). Based on its last three residues, we have termed this isoform "cystinosin-LKG" and will refer in the text to the exclusively lysosomal isoform as "cystinosin", to avoid ambiguity.

Analysis of cystinosin-LKG sequence indicates that its carboxy terminal tail contains the "SSLK" motif, which is shared by other ER membrane proteins (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/). In addition, four other identical human sequences, obtained from different tissues, have already been registered in protein sequence databases (NM_001031681.1, BG392867, CD171768, CA454595). Cystinosin-LKG homologous sequences have also been isolated from *macaca mulatta* (XM_001089495.1), *pan troglodytes* (XR_021668.1), and *canis familiaris* (XM_849427.1) mRNAs (Fig. 2).

As this isoform was not reported in mice, we have amplified with specific primer mouse kidney mRNA and found an alternative splicing of exon 12 that encompasses a longer RNA sequence and results in a protein product that has lower degrees of homology with the human sequence, compared with other primate isoforms (Fig. 2).

Localization of the cystinosin-LKG. HK2 cells expressing cystinosin-GFP and cystinosin-LKG-GFP fusion proteins showed colocalization in vesicular structures that are predominantly located in the perinuclear region (Fig. 3, *column 1*). In addition, cells overexpressing the cystinosin-LKG isoform exhibited a diffuse fine granular signal in the cytosol and a distinct signal in the plasma membrane or immediate submembrane space. Coimmunostaining with the lysotracker lysosomal marker and anti-ZO-1 antibodies showed partial colocalization with these markers (Fig. 3, *columns 3* and *4*), indicating that part of the protein is located in lysosomes and that a substantial portion of the protein is directed to the plasma membrane. Conversely, all of the fluorescence emitted by GFP-cystinosin fusion proteins colocalized with the lysotracker marker (Fig. 3, *column 2*). The pattern of expression of each isoform was not significantly modified, whether they were coexpressed or expressed separately.

By cell fractionation, cystinosin-GFP was mainly recovered in the P2 pellet (20,000 *g*) that contains the lysosome fraction, as demonstrated by staining with cathepsin D (Fig. 4). A less abundant part of the fluorescence was also recovered in the P4 fraction (120,000 *g*), which contains small microsomes, ER, and Golgi vesicles. The opposite pattern was observed with cystinosin-LKG (Fig. 4). Contrary to the nonfused GFP pro-

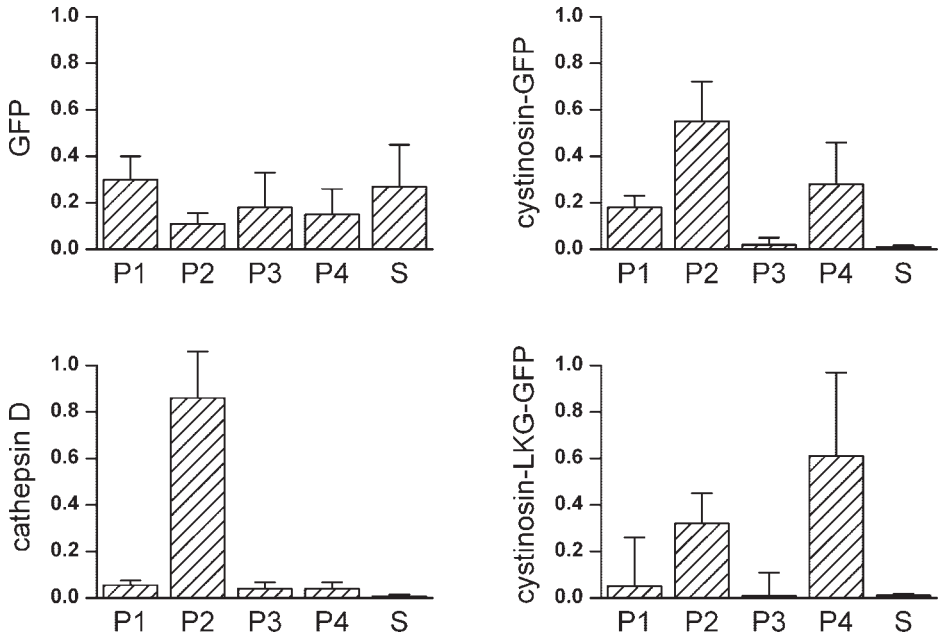

Fig. 4. Differential centrifugation of transfected cells overexpressing GFP or GFP proteins fused to cystinosin isoforms. Pellets (P) were obtained after centrifugation at 6,000 *g* (P1), 20,000 *g* (P2), 45,000 *g* (P3), and 120,000 *g* (P4). The relative expression of GFP proteins was assayed by fluorimetry, and the expression of cathepsin D, as a lysosomal marker, by immunoblotting. S, final supernatant. Experiments were performed in triplicates. Values are means ± SE ($N = 3$).

teins, no signal was recovered from the final supernatant, indicating that both cystinosin isoforms are not soluble in the cytosol.

To further investigate the localization of cystinosin-LKG, an affinity-purified rabbit antibody directed against a specific peptide sequences of cystinosin-LKG was generated. The antiserum specificity was verified on transfected cells. As shown in Fig. 5, the antiserum recognizes only cells overexpressing the LKG isoform.

The antiserum was then used for immunoelectron microscopy studies on transfected cells expressing cystinosin-LKG. In these cells, immunogold particles were observed predominantly in tubulovesicular structures distributed in the perinuclear and Golgi region, in multivesicular bodies, in the ER, within the plasma membrane, and in small cytosolic vesicles (Fig. 6*A*). These vesicles have the characteristics of lysosomes or small endosomes. Occasionally, immunogold particles were detected in mitochondria. Untransfected cells were not significantly labeled (Fig. 6*B*).

To demonstrate CTNS-LKG protein expression in the kidney, human renal sections were analyzed by immunoperoxidase staining performed with affinity-purified anti-cystinosin-LKG antiserum. As shown in Fig. 7, a diffuse, predominantly granular staining was observed in tubular cells, but not in glomeruli. Most tubular structures correspond to proximal tubules. Some smaller tubules, which are also stained by the antibody, have the morphological characteristics of distal tubules. The specificity of the observed staining was confirmed by peptide competition.

[^{35}S]cystine flux studies. The localization of cystinosin-LKG in the plasma membrane suggests that this isoform may mediate cystine transport between the cell and the extracellular milieu. Flux studies were, therefore, performed in cells transfected with cystinosin, cystinosin-LKG, or a truncated cystinosin protein lacking the GYDQL motif, which has been previously described by Kalatzis et al. (12). As shown in Fig. 8, cells transfected with the last two constructs demonstrated a dramatic increase in intracellular L-[^{35}S]cystine content, in the presence of a proton gradient. In the absence of a pH gradient, no significant uptake was observed.

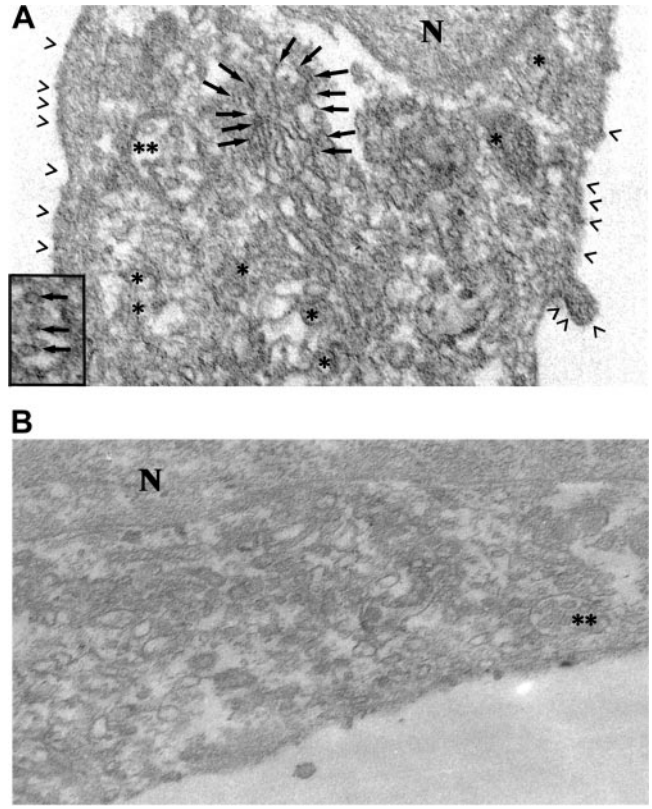

Fig. 6. Immunoelectron microscopy in cells overexpressing cystinosin-LKG. *A*: electron micrography of a transfected cell overexpressing cystinosin-LKG. Immunogold particles (5 nm) are localized in the membranes of small vesicles resembling endosomes or lysosomes (asterisks), in the ER and in the Golgi cisternae (arrows), and near or within the plasma membrane (arrowheads). The *inset* shows a higher magnification of a multivesicular body (double asterisks), where gold particles are localized in the membrane of lysosome (electron dense) and endosome (electron lucent) organelles. *B*: electron micrography of a nontransfected cell. Very poor staining was observed. Double asterisks indicate a multivesicular body. N, nuclei. Magnification: ×60,000.

DISCUSSION

The pathophysiology of renal damage in NC remains controversial. Current hypotheses include intracellular ATP depletion, decreased synthesis of glutathione, impaired activity of

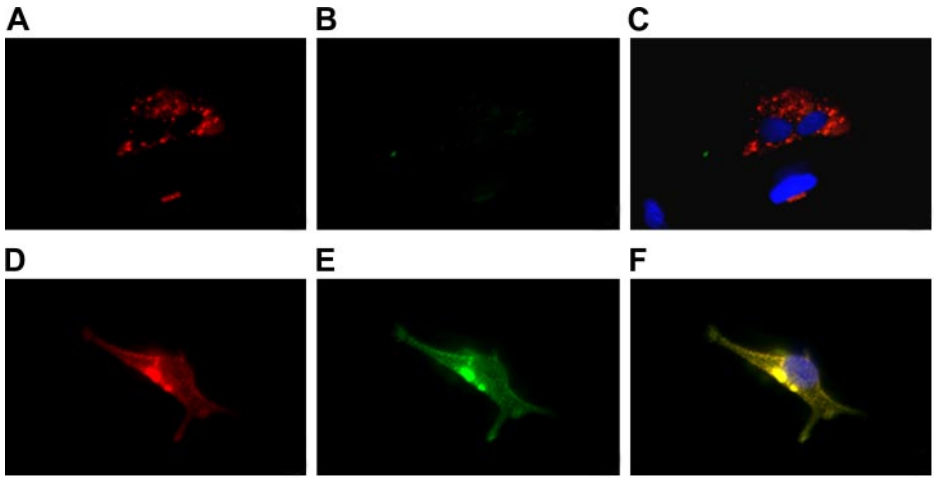

Fig. 5. Immunostaining with anti-cystinosin-LKG antiserum. Transiently transfected cells overexpressing GFP-cystinosin (*A*) or GFP-cystinosin-LKG (*D*) were stained with anti-cystinosin-LKG antibodies (*B* and *E*, respectively). Overlays are shown in *C* and *F*, respectively. As shown, the antiserum recognizes only cells overexpressing the LKG isoform (*E*), which demonstrates the specificity of the antiserum for this isoform. The overlap with the GFP-fused LKG protein is complete (*F*). Conversely, no signal was detected in cells overexpressing the lysosomal cystinosin isoform (*B*). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

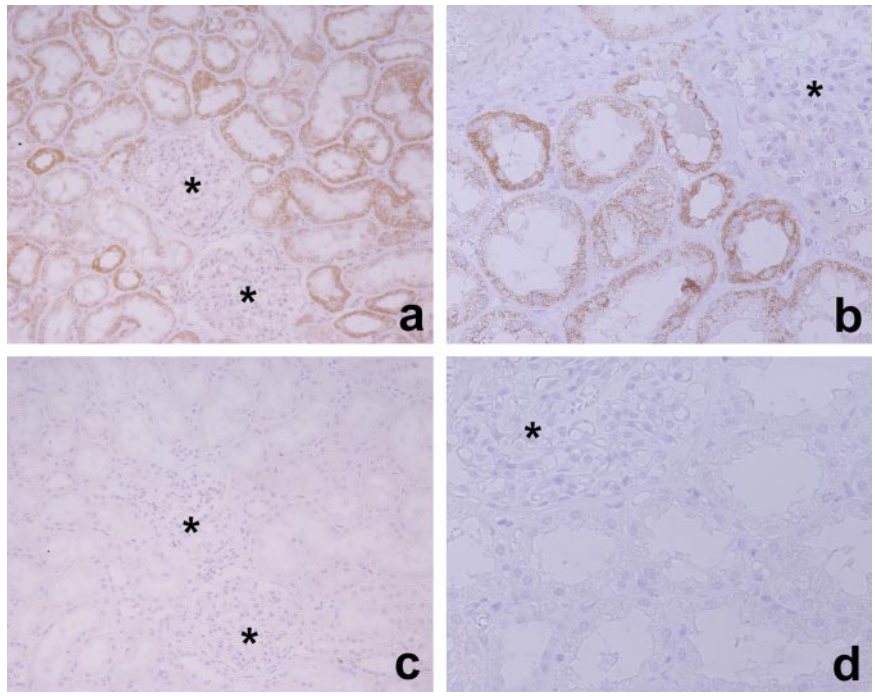

Fig. 7. Staining of normal human kidney sections. *a* and *b*: Immunoperoxidase staining was performed using affinity-purified anti-cystinosin-LKG antibodies on normal human kidney. Most tubular structures correspond to proximal tubules; some smaller tubules have the morphological characteristics of distal elements. All tubular cells were diffusely stained. No staining was observed in glomeruli (asterisks). *c* and *d*: Competition experiments with soluble peptide antigen, corresponding to amino acids 366–389 (ARTGSGSRLRQDWAPSLQPKALPQ) of cystinosin-LKG. No significant staining was observed. Magnification: ×20 (*a* and *c*) and ×40 (*b* and *d*).

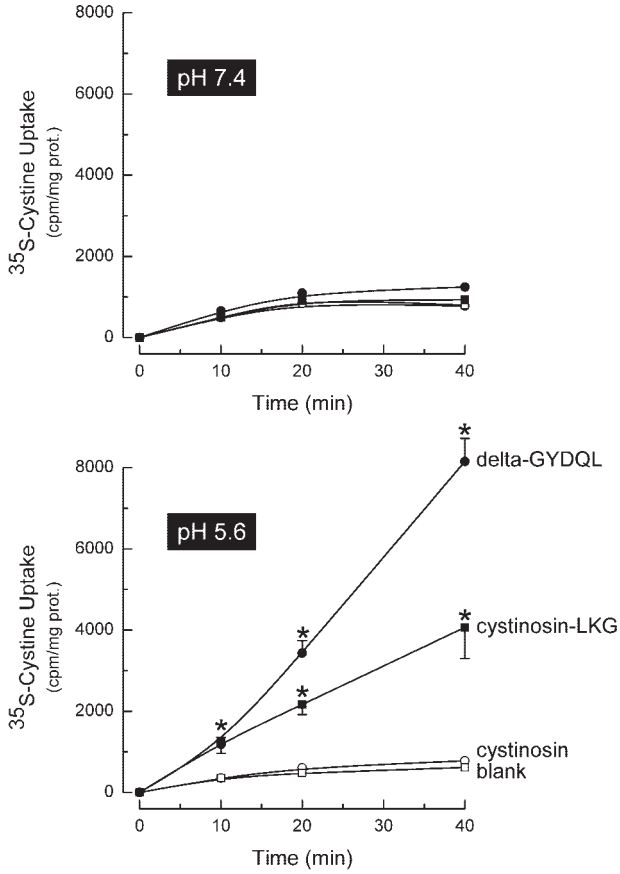

Fig. 8. L-[^{35}S]cystine fluxes. L-Cystine uptake was measured in COS-1 cells. Cells were transiently transfected with the pcDNA3 plamid (□, blank), or with plasmids expressing the cystinosin isoform (○), the cystinosin-LKG isoform (■), or a truncated cystinosin protein (indicated as "delta-GYDQL") lacking the last 6 amino acids (●). Flux experiments were performed in the absence (pH 7.4) or in the presence (pH 5.6) of a transmembrane proton gradient. Experiments were performed 3 times in quadruplicate. Values are means ± SE.

mitochondrial respiratory chain complexes, and increased rate of apoptosis (13, 14, 16, 20–22). These hypotheses are likely to coexist at various levels in the chain of events that lead to cell damage, but none allows the establishment of a convincing link between cystine retention in the lysosome compartment and the metabolic alterations that are reported in NC cells. This relationship has been further questioned by the generation of a CTNS knockout mouse, which reproduces lysosomal cystine accumulation in renal proximal cells, without evidence of Fanconi syndrome (6).

Herein we have reported the identification of a second product of the CTNS gene in the human cortex. Cystinosin-LKG is expressed in several cell types, as indicated by the expressed sequence tag sequences isolated from various human tissues. This isoform does not harbor the "GYDQL" sequence at its carboxy terminal tail and has a subcellular localization similar to the experimentally generated cystinosin variant described by Cherqui et al. (5). Part of the protein is still targeted to the lysosome compartment, as shown by colocalization with the more abundant cystinosin isoform, but a considerable proportion of the transcripts are located in other cytosolic vesicles, in the ER, in the Golgi apparatus, and in the plasma membrane.

The exact role of this isoform in these structures is not known at this stage. Our data indicate that this naturally occurring isoform is able to transport cystine similarly to the artificially generated mutant protein previously described (12). Cystinosin-LKG-mediated cystine transport, however, requires a proton gradient, which may question the exact relevance of this protein in cystine transport across the cell membrane.

Recently, other proton-coupled amino acid transporters that are expressed in the renal cortex have been identified. These include the imino acid proton/amino acid transporter 1 (PAT-1; also termed lysosomal amino acid transporter-1), which mediates the pH-dependent transport of small amino acids (mainly glycine, alanine, and proline) (2, 25). This transporter was

originally thought to be sodium dependent, which is explained by the fact that the luminal pH in the proximal tubule is, in part, set by the activity of the Na^+/H^+ exchanger (28). Similarly to the CTNS gene products, PAT-1 can have different subcellular localizations. In the central nervous system, it is located mainly in lysosomes, but has also been localized in the plasma membrane of axonal processes (25, 29). In the intestine Caco-2 cell line on the other hand, PAT-1 is mainly expressed in the apical plasma membrane (4). These subcellular localizations, however, do not seem to be related to alternative protein sequences.

PAT-2 is a second imino acid transporter that has very similar transport characteristics to PAT-1, including the H^+ dependence. It is mainly expressed in the ER and in recycling endosomes (23). This also resembles cystinosin-LKG, which is expressed in the ER and which contains the ER motif "SSLK" in its sequence. In addition, the present report indicates that cystinosin-LKG is also expressed in small intracytoplasmic vesicles that may correspond to recycling endosomes.

Taken together, these data suggest that the products of the CTNS gene and the imino acid transport systems share several important characteristics, including their ubiquitous pattern of expression, their pH dependence, and their expression in similar subcellular compartments.

The exact site of expression in the proximal tubule of these imino acid transporters and of cystinosin-LKG needs, however, to be further assessed. We have been successful in generating an affinity-purified antibody that has allowed the performance of immuno-electron studies in cultured cells overexpressing the protein, but we could not obtain convincing electron micrograph images in the renal cortex because of a significant background signal (data not shown). Conversely, the pH-dependent peptide transporter 2 (PEPT2) has recently been identified and located in the apical membrane of the proximal tubule, where it mediates proton-dependent transport of di- and tripeptides (3, 17, 24).

Antibody detection of cystinosin remains problematic. Currently, very limited data are available, despite several attempts by different groups to generate specific antibodies that would allow direct detection of the protein in tissues and cell extracts (10). Similar to previous studies, we had to overexpress GFP fusion proteins to obtain reliable signals in cell lines (5, 12) and could not detect a reproducible signal by Western blotting, even after overexpressing the protein. In the human kidney, we could only show the expression of cystinosin-LKG by immunoperoxidase staining. Immunofluorescence studies, which may have provided more information on the subcellular localization of the protein, were not successful.

Clearly, overexpression of cystinosin-LKG may have affected the distribution of the protein in the cell and may not entirely represent its physiological pattern of expression. On the other hand, overexpression of the lysosomal isoform shows a pattern of expression that remains restricted to the lysosome.

Cherqui et al. (5) have identified a second domain (YFPQA) of the protein located in the third cytoplasmatic loop (amino acids 281–285), which is equally important for targeting the protein to the plasma membrane. We have investigated the existence of a similar isoform, which could result from an additional alternative splicing of the mRNA, but did not find it.

The identification of cystinosin-LKG also raises the hypothesis that differences in the expression of this transcript between mouse and humans may explain the absence of FS in the CTNS knockout model (6). We have, therefore, searched this isoform in mice and have isolated a similar splicing variant. The murine sequence is significantly different from the human sequence, but shares complete identity in the amino acid string flanking the splice site and in the last residues containing the "SSLK" sequence. At the moment, no data are available on the expression pattern of the murine analog of cystinosin-LKG.

Even if the exact function of cystinosin-LKG remains unclear at this stage, it is unlikely, in our view, that this splicing variant, which selectively removes the lysosome targeting motif and replaces it with a new sequence, has been preserved during evolution, unless it plays a significant role in the cell, probably related to cystine homeostasis.

One of the major characteristics of FS associated with NC is, in fact, the presence of an overt low-molecular-weight proteinuria. This finding has been mainly reported in Dent disease and Lowe syndrome, which are both characterized by defects in endosome recycling. These are caused by mutations in the endosomal ClCN5 chloride proton exchanger in Dent disease (18, 19) and by a defect of phosphatidylinositol 4,5-bisphosphate 5-phosphatase, which regulates endosome trafficking between the Golgi apparatus and the plasma membrane, in Lowe syndrome (1). The expression pattern of cystinosin-LKG may, therefore, provide new insights to explain low-molecular-weight proteinuria in NC.

It has also been shown that cystinotic cells have enhanced apoptotic activity, which is mediated, at least in part, by cysteinylation of proteins, including PKC-δ (26). Cystinosin-LKG may also play a role in regulating the amount of free cystine in the cell, preventing the formation of mixed disulfide compounds.

In conclusion, the present data show, for the first time, that the expression of the CTNS gene products is not restricted to the lysosomal compartment. This may have relevant implications in understanding the mechanisms of cell dysfunction in NC.

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