Abstract. Cystinosis is an autosomal recessive disorder associated with excessive lysosomal cystine accumulation secondary to defective lysosomal cystine efflux. CTNS, the gene mutated in cystinosis, codes for the lysosomal membrane protein cystinosin. Antisera were raised in rabbits to a carboxy-terminal oligopeptide sequence from cystinosin. Antisera were screened by Western blotting and immunocytochemical analyses of transfected COS-7 cells expressing either human wild-type cystinosin, a wild-type cystinosin-green fluorescent protein (GFP) fusion protein, or a fusion protein of GFP and mutant human cystinosin with a carboxy-terminal deletion. In Western blots, bands corresponding to cystinosin or cystinosin-GFP were observed in transfected cells but no signal was detected in cells expressing the carboxy-terminal mutant; pre-immune sera yielded negative results in all three cases. In transfected cells expressing wild-type cystinosin, immunoreactivity appeared in subcellular vesicles. In cells expressing the wild-type cystinosin-GFP fusion protein, immunoreactivity colocalized with GFP fluorescence. Previous studies demonstrated that GFP fluorescence from this construct colocalized with immunostaining for a known lysosomal membrane protein, i.e., lysosome-associated membrane protein 2 (LAMP-2). Moreover, when the GYDQL motif is deleted from the fusion protein, fluorescence is partially relocalized to the plasma membrane, which confirms the role of this motif in lysosomal sorting of cystinosin (11). This observation was exploited to express cystinosin at the plasma membrane of COS-7 cells, to directly address its function. Kalatzis et al. (12) were thus able to demonstrate that cystinosin is a H⁺-driven, lysosomal, cystine transporter that is highly specific for L-cystine.

To date, anti-cystinosin antibodies have not been generated, which represents a hindrance to further experiments. In this study, we raised antisera to the carboxy-terminal end of cystinosin and tested transfected COS-7 cells expressing wild-type human cystinosin or cystinosin with a deletion of the GYDQL motif. We demonstrated colocalization of human cystinosin-GFP fluorescent and antiserum immunoreactivity. We also demonstrated that cystinosin was immunolocalized to tubule epithelia in normal human kidneys; furthermore, cystinosin immunoreactivity was absent in kidneys from patients with a CTNS deletion.

Cystinosis is an autosomal recessive disorder that results in renal proximal tubular dysfunction, renal failure, and, ultimately, multisystem disease (1). It is caused by a failure of cystine efflux from lysosomes (2–4), which results in intralysosomal accumulation of disulfides of the amino acid cysteine (5,6). Cystine efflux is inhibited by a defect in lysosomal membrane cystine transporter (which is carrier-mediated) (7,8).

CTNS, the gene mutated in cystinosis, maps to human chromosome 17p and codes for cystinosin, a lysosomal membrane protein (9). Cystinosin contains 367 amino acids and is thought to have seven transmembrane domains, with its amino terminus in the lysosomal lumen and its carboxy terminus in the cytoplasm (9). It is predicted to have seven transmembrane domains, with its amino terminus in the lysosomal lumen and its carboxy terminus in the cytoplasm (9). It is predicted to have seven potential glycosylation sites in its amino-terminal sequence; heavy glycosylation of luminal domains is a feature of other lysosomal membrane proteins (10). Moreover, the carboxy terminus of cystinosin contains an amino acid sequence, i.e., GYDQL, that resembles a targeting motif (GYXXΦ, where Φ is a hydrophobic residue) present in other lysosomal membrane proteins (10).

When MDCK or HeLa cells are transfected with a vector expressing a human cystinosin-green fluorescent protein (GFP) fusion protein, fluorescence colocalizes in vesicular structures with immunostaining for a known lysosomal membrane protein named lysosome-associated membrane protein 2 (LAMP-2) (11). Moreover, when the GYDQL motif is deleted from the fusion protein, fluorescence is partially relocalized to the plasma membrane, which confirms the role of this motif in lysosomal sorting of cystinosin (11). This observation was exploited to express cystinosin at the plasma membrane of COS-7 cells, to directly address its function. Emmerling and colleagues (12) were thus able to demonstrate that cystinosin is a H⁺-driven, lysosomal, cystine transporter that is highly specific for L-cystine.

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Materials and Methods

Reagents

BDH (Poole, Dorset, UK) or Sigma-Aldrich Co. (Poole, Dorset, UK) supplied all reagents, except as otherwise specified.

Cell Transfection

COS-7 monkey kidney epithelial cells were cultured and transiently transfected exactly as described previously (12). Cells expressed either (1) GFP alone (i.e., transfected with the pEGFP-N1 plasmid; Clontech, Le Pont de Claux, France); (2) wild-type human cystinosin alone (i.e., transfected with the pcDNA-CTNS construct (12)); (3) a fusion protein of wild-type human cystinosin and GFP [i.e., transfected with the pcDNS-EGFP construct (11)]; or (4) a fusion protein of mutated human cystinosin, with the GYDQL targeting motif deleted, and GFP [i.e., transfected with the pcDNS-DGYDQL-EGFP construct (11)].

Raising of Antibodies to Cystinosin

Whole cystinosin protein is not available. We therefore selected seven oligopeptides, spread throughout the human cystinosin protein, for antibody production in rabbits. The synthesis of oligopeptides and the production of antiserum were performed by Sigma-Genosys (Cambridge, UK). Two animals were immunized with each oligopeptide. Each blood sample from the rabbits was tested with an enzyme-linked immunosorbent assay against the relevant peptide, to ensure that the antiserum exhibited high titer and avidity (data not shown). In this study, we describe results generated with antiserum (MH1 and MH2) raised against the last 13 amino acids of cystinosin (CLYRKRPGYDQLN). The GYDQL lysosomal targeting motif is contained within this sequence; apart from this motif, this oligopeptide does not demonstrate homology with other known proteins. The antiserum MH1 and MH2 yielded identical results in these experiments and, for simplicity, in this report we have not distinguished between results obtained with the two antibodies.

Western Blotting

Transfected COS-7 cells (1.2 × 10⁵) were grown in 10-cm plates for 48 h. Cells were then washed with phosphate-buffered saline (PBS) and scraped in PBS containing protease inhibitors [1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin]. The scraped cells were centrifuged at 13,000 rpm for 10 min at 4°C, and the cell pellet was immediately frozen in liquid nitrogen. At the time of use, 100 μl of Laemmli sample buffer was added to resuspend each cell pellet. One microliter of the recombinant endonuclease Benzonsase (Merck, Poole, UK) was added to the cell suspension, to reduce viscosity. Samples were directly separated at 10% polyacrylamide gels and were then immunoblotted to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with 5% nonfat milk in 0.05% Tween/PBS, to reduce nonspecific binding. The membranes were then incubated for 1 h at room temperature with a 1:500 dilution of rabbit antiserum in blocking solution. As a negative control, blots were incubated with a 1:500 dilution of the corresponding preimmune serum. Membranes were washed with 0.05% Tween/PBS and incubated for 1 h at room temperature with a 1:100,000 dilution of horseradish peroxidase-conjugated secondary antibody (Dako Ltd., Ely, UK). After washing, immunolabeling was detected via incubation of the membranes with a 1:5 dilution of Lumi-LightPlus Western blotting substrate (Roche Diagnostics Ltd., Lewes, East Sussex, UK) for 5 min at room temperature and exposure to autoradiographic film.

Immunocytochemical Analyses

Transfected COS-7 cells (2 × 10⁵) were grown on glass coverslips in 15-mm wells for 48 h. Plates were washed twice with PBS containing 100 μM CaCl₂ and 100 μM MgCl₂. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing of the coverslips with PBS, nonspecific binding was blocked with 10 mM NH₄Cl for 10 min at room temperature. Cells were then washed and permeabilized for 10 min at room temperature with 0.5% Triton X-100. Washed coverslips were incubated for 1 h at room temperature with a 1:250 dilution of anti-cystinosin antiserum, in a humidified chamber. The coverslips were then washed and incubated for 1 h at room temperature with a 1:100 dilution of anti-rabbit Ig secondary antibody conjugated with tetramethylrhodamine B isothiocyanate (Dako). Coverslips were washed and mounted by using Fluoroprep (bioMérieux, Marcy-l’Etoile, France). Fluorescence was observed by using a Leica DMR microscope (Leica Microsystems AG, Heerbrugg, Switzerland) equipped with Leica Qfluoro software, with a ×60 objective. GFP fluorescence was observed between 490 and 520 nm, whereas tetramethylrhodamine B isothiocyanate was observed between 555 and 580 nm.

Immunohistochemical Analyses

Three normal (i.e., noncystinotic) kidneys (which were unsuitable for transplantation because of vascular problems) obtained from donors of ages 4, 41, and 51 yr were used for immunolocalization of cystinosin. Kidneys from two 9-yr-old cystinotic patients were also examined; both individuals exhibited the most common mutation observed among patients with cystinosis, i.e., a homozygous 57-kb deletion removing most of the gene. These null mutants constituted negative control samples for cystinosin immunohistochemical analyses. In addition, we studied renal biopsies from two children (both 14 yr of age) with active, histologically proven, minimal-change nephrotic syndrome. Tissue specimens were immediately snap-frozen in liquid nitrogen with Cryo-M-Bed compound (Bright Instrument Co., Huntington, England) and were stored at −80°C until use. Immunostaining was performed with anti-cystinosin and anti-LAMP-2 antibodies diluted 1:100. H4B4, directed against human...
Figure 2. Immunocytochemical analyses. COS-7 cells were observed at wavelengths suitable for the detection of tetramethylrhodamine B isothiocyanate fluorescence (A, B, D, F, and G), GFP fluorescence (C and H), or both (E). (A and B) Transfected cells expressing wild-type human cystinosin. In the cystinosin-immunoreactive cells (A), the labeled subcellular vesicles (arrows) adjacent to the nuclei (asterisks) should be noted. No specific immunostaining was detected with the preimmune antiserum in the same lot of transfected cells (B). (C to F) Transfected cells expressing
LAMP-2, was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Labeling was performed on 3-μm cryostat sections that had been fixed in acetone for 10 min, and labeling was detected by immunoperoxidase staining with amino-9-ethylcarbazole, using a Universal immunostaining kit (Beckman Coulter, Brea, CA). After washing with fresh Tris-buffered saline (TBS) (0.15 M, pH 7.4), endogenous biotin was blocked with the biotin-blocking agent, according to the instructions provided by the manufacturer (Beckman Coulter). Endogenous peroxidase was quenched by treatment of the sections with 3% hydrogen peroxide in methanol for 5 min and then washing in TBS for 20 min. The sections were incubated for 1 h at room temperature, in a moist chamber, with the primary antibodies diluted in TBS. After being washed with TBS, the sections were incubated with the biotinylated secondary antibody for 30 min. The sections were then incubated for 45 min with streptavidin-peroxidase reagent. After washing, the final staining of the sections with amino-9-ethylcarbazole was monitored with the microscope. Sections were briefly counterstained with hematoxylin, washed with running water, and mounted with aqueous medium. Tissue sections with amino-9-ethylcarbazole was monitored with the microscope. Sections were briefly counterstained with hematoxylin, washed with running water, and mounted with aqueous medium. Tissue sections incubated with the rabbit preimmune serum or incubated directly with the secondary antibodies served as control samples. Labeling was examined by using a Leica DMR microscope (see above).

Results

Western Blotting

A Western blot representative of several experiments is depicted in Figure 1. In the lane containing cells transfected with pEGFP-N1, no bands were detected after incubation with the anti-cystinosin antisemur. The predicted molecular mass of human cystinosin is approximately 41 kD without glycosylation and up to 55 kD if all potential sites are glycosylated; in cells transfected with pcDNA-CTNS, several bands within this range were detected with the antisemur, with the most prominent at approximately 55 to 60 kD. In cells transfected with pCTNS-EGFP, the antisemur identified a larger broad band, probably corresponding to the variably glycosylated human cystinosin–GFP fusion protein, which is predicted to have a molecular mass of 67 to 81 kD. No signal was observed for cells transfected with pCTNS-ΔGYDQL-EGFP, in which the carboxy-terminal targeting motif had been deleted. Finally, no signal was detected when we substituted preimmune serum for the corresponding antisemur.

Immunocytochemical Analyses

Representative immunocytochemical results from several experiments with COS-7 monkey cells are depicted in Figure 2. The two antisera raised against the carboxy terminus identified subcellular structures in pcDNA-CTNS-transfected COS-7 cells expressing wild-type human cystinosin (Figure 2A). No significant signal was observed in nontransfected cells (data not shown); similarly, no immunostaining above background levels was detected with preimmune antisemur in cells transfected with pcDNA-CTNS (Figure 2B). In pCTNS-EGFP-transfected cells expressing wild-type cystinosin–GFP fusion protein, antisera identified apparently vesicular structures, with immunostaining colocalized with GFP fluorescence (Figure 2, C to E). It should be noted that Cherqui et al. (11) previously observed colocalization of GFP fluorescence for this fusion protein with LAMP-2 immunoactivity. Cells transfected with pCTNS-EGFP exhibited no significant immunostaining with preimmune serum (Figure 2F). As expected, cells transfected with pCTNS-ΔGYDQL-EGFP exhibited no significant immunostaining with the anti-cystinosin antisemur (Figure 2G); in these cells, GFP fluorescence was detected with partial relocation to the plasma membrane (Figure 2H), as described previously (11).

Immunohistochemical Analyses

Figure 3, a to d, depicts representative results for normal human kidneys. Strong immunostaining of proximal tubular cells was observed with the antibody to LAMP-2, a lysosomal membrane marker (Figure 3, a and c). The staining, which demonstrated a microgranular appearance (best seen at high power in Figure 3c), was diffusely distributed within the cell cytoplasm, sparing the brush border and the nucleus. Mild microgranular staining of the distal tubules could also be observed, whereas very few structures were detected within the glomerular cells. A similar distribution was observed with the anti-cystinosin antibodies in normal kidneys (Figure 3, b and d). Histologically, marked heterogeneity in the shape and size of the proximal tubules and striking irregularities in epithelial cells were observed in the cystinotic kidneys (Figure 3, e and f); these lesions were associated with foci of interstitial fibrosis and tubular atrophy. LAMP-2 immunostaining was preserved in the cystinotic kidneys (Figure 3e), whereas no significant immunostaining could be detected with the anti-cystinosin antisemur (Figure 3f). No specific signal was detected with either preimmune anti-cystinosin serum in normal (Figure 3g) or cystinotic (Figure 3h) kidneys, and no signal was detected when anti-LAMP-2 and anti-cystinosin primary antibodies were omitted (data not shown). Finally, patterns similar to those in normal kidneys were observed in the minimal-change nephrotic syndrome biopsies (data not shown).

Discussion

Cystinosis is a lysosomal membrane transport disorder. Cystinosin, the protein defective in cystinosis, has several features of a lysosomal membrane protein, in particular, a targeting motif in the third cytoplasmic loop (9). Immunocytochemical studies with transfected MDCK and HeLa cells expressing a cystinosin–GFP fusion protein demonstrated colocalization of GFP fluorescence with LAMP-2, a known lysosomal membrane protein (11). Deletion of the GYDQL motif led to partial relocation of the fusion protein to the plasma membrane (11). Relocalization was complete if a second, novel, targeting motif in the third cytoplasmic loop was also deleted (11).
Figure 3. Immunohistochemical analyses. Immunoperoxidase staining of normal (a to d and g) and cystinotic (e, f, and h) human kidneys was performed with the anti-lysosome-associated membrane protein 2 (LAMP-2) antibody (a, c, and e), anti-cystinosin antibodies (b, d, and f), or preimmune anti-cystinosin serum (g and h). (a to d) Strong labeling of the proximal tubules was noted with anti-LAMP-2 and anti-cystinosin antibodies in normal kidney samples. (e and f) LAMP-2 labeling of tubular cells was preserved in the distorted proximal tubular cells of a kidney from a cystinotic patient, whereas no significant immunohistochemical signal was detected with the anti-cystinosin antibody. (g and h) No staining was observed with the preimmune serum. Magnification: ×120 in a, b, f, and g; ×300 in c, d, and e; ×150 in h.
This observation was used to deliberately redirect cystinosin to the plasma membrane of transfected COS-7 cells, to allow direct study of its function (12). COS-7 cells expressing cystinosin-ΔGYDQL selectively transported L-cystine across the plasma membrane into the cell at acidic pH (12). Transport was greatly reduced if the targeting motif was restored, and transport was inhibited if the transmembrane pH gradient was disrupted or if the cells were incubated in medium of neutral pH (12). These findings demonstrated that cystinosin is a novel, H+-driven transporter that is responsible for cystine export from lysosomes.

To date, no antibodies specific for cystinosin have been successfully generated. We therefore produced several oligopeptides of human cystinosin and used them to immunize rabbits. Of the resultant sera, two immune sera raised against the carboxy terminus detected cystinosin, in both Western blotting and immunocytochemical experiments, in transfected COS-7 monkey cells expressing wild-type human cystinosin or cystinosin-GFP fusion protein. In Western blotting assays, these antisera detected multiple bands (characteristic of a glycosylated protein) at the predicted molecular masses for both the wild-type and fusion proteins. The band pattern observed for cystinosin-GFP was consistent with that reported previously (11,12). Additional bands, with lower molecular masses, were detected for wild-type cystinosin; these may represent protein degradation products. Control immunoblotting experiments with transfected cells expressing GFP alone or human cystinosin with deletion of the GYDQL motif yielded negative results; furthermore, all experiments with preimmune sera yielded negative results. In normal human kidneys, prominent labeling of proximal tubular cells was observed with the antisera. Findings were similar to the labeling observed for LAMP-2, which is a specific lysosomal marker. This is particularly interesting because proximal tubulopathy is the first clinical sign of nephropathic cystinosis. No staining was observed for cystinotic patients with homozygous deletions of the CTNS gene, who continue to express LAMP-2 in their preserved tubules. Collectively, these data demonstrate the cystinosin specificity of our antisera. Moreover, the intracellular structures stained in the immunocytochemical experiments were vesicular, and staining patterns were the same in transfected cells expressing cystinosin or cystinosin-GFP. The previously reported colocalization of GFP fluorescence and LAMP-2 immunostaining in transfected cells expressing cystinosin-GFP demonstrated that these vesicular structures are lysosomes (11). Our observations thus confirm the previously reported lysosomal localization of cystinosin. In addition, our anti-cystinosin antisera will be of great benefit in investigations of the cell biologic features of cystinosis.

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References


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