The Targeting of Cystinosin to the Lysosomal Membrane Requires a Tyrosine-based Signal and a Novel Sorting Motif*

Cystinosin is a lysosomal transport disorder characterized by an accumulation of intra-lysosomal cystine. Biochemical studies showed that the lysosomal cystine transporter was distinct from the plasma membrane cystine transporters and that it exclusively transported cystine. The gene underlying cystinosis, CTNS, encodes a predicted seven-transmembrane domain protein called cystinosin, which is highly glycosylated at the N-terminal end and carries a GX-XX-F (where F is a hydrophobic residue) lysosomal-targeting motif in its carboxyl tail. We constructed cystinosin-green fluorescent protein fusion proteins to determine the subcellular localization of cystinosin in transfected cell lines and showed that cystinosin-green fluorescent protein colocalizes with lysosomal-associated membrane protein 2 (LAMP-2) to lysosomes. Deletion of the GX-XX-F motif resulted in a partial redirection to the plasma membrane as well as sorting to lysosomes, demonstrating that this motif is only partially responsible for the lysosomal targeting of cystinosin and suggesting the existence of a second sorting signal. A complete relocalization of cystinosin to the plasma membrane was obtained after deletion of half of the third cytoplasmic loop (amino acids 280–288) coupled with the deletion of the GX-DQ-L motif, demonstrating the presence of the second signal within this loop. Using site-directed mutagenesis studies we identified a novel conformational lysosomal-sorting motif, the core of which was delineated to YF-PQA (amino acids 281–285).

Lyosomes are a principal site of intracellular digestion in mammalian cells. Extracellular biological materials destined for degradation in the lysosomes are endocytosed and shuttled to the lysosomes via early and late endosomes (1). In contrast, endogenous cellular structures which are to be degraded in the lysosome are either selectively (macro- or micro-) transported independently of the endosomes via a process called autophagy (2, 3). The lysosomal membrane, due to the presence of a H^+ pump, is responsible for acidification of the interior and for sequestration of the lysosomal enzymes responsible for the degradation process (4). Furthermore, the lysosomal membrane contains transport proteins that mediate the transport of degradation products such as amino acids, sugars, and nucleotides from the lysosomal lumen to the cytosol, where they are excreted or reused by the cell. A defect in either the degradation or transport process can result in an accumulation of the undegraded substrate or the degradation product within the lysosomes, impairing the physiology of the cell and leading to a lysosomal storage disorder.

Several such disorders have been described and are usually classified according to the molecule that has accumulated intra-lysosomally (5). Most of these disorders are due to a defect in one of the lysosomal hydrolases; however, a subset exists that arises from a defect in one of the membrane transporters (6). One such example is the autosomal recessive disorder cystinosis (MIM 21980), which is characterized by an accumulation of intra-lysosomal cystine (7). Various biochemical studies over the years show that cystine transport across the lysosomal membrane is carrier-mediated, that the carrier is located in the membrane itself, and that it is distinct from the plasma membrane cystine transporters (8), which in contrast do not exclusively transport cystine (9, 10). Moreover, the lysosomal cystine transporter is stimulated by the acid pH of the lysosome, which is ATP-dependent (11). Cystine is the disulfide of the amino acid cysteine, and cysteine has been found to exit the lysosome freely in cystinotic cells, implying that the cysteine transporter is independent of the cystine transporter (12). This finding has been exploited for the treatment of cystinosis as the currently used drug, cysteamine, is an aminothiol that reacts with cysteine to form cysteine and a cysteine-cysteamine mixed disulfide that can also readily exit the cystinotic lysosome (13). Thus, cysteamine, if used early and in high doses, delays the progression of cystinosis in affected individuals by reducing intra-lysosomal cystine levels (14).

We recently identified the gene underlying cystinosis, CTNS, (15) which is composed of 12 exons and extends over 23 kilobases of genomic sequence. The identification of mutations in the CTNS-coding sequence of affected individuals, the most common of which is a 57-kilobase deletion (15, 16), confirmed this gene as underlying cystinosis. Three allelic clinical forms have been defined based on the severity of symptoms and age of onset (11); the infantile form is the most severe and is characterized initially by the appearance of a proximal renal tubule dysfunction at 6–8 months, leading to end-stage renal failure by 10 years of age, and, eventually, by damage to most tissues due to widespread cystine accumulation. The juvenile form is less severe and characterized by a glomerular renal impairment appearing at a later age. Finally, the ocular nonnephropathic form is characterized by the presence of corneal cystine-crystal deposits without renal anomalies.

The 2.6-kilobase CTNS transcript encodes a novel 367 amino acid protein named cystinosin, which shows homology with a 55.5-kDa Caenorhabditis elegans protein (C41C4.7) and a yeast transmembrane protein, ER51 (15). Computer-aided sequence

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analysis of cystinosin predicts that it is composed of seven transmembrane domains, suggesting that it is also a membrane protein. These domains are preceded by seven potential glycosylation sites at the N-terminal end, characteristic of lysosomal membrane proteins that are highly glycosylated to help protect them from the lysosomal proteases in the lumen (17). Finally, following the seventh transmembrane domain is a 10-residue C-terminal tail. Within this tail lies a 5-residue sequence, GY-DQ-L. This sequence is reminiscent of the tyrosine-based sorting motif, GY-XX-Φ (where Φ is a hydrophobic residue), which was identified in the 10–20 amino acid carboxy-tails of lysosomal-associated membrane protein (LAMP)1 -1, -2, and -3 and in lysosomal acid phosphatase and shown to be responsible for their correct targeting to the lysosome (for review see Ref. 18). Taken together these results suggest that cystinosin is a lysosomal membrane protein. We constructed cystinosin-green fluorescent protein (GFP) fusion proteins and cystinosin-c-Myc epitope-tagged proteins to study the subcellular localization of cystinosin in vitro by transient and stable transfections of various cell lines. This study allowed the determination of the subcellular localization of cystinosin and resulted in the identification of a novel motif required for lysosomal sorting.

EXPERIMENTAL PROCEDURES

**Protein Fusion Constructs**—To create a fusion construct with the CTNS sequence at the 5’ end of and in-frame with the GFP sequence, the CTNS cDNA sequence was polymerase chain reaction-amplified (Advantage cDNA polymerase mix, CLONTECH) using a forward primer situated in the 5’-non-coding region (5’-AGT CTA GCC GGG CAG GGG AA-3’) and a reverse primer (5’-GGG GAT CCT GAG CTC TGC CTC TTC C-3’) restriction enzyme-digested, and subcloned into the expression vector GG-10 phage clone (Invitrogen). Site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit according to the manufacturer’s recommendations and verified by sequencing the region containing the introduced mutation. Primers used are not listed here). The amplified product was digested by Xhol (situated 250 base pairs upstream of the ATG initiator codon) and KpnI and inserted into the Xhol/KpnI-digested pEGFP-N1 (CLONTECH) (constructing the pcTNS-EGFP). Clones containing the CTNS insert were checked by sequencing (Applied Biosystems 373XL). To generate a fusion construct containing the CTNS sequence at the 3’ end of the GFP sequence, the CTNS-coding sequence was polymerase chain reaction-amplified using a forward primer (5’-GGG CTC GTC GGG GAG CTA GCT CTA GTC TCT TAC GTA CTA T-3’) and a reverse primer situated in the 5’-end of the coding region, which deleted the stop codon and added a KpnI restriction site. The amplified product was digested by Xhol (situated 250 base pairs downstream of the stop codon) and inserted into the Xhol/KpnI-digested pEGFP-N1 (CLONTECH) (constructing the pCTNS-EGFP). Clones containing the CTNS insert were checked by sequencing (Applied Biosystems 373XL). To generate a fusion construct containing the CTNS sequence at the 3’ end of the GFP sequence, the CTNS-coding sequence was polymerase chain reaction-amplified using a forward primer (5’-GGG CTC GTC GGG GAG CTA GCT CTA GTC TCT TAC GTA CTA T-3’) and a reverse primer situated in the 5’-end of the coding region, which deleted the stop codon and added a KpnI restriction site. The amplified product was digested by Xhol (situated 250 base pairs downstream of the stop codon) and inserted into the Xhol/KpnI-digested pEGFP-N1 (CLONTECH) (constructing the pCTNS-EGFP). Clones containing the CTNS insert were checked by sequencing (Applied Biosystems 373XL).

**Mutagenesis of the pCTNS-EGFP Construct**—All the modifications of pCTNS-EGFP listed under “RESULTS” were carried out using the Strategene QuickChange Site-Directed Mutagenesis Kit according to the manufacturer’s recommendations and verified by sequencing the region surrounding the introduced mutation. Primers used are not listed here but are available upon request.

**Cell Culture and Transfection**—MDCK and HeLa cells were grown in minimal essential medium (MEM; Life Technologies, Inc.) supplemented with 10% fetal calf serum (Vabliotek), 100 units/ml penicillin/streptomycin (Life Technologies, Inc.), and 2 mM l-glutamine (Life Technologies, Inc.). Confluent cells were passaged the day before transient transfection, and 2 × 105 cells were distributed into 35-mm wells containing 3 sterile glass coverslips. Once cells reached 60% confluency, the supplemented MEM media was replaced by 1 ml of OPTI MEM (Life Technologies, Inc.), and cells were incubated at 37 °C for 24 h. Transfected cells were washed with phosphate-buffered saline (PBS), overlaid with supplemented MEM, and left to incubate for 48 h. For the stable transfections, cells were distributed into 35-mm wells without coverslips and treated as above. After the 48-h incubation, the culture medium was supplemented with 2 mg/ml G418 (Invitrogen).

**Immunofluorescence**—Transfected cells were washed with PBS and fixed with 2% paraformaldehyde (Merck) for 20 min at room temperature. After washing with PBS, cells were blocked with 10% normal horse serum (NH;CL for 10 min, washed, and permeabilized using 0.5% Triton X-100 (Sigma) for 10 min. Coverslips were then either RNase-treated for 10 min and the cell nuclei stained with 0.5 μg/ml propidium iodide (Sigma) for 3 min or incubated with the first antibody for 1 h, washed in PBS, and incubated with the second antibody for 1 h. Finally, coverslips were washed in PBS, incubated with 100 ng/ml diazabicyclo[2.2.2]octane (Sigma) for 10 min to maintain the fluorescence, and mounted using Glycergel (DAKO). Stained coverslips were visualized using a Zeiss Axiosvert 100 microscope equipped with LSM 510 software and Ar/Kr (458 and 488 nm) and 2 × HeNe (543 and 633 nm) lasers with a 100 × oil immersion objective. All images correspond to a 1-μm cell section. Photographs were processed with Adobe Photoshop 5.5. For the transferrin internalization studies, transiently transfected HeLa cells were washed three times in PBS and incubated for 30 min at 37 °C with 4 μg/ml Texas Red-conjugated human transferrin (Molecular Probes) in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 2 mg/ml HEPES (pH 7.4) and 2 mg/ml bovine serum albumin. Cells were washed three times with PBS and fixed, mounted, and visualized as above.

**Antibodies**—The mouse monoclonal antibodies used were as follows: AC17, directed against canine LAMP-2 (19), was kindly provided by A. L. Mevelcq; gt10, directed against canine LAMP-2 (19), was kindly provided by A. L. Mevelcq; and 9E10, directed against the c-Myc epitope, was obtained from Santa Cruz Biotechnology and used at a dilution of 1:100; anti-GFP was purchased from Roche Molecular Biochemicals and used at a dilution of 1:250; 9E10, directed against the c-Myc epitope, was obtained from Santa Cruz Biotechnology and used at a dilution of 1:100; and EMA, directed against epithelial membrane antigen (EMA), was obtained from DAKO and used at a dilution of 1:500. Secondary donkey anti-mouse antibodies for the immunofluorescence studies conjugated to either fluorescein isothiocyanate or tetramethylrhodamine B isothiocyanate were purchased from Jackson Laboratories and used at a dilution of 1:200.

**Western Blot Analysis**—Western blots were prepared from whole cell lysates. 293 cells, non-transfected or transiently transfected with the pcTNS-EGFP, were lysed in PBS, centrifuged for 5 min at 2500 rpm at 4 °C, and resuspended in lysis buffer (150 mM NaCl, 1% Nonidet P-40, 100 mM Tris HCl (pH 8), 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cells were lysed for 20 min on ice and centrifuged for 2 min at 12,000 rpm at 4 °C. Cell lysates were mixed with Laemmli’s sample buffer and loaded directly without boiling onto an 8% SDS-polyacrylamide electrophoresis gel. The separated proteins were electro-transferred to nitrocellulose filters (Bio-Rad) and, after blocking for 1 h in 0.05% Tween/PBS + 1% polyvinylpyrrolidone (Sigma), the membrane was incubated for 1 h at room temperature with a 1/1000 dilution of the anti-GFP antibody. After three washes in 0.05% Tween/PBS, the filter was incubated for 1 h at room temperature with 1/12000 horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Pharmacia Biotech). The final detection was performed using the Amersham Pharmacia Biotech ECL reagents according to the manufacturer’s recommendations.

**RESULTS**

**Subcellular Localization of Wild-type Cystinosin**—The CTNS-coding region was cloned 5’ to the GFP cDNA in the plasmid pEGFP-N1, generating pcTNS-EGFP. In this orientation, the GFP end of the fusion protein was extra-lysosomal (Fig. 1). pcTNS-EGFP was transiently transfected into MDCK and HeLa cells, and the GFP fluorescence signal was observed 48 h post-transfection. In both cell types, cystinosin-GFP was...
Lysosomal-targeting Signals of Cystinosin

To verify that the fusion of the GFP to the C terminus of cystinosin did not interfere with its native localization, two additional constructs were made. First, the CTNS-coding region was subcloned downstream of the GFP gene in the plasmid pEGFP-C1 (pEGFP-CTNS) such that the GFP of the fusion protein would be predicted to lie intra-lysosomal. However, no fluorescence could be observed upon transfection of this construct, cells were fixed, permeabilized, and incubated with propidium iodide. The fluorescent signal from the cystinosin-GFP fusion protein can be seen to be localized to small discrete intracellular vesicles and to large vesicular structures.

Deletion and Mutagenesis of the Lysosomal-targeting Signal—Previous studies on other lysosomal membrane glycoproteins have delineated one of the lysosomal-targeting signals as the glycosylated form of cystinosin and a smaller band with an apparent size of 70 kDa could also be seen that could presumably correspond to the unglycosylated form of the protein. Alternatively, this band could arise from proteolytic cleavage of cystinosin, although no known cleavage sites were detected. These same bands were not detected in nontransfected 293 cells.

To determine whether the labeled vesicles were indeed lysosomes, transfected cells were immunofluorescently labeled using antibodies directed against the late endosomal and lysosomal membrane protein LAMP-2. As seen in Fig. 4, in both MDCK (Fig. 4, A–C) and HeLa (Fig. 4, D–F) cells, the cystinosin-GFP signal colocalized with that of AC17, directed against canine LAMP-2, and H4B4, directed against human LAMP-2, respectively. The exact overlap between the LAMP-2 and cystinosin localization patterns demonstrates that cystinosin is a lysosomal protein. Interestingly, the above-mentioned large vesicular structures were also labeled by antibodies directed to LAMP-2 in cystinosin-overexpressing cells (Fig. 4, A and B), indicating that these large vesicles are also lysosomal structures; immunostaining of nontransfected cells with the same antibodies did not reveal the presence of these structures (data not shown). Finally, colocalization studies using fluorescently labeled transferrin uptake ruled out the possibility that cystinosin also localizes to the early endosomes (Fig. 4, G–I).

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found at the plasma membrane that colocalized with that of the epithelial membrane antigen antibody, EMA (Fig. 5, D–F). These results confirm that the GY-DQ-L motif is involved in targeting cystinosin to the lysosomes.

By site-directed mutagenesis, each codon of the lysosomal-targeting sequence was individually mutated to an A residue to determine the critical residues of this sequence. Substitution of the Gly, Asp, or Gln residues for an Ala residue did not alter the lysosomal localization of cystinosin (Fig. 6, A, C, and D). In contrast, mutation of the Tyr or Leu residues resulted in the same localization profile as that obtained when the entire lysosomal-targeting sequence was deleted (Fig. 6, B and E). The Asn residue after the lysosomal-targeting motif was also mutated to Ala to ensure that this amino acid was not part of the targeting sequence. This substitution resulted in the same localization pattern as that seen with the wild type (Fig. 6F).

Taken together these results demonstrate that it is the Tyr and Leu residues of the tyrosine-based sorting motif that are critical for the targeting of cystinosin to the lysosomes.

**Existence of a Second Lysosomal-targeting Signal—**Our mutational analysis of the GY-DQ-L motif demonstrated that it is only partially responsible for the targeting of cystinosin. These data strongly suggested the presence of a second lysosomal-targeting signal. In the first instance, to determine whether the remainder of the cytoplasmic tail, which is not included in the defined targeting signal, may also play a role in lysosomal targeting, the entire cytoplasmic tail was deleted (amino acids 358–367) from the pCTNS-EGFP construct. This resulted in the same subcellular localization of cystinosin-GFP as obtained after deletion of the lysosomal-targeting signal (data not shown).

A sequence analysis of the other three cytoplasmic domains was then performed to identify a possible motif resembling one of the documented lysosomal-targeting signals. The third cytoplasmic loop, located between the fifth and sixth potential transmembrane domains, is tyrosine-rich and, hence, seemed to be a good candidate for containing a lysosomal-targeting signal. In the first instance, we deleted the first nine amino acids (amino acids 280–288) of this 19-residue cytoplasmic domain (∆A) from the construct pCTNS-EGFP and observed the same cystinosin-GFP localization pattern as when the GY-DQ-L motif was deleted alone, with the exception that no large labeled vesicles were seen (data not shown). Subsequently, the remaining 10 amino acids (amino acids 289–298) were deleted (∆B) from pCTNS-EGFP, resulting in a localization of cystinosin-GFP to the lysosomes but, again, not to the large lysosomal structures without an obvious signal at the plasma membrane (data not shown). Each of these deletions, ∆A and ∆B, was then included with ∆GY-DQ-L in pCTNS-EGFP. Transfection of the ∆A/∆GY-DQ-L construct resulted in the exclusive localization of cystinosin-GFP to the plasma membrane (Fig. 7A). In contrast, transfection of the ∆B/∆GY-DQ-L construct gave a similar pattern (Fig. 7B) to that obtained using ∆GY-DQ-L alone, with the exception of the large labeled vesicles. In MDCK and HeLa cells transfected with the construct containing ∆A/∆GY-DQ-L, there was no longer a colocalization between cystinosin-GFP and LAMP-2 (Fig. 8, A–C), whereas there was a colocalization between cystinosin-GFP and epithelial membrane antigen (Fig. 8, D–F). In contrast, in cells transfected with the construct containing ∆B/∆GY-DQ-L, the cystinosin-GFP signal colocalized with both that of AC17 and epithelial membrane antigen (EMA), demonstrating that this double deletion still directed the fusion protein to the lysosomes (data not shown). Taken together, these results suggest that the third cytoplas-
mic loop, in particular the first half, plays a role in the targeting of cystinosin to the lysosome.

As seen here and reported by others (22, 23) that a Tyr plays a critical role in lysosomal targeting, we individually replaced the two Tyr residues in the first half of the third cytoplasmic domain by an Ala residue using site-directed mutagenesis of pCTNS-EGFP. Each of these mutations coupled with ΔGY-DQ-L resulted in the same localization pattern (data not shown) as that observed with ΔGY-DQ-L alone (Fig. 2A), indicating that this loop does not play a role in lysosomal targeting via a tyrosine-based motif. Subsequently, blocks of four and five amino acids beginning at each of the aforementioned tyrosine residues were either deleted (designated Δa and Δb, respectively, see Fig. 1) or mutated (μa and μb, respectively) and

![Figure 5](image)

**FIG. 5.** Subcellular localization of cystinosin following deletion of the C-terminal GY-DQ-L signal. A, when MDCK cells were transiently transfected with the ΔGY-DQ-L construct, a signal from the cystinosin-GFP fusion protein could be seen at the plasma membrane as well as in small and abundant large intracellular vesicles (visualized in green). B, after incubation of these cells with AC17, a signal could be seen only in intracellular vesicles (visualized in red). C, a superimposition of A and B identifies the intracellular vesicles seen in A as the lysosomes. D, upon transient transfection of HeLa cells with the ΔGY-DQ-L construct, the same fluorescent pattern as that in panel A could be seen (visualized in green). E, after incubation of these cells with a mouse monoclonal antibody directed against EMA followed by a tetramethylrhodamine B isothiocyanate-conjugated donkey anti-mouse secondary antibody, a labeling of the plasma membrane can be seen (visualized in red). F, a superimposition of D and E confirms that the cystinosin-GFP fusion protein, upon deletion of the GY-DQ-L signal, is directed to the plasma membrane as well as to lysosomes.

![Figure 6](image)

**FIG. 6.** Subcellular localization of cystinosin after site-directed mutagenesis of the GY-DQ-L-targeting signal. By site-directed mutagenesis, each residue of the GY-DQ-L-sorting motif in the construct pCTNS-EGFP was sequentially changed to an alanine residue. MDCK cells transiently transfected with the construct containing a mutation of the Gly (A), Asp (C), or Gln (D) residue results in a GFP fluorescence pattern identical to that of the wild-type cystinosin-GFP fusion protein. Transient transfection with the construct containing a mutation of the Tyr (B) or Leu (E) residue results in a fluorescent signal in intracellular vesicles but also in a redirection of the mutated cystinosin-GFP fusion protein to the plasma membrane. Mutation of the Asn residue (F) following the targeting motif resulted in the same subcellular localization pattern as the wild-type cystinosin-GFP protein.
assayed with ΔGY-DQ-L. Transfection of the Δα/ΔGY-DQ-L construct resulted in the same plasma membrane localization of cystinosin-GFP as seen with the Δα/ΔGY-DQ-L construct (Fig. 9A), whereas transfection of the μα/ΔGY-DQ-L construct resulted in the appearance of a distinct labeling of the lysosomes with no large lysosomal structures seen (Fig. 9B). In contrast, transfection of both the Δβ/ΔGY-DQ-L and μβ/ΔGY-DQ-L constructs resulted in the same localization pattern as obtained with μα/ΔGY-DQ-L (data not shown). These results indicate that the beginning of the third cytoplasmic loop, from the Tyr to the Ala residue, is crucial for the targeting of cystinosin to lysosomes and that the rest of the loop also plays a minor role.

**DISCUSSION**

Cystinosin is a novel integral membrane protein that, when defective, is responsible for the lysosomal transport disorder cystinosis, characterized by an accumulation of intralysosomal cystine (15). The disease phenotype led to the prediction that cystinosin is a cystine transporter localized in the lysosomal membrane (8). The construction of a cystinosin-GFP fusion protein has now allowed us to confirm the predicted subcellular localization of cystinosin-GFP to the lysosomes. This is in contrast to the situation for LAMP-1, where it has been shown that mutagenesis of the Tyr or Φ residues from its GY-QT-I signal results in the exclusive detection of LAMP-1 on the cell surface and not in intracellular vesicles (21). These results imply that there may be a second sorting signal at play for cystinosin. Such a situation has been described for tyrosinase, which contains both a di-leucine motif as well as a tyrosine-based motif in its cytoplasmic tail, and mutagenesis studies show that it is the di-leucine motif that is necessary for the efficient sorting of this protein to late endosomes and lysosomes, whereas the Υ-XX-Φ motif seems to be part of a weak secondary sorting signal (24). Along this line, we deleted the entire cytoplasmic tail from the cystinosin-GFP fusion protein to determine whether, as is the situation for tyrosinase, a second targeting signal resided in the tail even though a di-leucine motif was not detected. However, transfection of this construct resulted in the same localization pattern as obtained when just the GY-DQ-L signal was deleted. Because a second signal was not detected in the cytoplasmic tail of cystinosin, we focused our attention on the three predicted cytoplasmic loops, which would be the most susceptible to an interaction with adaptor proteins involved in the sorting of proteins to their fated location. None of these loops contain a di-leucine motif, but the third cytoplasmic loop, composed of 19 amino acids, was tyrosine-rich. Deletion of the first nine amino acids of this loop (amino acids 280–288) coupled with the deletion of the defined carboxyl GY-DQ-L motif, resulted in the complete relocalization of cystinosin-GFP to the plasma membrane without a signal remaining in the lysosomes. This redirection was due to a novel sorting motif, the core of which was defined as YFPQA (amino acids 281–285) by mutagenesis studies.

This novel sorting motif does not resemble any of the lysosomal sorting motifs so far defined. In addition, serial deletions of the remaining third cytoplasmic loop, although they do not provoke as drastic an effect, do seem to indicate that this region also has a minor role in lysosomal targeting. This is reminiscent of the situation involving the epidermal growth factor receptor. Epidermal growth factor receptor is a transmembrane domain protein with three lysosomal sorting signals in its cytoplasmic tail: a tyrosine-based motif (YLVI), a tyrosine kinase domain, and an uncharacterized region situated between amino acids 1022 and 1123 (25–27). Within this third 1022–1123 amino acid-targeting domain of epidermal growth factor receptor, it has been shown that the region 1022–1063 appears to contribute more significantly to sorting than residues 1063–1123 (27). Moreover, it is noteworthy, that mutating the core of the second sorting motif in cystinosin to alanine residues does not have as a dramatic effect on the relocalization of cystinosin as compared with the complete deletion of this pentapeptide. Thus, the YFPQA region of cystinosin seems to act as a conformational motif that is recognized as part of a particular secondary structure formed by the rest of the third cytoplasmic loop.

Other proteins that have a nonclassical lysosomal-sorting signal have been described. For example, P-selectin does not contain a classic lysosomal-targeting signal but rather a novel sequence, KCPL, in its C-terminal tail that has been shown to be responsible for its lysosomal targeting (28). The one striking difference between cystinosin and other proteins targeted to the lysosome is that, for the latter, the lysosomal-targeting signal(s) has been found to be situated in the carboxyl tail, whereas the second putative lysosomal-targeting signal for cystinosin is localized in one of its cytoplasmic loops. This seems to be a unique situation, although a recent study has
shown that the GY-DQ-L motif is active in cystinosin, one of the pathways by which this protein is sorted to the lysosome must involve one or more of these three adaptor proteins. On the other hand, we do not know which pathway the second sorting motif would require an increase in activity of the second sorting motif and, thereby, in the number of large vesicles. Thus, it is tempting to speculate that this second sorting motif may act to bring cystinosin to the lysosome via the intermediate of these large structures but just small discrete lysosomes. Hence, the pathway used by this second and novel sorting motif may represent a previously uncharacterized pathway for the sorting of lysosomal proteins to their final destination.

Cystinosin has been shown to be homologous to a 55.5-kDa protein of \textit{C. elegans} (C41C4.7) as well as to a yeast transmembrane protein ERS1 (15). Surprisingly the C-terminal lysosomal-targeting signal of cystinosin, GY-DQ-L, is not found in either of these two other organisms, whereas the YFPQA motif is conserved in both \textit{C. elegans} (YFPQV) and yeast (YIPQV), suggesting an important role for this motif. Moreover, there has been a splice site mutation (IVS11−2T>C) reported as associated with the less severe juvenile cystinosis, which has been predicted to lead to a truncated protein missing exon 11. This splice site mutation alters the reading frame, producing a missense mutation after amino acid residue 284 and introducing a new termination codon at position 289 (34). Although the encoded product lacks the GY-DQ-L motif, a CTNS-GFP fusion construct carrying the IVS11−2T>C mutation transfects into \textit{C. elegans} cells results in a partial rescue of the cellular phenotype, implying the truncated cystinosin protein is still targeted to lysosomes (34). These observations are consistent with our results demonstrating the existence of a second lysosomal sorting signal for cystinosin. Infantile cystinosis is a multisystemic disease that can lead to death at 10 years of age due to the widespread accumulation of intralysosomal cystine,
highlighting the crucial role of cystinosin in the normal metabolic functions of the body. Due to this indispensable role of cystinosin in the lysosomes of all cells, it is thus not surprising to find that this protein has a rescue pathway by which it can be sorted to the lysosome. In conclusion, the identification of this novel lysosomal-targeting motif not only provides an insight into the way cystinosin functions but also represents an addition to the ever-growing list of targeting motifs required for the correct sorting of proteins to their fated cellular location.

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REFERENCES