BIOCHEMISTRY


The authors note that within Figure 6A, a structural formula of the mixed disulfide appeared incorrectly. The corrected Figure 6 and its legend appear below. This error does not affect the conclusions of the article.

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**Fig. 6.** PQLC2 exports a key chemical intermediate in cysteamine therapy of cystinosis. (A) Chemical structure of the MxD resembles that of lysine. (B) Current traces evoked by MxD and arginine (10 mM each) on a representative PQLC2-L/JAA-EGFP oocyte at −40 mV and pH 5.0. (C) Saturation kinetics of paired MxD and arginine responses (means ± SEMs of five oocytes from two batches). Kᵣ and Iₘₐₓ values are reported in the main text. I/S, current/substrate concentration ratio. (D) Kinetics of PQLC2 mRNA knockdown in human cystinotic fibroblasts after two rounds of siRNA transfection. Two PQLC2-targeted siRNAs are compared with a luciferase-targeted negative control. Means ± SEMs of four measurements are shown. (E and F) PQLC2 gene silencing decreases the clearance of lysine from an intracellular compartment. (E) Scheme depicts how lysosomes are preferentially loaded with amino acids in whole cells using a methyl ester precursor. After loading human fibroblasts with [³H]LysOMe, the fate of the resulting intracellular [³H]Lys pool was monitored by TLC. (F) Plots show representative chromatograms (Left) and representative [³H]Lys clearance kinetics (Right), respectively. PQLC2 gene silencing increases the intracellular [³H]Lys pool. (G) Effect of PQLC2 gene silencing on intracellular cystine and MxD levels. PQLC2 knockdown exacerbates cystine storage (Left) and dramatically increases the level of MxD induced by cysteamine (Right) in human cystinotic fibroblasts, as illustrated in this representative experiment (means ± SEMs of three measurements). luc, luciferase; no, untreated.
PLANT BIOLOGY

The authors note that in Fig. 3, all three panels in the first row (for CrCAS CrPGRL1) appeared incorrectly. The corrected figure and its corresponding legend appear below. This error does not affect the conclusions of the article.

![Fig. 3. C. reinhardtii CAS, ANR1, and PGRL1 interact with each other in vivo. BiFC interaction analysis of codon-optimized heterologously expressed C. reinhardtii proteins in N. benthamiana epidermal cells transiently expressing the plasmid combinations indicated at the left.](www.pnas.org/cgi/doi/10.1073/pnas.1300172110)

PSYCHOLOGICAL AND COGNITIVE SCIENCES, NEUROSCIENCE

The authors note the following: “Response times and key strokes were erroneously analyzed for 4 of the 13 control participants and 1 of 10 blind participants in experiment 2. Trials from condition 1 were coded as condition 2, and so on. This coding error resulted in inaccurate condition means for these individuals and inaccurate group averages. This error also obscured a group-by-condition interaction in the reaction time data. In the original article, the interaction is not significant. Once we corrected the coding error, the interaction became significant.”

Because of this change in the behavioral results, the authors note that on page 11313, right column, the second and third full paragraphs about experiment 2 should instead appear as “Experiment 2. There was no difference in reaction times for seeing and hearing stories in EB participants [t(9) = 1.45, P = 0.18]. Sighted participants were marginally faster on the seeing trials (t(12) = −2.11, P = 0.06). We performed a 2 × 2 ANOVA using condition (seeing/hearing) as a within-subjects factor and group (EB/sighted) as a between-subjects factor. No main effects reached significance (P > 0.25). However, there was a condition (seeing vs. hearing) by group (sighted vs. blind) interaction [F(1,21) = 6.13, P = 0.02].”

“We then looked for reaction time differences among belief, feeling, and control conditions among sighted and EB participants, as well as group-by-condition interactions. EB and sighted participants did not differ in reaction time (P > 0.30). There was a main effect of condition (belief/feeling/control) [F(2,42) = 7.16, P = 0.002] but no group-by-condition interaction (P > 0.30). In post hoc comparisons, participants were reliably faster to respond in the control condition than in the belief condition (Tukey’s honestly significant differences test, P < 0.05). No other differences were reliable (see Table S1 for reaction time data).”

These errors do not affect the conclusions of the article.

![Fig. 3.](www.pnas.org/cgi/doi/10.1073/pnas.1221828110)
Heptahelical protein PQLC2 is a lysosomal cationic amino acid exporter underlying the action of cysteamine in cystinosis therapy

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Cystinosin, the lysosomal cystine exporter defective in cystinosis, is the founding member of a family of heptahelical membrane proteins related to bacteriorhodopsin and characterized by a duplicated motif termed the PQ loop. PQ-loop proteins are more frequent in eukaryotes than in prokaryotes; except for cystinosin, their molecular function remains elusive. In this study, we report that three yeast PQ-loop proteins of unknown function, Ypq1, Ypq2, and Ypq3, localize to the vacuolar membrane and are involved in homeostasis of cystatic amino acids (CAAs). We also show that PQLC2, a mammalian PQ-loop protein closely related to yeast Ypq proteins, localizes to lysosomes and catalyzes a robust, electrogenic transport that is selective for CAAs and strongly activated at low extracellular pH. Heterologous expression of PQLC2 at the yeast vacuole rescues the resistance phenotype of an ypq2 mutant to caravamine, a toxic analog of arginine efficiently transported by PQLC2. Finally, PQLC2 transports a lysine-like mixed disulfide that serves as a chemical intermediate in cysteamine therapy of cystinosis, and PQLC2 gene silencing trapped expression of PQLC2 at the yeast vacuole rescues the resistance phenotype of a PQLC2 mutant to the poisoning by caravamine, a toxic analog of arginine efficiently transported by PQLC2. Two PQ loops are key functional elements that probably interact with each other, and they raised the possibility that other PQ-loop proteins may transport solutes across membranes.

In this study, we addressed this hypothesis and identified another PQ-loop amino acid transporter using yeast genetics and flux measurements in Xenopus oocytes. The function of this transporter in the lysosomal/vacuolar membrane of eukaryotic cells is conserved from yeast to mammals. Moreover, we show that the human transporter plays a key role in the treatment of cystinosis with the aminothiol drug cysteamine. Cystinosis is a rare autosomal recessive disease caused by loss-of-function mutations in the cystinosin gene, CTNS. As a consequence, large amounts of cystine accumulate in a patient’s lysosomes and progressively impair the function of multiple organs, including the kidneys, endocrine glands, muscles, and CNS (13, 19). Cysteamine depletes cystine from cystinotic lysosomes and, on lifelong treatment, alleviates symptoms. According to an early biochemical model (19, 20), cysteamine reacts with lysosomal cystine and forms a chemical intermediate that leaves lysosomes through a distinct, unaffected transporter. Our study now provides molecular evidence for this model.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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See Author Summary on page 20184 (volume 109, number 50).

1This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1211198109/-/DCSupplemental.

The transport of solute across membranes is crucial to eukaryotic cell physiology, as illustrated in the human species by the existence of diverse diseases associated with defective transport (1–3) and the presence of ~400 solute transporter genes grouped into 51 families in the human genome (www.bioparadigms.org/slc/menu.asp) (4, 5). However, this inventory is far from being complete, because the frequency of many putative transporters remains unknown and, for technical reasons, the repertoire of elucidated activities is biased in favor of cellular uptake at the expense of less tractable activities, such as cellular export or intracellular solute compartmentalization. For instance, most of the proteins responsible for the export of lysosomal catabolites remain unknown (3), and the lysosomal chloride transporter, CIC-7, was functionally characterized (6, 7) long after its identification. Even a key protein, such as the pyruvate transporter that fuels mitochondria and links glycolysis to the citric acid cycle, has long remained elusive (8, 9). A novel family of transporters that export sugars from plant and animal cells has also been only recently unveiled (10, 11).

In this study, we focus on a poorly characterized, mostly eukaryotic protein family defined by cystinosin, the lysosomal cystine transporter defective in human cystinosis (12, 13). This family [Pfam no. PF04193 (14); Transporter Classification Database no. 2.A.43.1.1 (15)], is characterized by a seven-helix membrane topology, a distant relationship with bacteriorhodopsin, and the presence of a duplicated motif termed the “PQ loop” (16, 17). Although the transport activity of cystinosin is well established (12, 18) and consistent with human pathological findings (19), the PQ-loop protein family is usually absent from transporter inventories because the molecular activity of other PQ-loop proteins remains unknown. In a recent study, we showed that cystinosin has a proton/cystine symport activity and we identified the cystine-coupled proton-binding site (D305) underlying this symport within the second PQ loop (18). Moreover, analysis of a large, diverse set of PQ-loop proteins revealed amino acid correlations between the two PQ-loop sequences. Taken together, these data suggested that PQ loops are key functional elements that probably interact with each other, and they raised the possibility that other PQ-loop proteins may transport solutes across membranes (18).

In this study, we addressed this hypothesis and identified another PQ-loop amino acid transporter using yeast genetics and flux measurements in Xenopus oocytes. The function of this transporter in the lysosomal/vacuolar membrane of eukaryotic cells is conserved from yeast to mammals. Moreover, we show that the human transporter plays a key role in the treatment of cystinosis with the aminothiol drug cysteamine. Cystinosis is a rare autosomal recessive disease caused by loss-of-function mutations in the cystinosin gene, CTNS. As a consequence, large amounts of cystine accumulate in a patient’s lysosomes and progressively impair the function of multiple organs, including the kidneys, endocrine glands, muscles, and CNS (13, 19). Cysteamine depletes cystine from cystinotic lysosomes and, on lifelong treatment, alleviates symptoms. According to an early biochemical model (19, 20), cysteamine reacts with lysosomal cystine and forms a chemical intermediate that leaves lysosomes through a distinct, unaffected transporter. Our study now provides molecular evidence for this model.
Results

Yeast Ypq1–3 Proteins Are Vacuolar Membrane Proteins Associated with Homeostasis of Cationic Amino Acids. Six PQ-loop proteins have been inventoried in the yeast *Saccharomyces cerevisiae* (21). One of these proteins, Ers1, was reported to encode a functional homolog of human cystinosin (22), with which it shares 28.7% identity. The function of the five other PQ-loop proteins is unknown. At least two of them, Yol092p and Ydr352p (hereafter called Ypq1 and Ypq2), were reported in proteomic and genome-scale protein localization studies to be located at the membrane of the vacuole, the lysosome of yeast (23, 24). By colabeling with fluorescent FM4-64 dye, we observed that Ypq1-GFP and Ypq2-GFP fusion proteins are indeed located at the vacuolar membrane and found that the same is true for another member of the family, Ybr147p, hereafter called Ypq3 (Fig. 1A). These three PQ-loop proteins might thus transport compounds across the vacuolar membrane.

Interestingly, the YPQ3 gene has been predicted by a recent bioinformatic analysis of promoter signatures (25) to be under the control of the Lys14 transcription factor. Lys14 activates expression of the lysine-repressible *LYS* genes involved in lysine biogenesis (26), and its positive action is highly stimulated in cells lacking the Lys80/Mks1 regulatory protein (27) or when lysine biosynthetic enzymes encoded by the *LYS20* and *LYS21* genes are resistant to feedback inhibition (28). We monitored expression driven by the upstream control region of the YPQ3 gene and confirmed that it is under the positive control of Lys14, repressed by excess lysine, and derepressed in *lys80* and *lys20*/*lys21* mutant cells (Fig. 1B). This expression is similar to that of *LYS9*, a well-studied target gene...
of Lys14 (Fig. S1). The YPQ3 gene thus encodes a putative vacuolar membrane transporter repressed by excess lysine. Because lysine is stored at a high concentration in the yeast vacuole (29, 30), Ypq3 might export lysine to the cytosol, with its expression being inhibited when lysine is abundant in the cytosol (Fig. 1C).

Because Ypq1 and Ypq2 are closely similar in sequence to Ypq3 (21) (Fig. 1G), they might perform a similar transport function (i.e., catalyze export of other cationic amino acids (CAAs; arginine and/or histidine) that are also highly concentrated in the vacuole) (29, 30). We isolated ypq1Δ, ypq2Δ, and ypq3Δ mutant strains, as well as a triple ypq mutant, and tested their growth on various media containing toxic analogs of CAAs. These experiments revealed that the ypq2Δ mutant is resistant to canavanine (Fig. 1D); a natural analog of arginine that is misincorporated into proteins and is highly toxic to diverse species, including yeast (31, 32). The ypq1Δ mutant also displays resistance to canavanine, but to a lesser extent than the ypq2Δ strain (Fig. 1D). A previous study reported that uptake of the three proteinogenic CAAs into the yeast vacuole is mediated by the Vba1, Vba2, and Vba3 transporters from the major facilitator superfamily (33). In a triple vba mutant, the Ypq2-dependent canavanine resistance phenotype is abolished (Fig. 1E). A tentative interpretation of these observations is that Ypq2 and, to a lesser extent, Ypq1 export canavanine (and presumably other CAAs) from the vacuole. In the ypq2 mutant, canavanine would thus be sequestered in the vacuolar lumen, reducing its toxicity, provided that its accumulation in the vacuole via the Vba proteins is normal (Fig. 1F).

The canavanine resistance phenotypes of the ypq1 and ypq2 mutants, and the fact that the YPQ3 gene is repressed at the transcriptional level by excess lysine, thus demonstrated that Ypq1–3 proteins are involved in homeostasis of CAAs, present at high concentrations in the vacuole, presumably through a vacuolar export mechanism.

**Mammalian Homolog PQLC2 Is a Resident Lysosomal Membrane Protein.** Interestingly, mammalian genomes contain a gene, PQLC2, encoding a protein more closely related in sequence to yeast Ypq1–3 proteins than to cystinosin (Fig. 1G). Like cystinosin and Ypq1-3, PQLC2 is predicted to possess seven transmembrane

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**Fig. 2.** PQLC2 is a ubiquitous lysosomal membrane protein. (A) After purification from rat liver by isopycnic centrifugation on Nycodenz gradients, lysosomes and lysosome-depleted fractions were subjected to hydrophobic protein extraction, SDS/PAGE, and comparative semiquantitative proteomic analysis. (B) Relative protein abundance in the two subcellular fractions was assessed by calculating a lysosomal spectral index ranging from −1 (fully excluded) to +1 (fully included), based on normalized spectral counts and the number of positive replicates. The spectral index of PQLC2 is similar to those of lysosomal markers (LAMP1, LAMP2) and well above those of mitochondrial (SLC25A4), peroxisomal (PXMP2), cytoskeleton (tubulin α2), endoplasmic reticulum (SERCA), and plasma membrane (Na,K ATPase) markers. The dotted line represents the 5% significance threshold. (C) Putative membrane topology of PQLC2. PQ-loop motifs are highlighted in blue. The peptides identified by MS are shown in red, along with their spectral counts. (D) WT EGFP-tagged rat PQLC2 (green) was transiently expressed in HeLa cells and compared with LAMP1 immunostaining (red) by deconvolution fluorescence microscopy. EGFP-stained puncta overlap with LAMP1-positive lysosomes and late endosomes in the deconvoluted optical slice. (Lower) Enlarged views of the boxed areas are shown. Arrows indicate colocalization. (Scale bar: 10 μm.) (F) Mutation of a C-terminal dileucine-type sorting motif (underlined in red) prevents PQLC2 delivery to the lysosome. The epifluorescence images highlight the diffuse distribution of the LL290/291AA mutant on the plasma membrane, including microvilli. (Scale bar: 10 μm.) (F) PQLC2 mRNA was quantified in diverse mouse tissues by real-time RT-PCR. Expression levels are compared with the GAPDH transcript using the comparative Ct method. Means ± SEMs of six mice are shown. sm., small.
α-helices, with an ∼40-residue, N-glycosylated N terminus in the lysosomal lumen and a shorter, cytosolic C terminus. The two P-loop motifs cover the second and fifth transmembrane helices and their connecting cytosolic loops (Fig. 2C).

Using a semiquantitative MS analysis of proteins in highly enriched lysosomal membranes from rat liver cells (34), we found that PQLC2 is present at the lysosomal membrane (Fig. 2A). A comprehensive description of the proteins identified in these lysosomal membranes will be provided elsewhere. The mutual significance of the association of PQLC2 with lysosomes was assessed by calculating for each identified protein a spectral index ranging from −1 to +1 for proteins exclusively detected in lysosome-depleted and lysosome-enriched fractions, respectively. This index combines the relative peptide abundance in tandem MS (MS/MS) spectra (spectral counts) and the number of samples with detectable peptides to provide an estimate of protein abundance (35). Across three biological replicates, we detected three peptides matching the rat PQLC2 sequence. The spectral index value of PQLC2 (0.892) was high and similar to that of the late endosomal/lysosomal markers lysosome-associated membrane protein 1 (LAMP1; 0.755) and LAMP2 (0.748), but well above that of proteins from other organelles and the 5% confidence threshold (0.594) (Fig. 2B and C).

To confirm the subcellular localization, we tagged rat PQLC2 with EGFP at its C terminus and expressed the fusion protein in HeLa cells. Under fluorescence deconvolution microscopy, PQLC2-EGFP displayed a punctate distribution that extensively overlapped with LAMP1 (Fig. 2D), thus confirming the proteomic data. Resident membrane proteins are targeted to lysosomes by virtue of short cytosolic motifs that interact with adaptor protein complexes. These adaptors, in turn, interact with protein coats that ensure cargo selection and membrane fusion in the endocytic pathway (36). We thus scrutinized the PQLC2 sequence for potential sorting motifs and identified an evolutionarily conserved, dileucine-type consensus sequence (285-EREPLL-291) in the C terminus (Fig. 2C). Mutation of the critical leucine pair of this motif (LL290/291AA) dramatically reduced protein a spectral index (0.594) (Fig. 2B and C).

To assess this hypothesis because it allows replacing the poorly tractable lysosomal activity by a classic, whole-cell influx equivalent to lysosomal efflux. Several lysosomal transporters have been successfully characterized using this whole-cell approach (6, 12, 18, 37, 38). In preliminary experiments, we expressed PQLC2-LL/AA-EGFP in HEK-293 cells and examined their ability to take up [3H]arginine (l[3H]Arg) or [3H]-lysine (l[3H]Lys) from acidic extracellular medium (which is topologically equivalent to the lysosomal lumen in our assay). Interestingly, PQLC2-LL/AA-EGFP modestly increased the uptake of CAA relative to WT PQLC2 and mock-transfected cells (Fig. S2). However, the PQLC2-dependent PQLC2 Transports CAAs. The above yeast and mammalian data prompted us to examine whether PQLC2 is a CAA transporter. The LL/AA sorting mutant provided favorable conditions for testing this hypothesis because it allows replacing the poorly tractable lysosomal activity by a classic, whole-cell influx equivalent to lysosomal efflux. Several lysosomal transporters have been successfully characterized using this whole-cell approach (6, 12, 18, 37, 38).

Fig. 3. PQLC2 is a CAA transporter. cRNA-injected Xenopus oocytes were analyzed by epifluorescence microscopy (A) and radiotracer flux measurements (B–F). (A) Fluorescence is detected at the plasma membrane for the PQLC2-LL/AA-EGFP construct (arrows), but not for WT PQLC2-EGFP (Upper Right) or free EGFP (Lower Left). The focus was adjusted in the equatorial plane, and images were acquired under identical conditions. (Scale bar: 0.2 mm.) (B) and (C) Oocytes expressing PQLC2-LL/AA-EGFP, but not WT PQLC2-EGFP or free EGFP, accumulate l-arginine, l-lysine, and l-histidine (0.1 mM) at extracellular pH 5.0. Means ± SEMs from representative pools of five oocytes are shown. (C) Time course of arginine (1 mM) uptake. (D) Arginine (0.1 mM) uptake was measured at distinct pH values. PQLC2-LL/AA is activated in extracellular acidic medium, a condition mimicking the natural environment in the lysosome. (E) Saturation kinetics of l-arginine uptake at pH 5.0. (Right) Graph (Eadie–Hofstee plot) shows that arginine uptake follows Michaelis–Menten kinetics. In this experiment, K_C = 3.8 mM and V_max = 152 pmol/min per oocyte (r^2 = 0.901). Means ± SEMs of five to seven oocytes are shown. (F) Substrate selectivity. Inhibitors (10 mM) were added simultaneously to [3H]-Arg (40 nM) at pH 5.0. Proteinogenic amino acids are indicated by their three-letter code. Cit, citrulline; Orn, l-ornithine. Means ± SEMs of the number of oocytes indicated in parentheses are shown.
signal was low or undetected in some experiments, presumably because the strong endogenous uptake of CAAs into mammalian cells masked PQLC2 activity.

We thus chose *Xenopus laevis* oocytes as an alternative expression system owing to their low endogenous uptake of amino acids, including cationic ones. When cRNA-injected oocytes were observed under epifluorescence microscopy, PQLC2-LL/AA-EGFP displayed a robust fluorescence at the plasma membrane, whereas staining was intracellular with free EGFP or WT PQLC2-EGFP (Fig. 3A). On incubation in acidic medium (pH 5.0), PQLC2-LL/AA-EGFP oocytes, but not PQLC2-EGFP oocytes, accumulated $[^{3}H]$Arg, $[^{3}H]$Lys, and $[^{3}H]$histidine ($[^{3}H]$His) over the background levels (Fig. 3B), in agreement with our transporter hypothesis and the presence of PQLC2-LL/AA at the oocyte surface. Mean uptake values of 33.1 ± 3.4 (n = 12 oocyte batches), 19.9 ± 2.5 (n = 8 oocyte batches), and 10.1 ± 2.4 pmol per 20 min per oocyte (n = 6 oocyte batches), representing increases of 6.7 ± 1.8, 3.7 ± 0.6-, and 1.8 ± 0.6-fold over background, were obtained for 100 μM $[^{3}H]$Arg, $[^{3}H]$Lys, and $[^{3}H]$His, respectively.

$[^{3}H]$Arg uptake was time-dependent and remained linear for ~10 min (Fig. 3C). It was also strongly pH-dependent, with no detectable activity at an extracellular pH ≥7.0 (Fig. 3D and Fig. S3), in agreement with the lysosomal/late endosomal localization of the native protein. PQLC2 should thus be exclusively active in the endocytic pathway. Saturation kinetics studies showed that $[^{3}H]$Arg transport by PQLC2 follows Michaelis–Menten kinetics (Fig. 3E), with mean $K_{m}$ and $V_{max}$ values of 3.36 ± 0.26 mM and 112 ± 28 pmol/min per oocyte (three independent experiments).

To characterize the substrate selectivity of PQLC2, we applied unlabeled amino acids (10 mM) simultaneously with $[^{3}H]$Arg. Among proteinogenic amino acids, only the cationic ones inhibited $[^{3}H]$Arg transport, whereas other compounds had no effect (Fig. 3F). L-ornithine inhibited PQLC2 so efficiently as arginine and histidine, whereas L-citrulline had no effect, thus confirming the requirement for a positively charged side chain. Lysine was slightly less efficient than arginine and histidine. We concluded from the above data that PQLC2 is a pH gradient-driven transporter that displays marked selectivity for CAAs.

**Electrophysiological Characterization of PQLC2.** To characterize further the transport activity of PQLC2, we applied CAAs (10 mM) to voltage-clamped oocytes and recorded their currents at −40 mV and pH 5.0. Arginine, histidine, lysine, and ornithine, but not citrulline, evoked a robust inward current in PQLC2-LL/AA-EGFP oocytes (Fig. 4A and B). Non-CAAs had no effect on PQLC2-LL/AA-EGFP oocytes, nor had CAAs applied to water-injected oocytes. PQLC2 transport activity is thus electrogenic, as might be expected from the positive charge of its small-molecule substrates. Mean steady-state current values of −237 ± 13, −246 ± 43, −299 ± 44, and −151 ± 12 nA were obtained with 10 mM arginine (n = 43 oocytes), lysine (n = 15 oocytes), histidine (n = 19 oocytes), and ornithine (n = 11 oocytes). When responses were normalized for each oocyte to the arginine signal, the first three compounds yielded identical responses (Fig. 4B), suggesting that they are translocated with similar velocities. The PQLC2 evoked current was strongly activated in acidic media (Fig. 4C). In agreement with the radio-tracer flux data, application of increasing arginine concentrations showed that the steady-state evoked current follows Michaelis–Menten kinetics (Fig. 4D), with mean $K_{m}$ and maximal current intensity ($I_{max}$) values of 2.1 ± 0.2 mM and −212 ± 19 nA, respectively, at −40 mV and pH 5.0 (28 oocytes from four batches).

Early biochemical studies on isolated lysosomes reported that the lysosomal transport pathway for CAAs (“system c”) is sensitive to analogs, such as Nα-methyl-l-arginine (NMe-Arg) and ε-N-trimethyl-l-lysine (3Me-Lys), which are not, or are poorly, accepted by the plasma membrane pathway (“system y”) (39). We thus tested whether these compounds (10 mM) interact with PQLC2. For comparison, the system y+ transporter CAT-1 was expressed in oocytes and assayed for $[^{3}H]$Arg transport under the same conditions (pH 5.0). Interestingly, whereas L-arginine preferentially inhibited CAT-1 relative to PQLC2-LL/AA, NMe-Arg and 3Me-Lys interacted more efficiently with the lysosomal transporter (Fig. S4A). Both analogs also evoked an inward current in PQLC2-LL/AA-EGFP oocytes, albeit to a lesser extent than L-arginine (Fig. S4 B and C). We concluded that the functional properties of PQLC2 resemble those reported for the native lysosomal transporter and that the methylated analogs are substrates, rather than inhibitors, of PQLC2.

**Vacuolar Export of Canavanine Accounts for the Yeast Drug-Sensitization Phenotype.** Expression of Ypq4 proteins in oocytes yielded poor or undetectable levels, thus preventing their functional characterization. To assess whether the transport function of PQLC2 is conserved between yeast and mammals, we expressed the mammalian protein in yeast and examined whether it functionally complements the ypq4 mutant. Interestingly, rat PQLC2-EGFP

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**Fig. 4.** Electrophysiological characterization of PQLC2. PQLC2-LL/AA-EGFP oocytes and water-injected oocytes were recorded under two-electrode voltage clamp at −40 mV and perfused with $\omega$-amino acids at pH 5.0, unless otherwise stated. (A) Raw traces from two representative oocytes. In the PQLC2-LL/AA oocyte, CAAs (10 mM), but not isoleucine, evoked an inward current that was absent from the noninjected oocyte. Orn, $\omega$-ornithine. (B) Mean steady-state currents ± SEM evoked by various amino acids (10 mM). The number of oocytes analyzed is shown above the bars. Values were normalized for each oocyte to the corresponding L-arginine signal. (C) Extracellular pH dependence of the arginine-evoked current. Means ± SEMs of 9–12 oocytes from three experiments are shown. Where not visible, error bars are smaller than symbols. (D) Saturation kinetics of the arginine response. The steady-state current mediated by PQLC2 follows Michaelis–Menten kinetics. In this experiment, $K_{m}$ = 2.49 mM, $I_{max}$ = −110 nA, and $R^2$ = 0.994. Means ± SEMs of 7 oocytes from the same batch are shown.
similarly able to export canavanine (and presumably other CAAs) from the yeast vacuole. Conversely, the lack of the canavanine-sensitivity phenotype in ypq3 yeasts suggests that Ypq3 does not transport this analog, possibly because evolutionary pressures have narrowed its substrate selectivity toward lysine.

**Role of PQLC2 in Cysteamine Therapy of Cystinosis.** Cysteamine therapy remains the most effective treatment for cystinosis (40–42). The current model, based on early biochemical data (20, 43), posits that the compound enters the lysosome and condenses with lysosomal cystine, thus generating a cysteamine-cysteine mixed disulfide (MxD) that resembles lysine (Fig. 6A). MxD is then exported from the lysosome through the system C CAA pathway (20). The identification of PQLC2 as a lysosomal CAA transporter thus prompted us to examine its potential role in this cystine-depleting mechanism.

MxD (10 mM) efficiently inhibited [3H]Arg transport by PQLC2 (Fig. 3F). It also evoked a robust inward current in voltage-clamped PQLC2-LL/AA-EGFP oocytes (Fig. 6B), showing that it is translocated by the lysosomal transporter. Paired application of increasing MxD and arginine concentrations at −40 mV and pH 5.0 showed that MxD is transported as rapidly as arginine with an affinity only twofold lower (Fig. 6C). Mean I_{max} values of −246 ± 24 nA and mean K_{m values} of 0.7 ± 0.7 mM and 3.4 ± 0.3 mM were obtained for MxD and arginine, respectively (five oocytes from two batches). We concluded that MxD is an efficient substrate of PQLC2.

We thus performed gene silencing on human cystinotic fibroblasts to test the role of PQLC2 in cysteamine therapy. Application of two different PQLC2 siRNAs (ON-TARGETplus (Dharmacon) reagent no. J-020760-18 or no. J-020760-19 [hereafter named no. 18 and no. 19]) efficiently and durably reduced the PQLC2 mRNA level in human cystinotic fibroblasts (Fig. 6D). After two rounds of transfection, siRNAs no. 18 and no. 19 decreased the PQLC2 mRNA level, on average, to 39 ± 8% and 18 ± 3% of the untreated cell level, respectively, whereas a control (luciferase-targeted) siRNA had no effect (112 ± 8%, eight independent transfections). Due to the lack of good antibodies, we used an in situ functional assay based on lysine methyl ester to assess the impact of gene silencing at the protein level. When amino acid methyl esters are applied to intact cells, a significant proportion is converted to amino acid within the lysosome due to the high esterase activity of this organellar relative to other cell compartments (44) (Fig. 6E). L-[3H]lysine methyl ester ([3H]LysOMe) applied to human fibroblasts was almost fully converted to lysine (Fig. S5), and this LysOMe-labeled [3H]Lys pool increased twofold when fibroblasts were transfected with siRNA no. 18 or no. 19, but not with the control siRNA (Fig. 6F). We concluded that gene silencing significantly decreases endogenous PQLC2 activity and, consequently, increases retention of [3H]Lys in the protected lysosomal environment.

Finally, we transfected normal and cystinotic human fibroblasts with the siRNAs and tested their response to cysteamine. After gene silencing, cells were treated or not treated with cysteamine and cellular levels of cystine and MxD were measured by liquid chromatography (LC)-MS/MS. PQLC2 gene silencing specifically and dramatically increased the level of MxD in cystinotic fibroblasts (Fig. 6G), with mean ratios of 15 ± 6-fold and 7.6 ± 2.1-fold relative to untreated cells for siRNA no. 18 and no. 19, respectively (three independent experiments). Only part (~10%) of the initial cystine was “trapped” as MxD by the combined siRNA and cysteamine treatments (compare plots in Fig. 6G), in agreement with the presence of residual PQLC2 activity after gene silencing (Fig. 6F, Right). PQLC2-targeted siRNAs, but not a control siRNA, also exacerbated cystine storage in patient cells (Fig. 6G) for an unknown reason. However, this increase in cystine was limited (2.06 ± 0.16-fold and 2.12 ± 0.11-fold relative to untreated cells for siRNA no. 18 and no. 19, respectively; n = 3), and thus could not account for the increase in MxD after the cysteamine

Fig. 5. Defective drug export from the vacuole may account for the yeast canavanine-sensitivity phenotype. (A) PQLC2 localizes to the vacuolar membrane of yeast cells. The 23344c (ura3) strain transformed with a URA3 plasmid expressing the rPQLC2-GFP fusion gene under a galactose-inducible promoter was grown on galactose (3%)/proline (10 mM) medium. Glucose (3%) was added to the cell culture for 2 h before staining with the vacuolar membrane marker FM4-64 and fluorescent microscopy analysis. (Scale bar: 5 μm.) (B) PQLC2 complements the growth phenotype of the ypq2Δ mutant. Strains 23344c (ura3) and EL031 (ypq2Δ ura3) transformed with URA3 plasmids expressing or not expressing the Ypq2-GFP and rPQLC2 fusion genes under a galactose-inducible promoter were spread on a minimal glucose/ammonium medium with or without L-canavanine (Can) and grown for 6 d at 29 °C. (C–E) PQLC2 transports canavanine. Raw current traces evoked by arginine or canavanine and the resulting Eadie–Hofstee plots are shown for a single representative oocyte in C and D, respectively. IS: current substrate concentration ratio. (E) Distribution of K_{m} and I_{max} values determined from paired applications of the two compounds to eight oocytes from two batches is shown. Canavanine shows higher K_{m} (P < 10^{-4}, paired Student’s t test) and I_{max} (P < 10^{-4}) values than arginine. Mean values (horizontal marks) are given in the main text.

localized to the peripheral membrane of the vacuole (Fig. 5A and restored canavanine sensitivity in ypq2 cells (Fig. 5B). Ypq2 is thus a functional ortholog of PQLC2. According to our working hypothesis (Fig. 1F), vacuolar export of canavanine by PQLC2 may underpin its canavanine-sensitizing effect. To test this prediction, we applied canavanine to voltage-clamped PQLC2-LL/AA-EGFP oocytes and found, indeed, that the toxic analog elicits a robust inward current (Fig. 5C). Paired experiments with increasing concentrations of arginine and canavanine revealed that the toxic analog is translocated by PQLC2 with a lesser affinity (K_{m} = 5.6 ± 0.2 mM) but a higher capacity (I_{max} = −596 ± 64 nA, n = 8 oocytes) than arginine (K_{m} = 2.5 ± 0.2 mM, I_{max} = −430 ± 46 nA) (Fig. 5D and E). This efficient transport of canavanine implies that overexpression of PQLC2 should increase the canavanine-to-arginine ratio in the cytosol, in agreement with the observed drug-sensitization phenotype.

These data show that the molecular function of PQLC2 is conserved among eukaryotes, and suggest that Ypq2 and Ypq1 are
PQLC2 exports a key chemical intermediate in cysteamine therapy of cystinosis. (A) Chemical structure of the MxD resembles that of lysine. (B) Current traces evoked by MxD and arginine (10 mM each) on a representative PQLC2-LL/AA-EGFP oocyte at −40 mV and pH 5.0. (C) Saturation kinetics of paired MxD and arginine responses (means ± SEMs of five oocytes from two batches). \( K_a \) and \( I_{\text{max}} \) values are reported in the main text. \( I/S \), current/substrate concentration ratio. (D) Kinetics of PQLC2 mRNA knockdown in human cystinotic fibroblasts after two rounds of siRNA transfection. Two PQLC2-targeted siRNAs are compared with a luciferase-targeted negative control. Means ± SEMs of four measurements are shown. (E and F) PQLC2 gene silencing decreases the clearance of lysine from an intracellular compartment. (F) Scheme depicts how lysosomes are preferentially loaded with amino acids in whole cells using a methyl ester precursor. After loading human fibroblasts with \([^{3}H]\text{LysOMe}\), the fate of the resulting intracellular \([^{3}H]\text{Lys}\) pool was monitored by TLC. (F) Plots show representative chromatograms (Left) and representative \([^{3}H]\text{Lys}\) clearance kinetics (Right), respectively. PQLC2 gene silencing increases the intracellular \([^{3}H]\text{Lys}\) pool. (G) Effect of PQLC2 gene silencing on intracellular cystine and MxD levels. PQLC2 knockdown exacerbates cystine storage (Left) and dramatically increases the level of MxD induced by cysteamine (Right) in human cystinotic fibroblasts, as illustrated in this representative experiment (means ± SEMs of three measurements). luc, luciferase; no, untreated.

Discussion

In this study, we characterized a set of heptahelical PO-loop proteins and elucidated their molecular function using a combination of yeast genetics and flux measurement studies. In addition, we show that PQLC2 plays a key role in the cystine-depleting mechanism underlying cysteamine therapy of cystinosis. Mammalian PQLC2 and its yeast homologs Ypq1–3 localize to animal lysosomes and fungal vacuoles, respectively. Using a mutant construct misrouted to the plasma membrane, we clearly established that PQLC2 is able to export CAAs from acidic compartments. PQLC2 transport activity is strongly activated at low extracytosolic pH values, and it shows narrow selectivity for cationic side chains because it recognizes arginine, but not its neutral analog citrulline, as well as lysine and histidine among proteinogenic amino acids. It may be noted that the guanidinoxy group of l-canavanine, which is also efficiently translocated, has a \( pK_a \) of 7.45 in contrast to the side chain \( pK_a \) of 12.5 for arginine. Canavanine is thus partially charged in neutral compartments. However, it is fully protonated in the lysosomal/vacuolar lumen and under the conditions of our transport assay (pH 5.0).

To compare the properties of PQLC2 with those of the native transporter from lysosomes (system c), we took advantage of the discriminating effect of natural (3Me-Lys) and synthetic (N-oMe-Arg) methylated analogs relative to CAA transport at the plasma membrane (39). In agreement with the earlier study, these compounds strongly interacted with PQLC2, but not, or more...
weakly, with the plasma membrane transporter CAT-1. Therefore, PQLC2 should play a major role in recycling CAAs generated in lysosomes and autolysosomes into the metabolic network. Because PQLC2 is also able to transport ornithine and 3Me-Lys, this cellular role probably extends to the modified amino acids issued from the degradation of methylated and ornithylated proteins.

The precise transport mechanism of PQLC2 remains unclear because attempts to measure the charge/substrate coupling ratio by applying \([H]^+\)Arg to voltage-clamped oocytes yielded variable results across oocyte batches. It is thus unknown whether the transport current recorded in PQLC2 oocytes is exclusively carried by CAAs (uniport mechanism) or shared by the CAA substrate with an inorganic ion (for instance, \(H^+\)/CAA symport). This issue thus deserves further investigation. It is, however, noteworthy that the two PQ loops of PQLC2 harbor neutral side chains (W and M, respectively, in mammals) at the position equivalent to the substrate-bound, proton-bridging site of cystinosin (18), but this does not exclude the existence of a proton-bridging site elsewhere in PQLC2.

Our study also provides indirect evidence that yeast Ypq1 and Ypq2 proteins similarly act as vacuolar CAA exporters because (i) their genetic inactivation induces a canavanine-resistance phenotype that requires the vacuolar CAA importers Vba1–3 and (ii) heterologous expression of PQLC2 at the vacuolar membrane functionally complements the ypq2 mutation. The simplest explanation for these data is that the broadly specific Vba transporters (33) accumulate canavanine into the vacuole, thus reducing its cytosolic availability, whereas, in contrast, Ypq1 and Ypq2 export this toxic CAA from the vacuole (Fig. 1F), as does PQLC2 (Fig. 5 C–E). Because canavanine naturally occurs solely in leguminous plants and their predators, a reasonable interpretation is that Ypq1 and Ypq2 also export proteinogenic CAAs from the vacuole under physiological conditions. The evidence supporting a similar role (presumably restricted to lysine) for Ypq3 (34) is more indirect and based on the coordinated transcriptional regulation of the Ypq3 gene and those encoding lysine biosynthesis enzymes, thus suggesting a common role in the cytosolic availability of lysine.

In contrast to these conclusions, a previous study had suggested that the Schizosaccharomyces pombe homolog Stm1, which shares 36% and 28% sequence identity with S. cerevisiae Ypq1 and mammalian PQLC2, respectively, acts as a G protein-coupled receptor (68). However, the inductive cell growth and invasive cell growth in response to nitrogen starvation (46). However, mechanistic evidence for a GPCR function of Stm1 is weak. The conclusion that it physically interacts with the GTPase Gpa2 was based on the use of protein fragments in two-hybrid and pull-down assays suitable for soluble proteins, but not membrane proteins, and the argument that a reversed stretch of the Stm1 sequence is homologous to a motif found in known yeast GPCRs is evidently untenable.

Therefore, fusion yeast Stm1 may act as a vacuolar CAA exporter similar to its budding yeast homologs, a role consistent with the fact that STM1 transcription is induced under nitrogen, but not glucose starvation (46).

The existence of another small-molecule transporter in the cystinosin protein family strongly suggests that membrane transport is a conserved functional feature of PQ-loop proteins, in agreement with our previous demonstration that PQ loops have a functional significance in the case of cystinosin (18). For another heptahelical PQ-loop protein, termed MPU1, associated with a congenital disorder of glycosylation (47, 48), a transport function for soluble proteins, but not membrane proteins, and the argument that a reversed stretch of the Stm1 sequence is homologous to a motif found in known yeast GPCRs is evidently untenable. Therefore, fusion yeast Stm1 may act as a vacuolar CAA exporter similar to its budding yeast homologs, a role consistent with the fact that STM1 transcription is induced under nitrogen, but not glucose starvation (46).

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Proteomic Analysis of Lysosomal Membranes. Subcellular fractions from rat liver were prepared by differential centrifugation, followed by isopycnic centrifugation of the resulting L Fraction on a Nycodenz gradient (34). The lysosome-enriched (fraction 2) and lysosome-depleted (rest of the gradient) fractions were subjected to hypomosotic shock in 10 mM Hepes, pH 7.8, supplemented with protease inhibitors. Organellar membranes were recovered by ultracentrifugation (100,000 × g at 4 °C for 1 h) and treated by chloroform/methanol extraction (62) or Triton X-114 phase separation (63). All resulting samples were prepared by SDS/PAGE and subjected to LC/MS/MS analysis as described (64). Database searching was carried out on the IPi_rat_decoy database (IPI_Rat v3.48). Spectral count data from lysosome-enriched or lysosome-depleted samples were merged for semiquantitative analysis of the fractions (65), and statistical analysis was carried out according to the method of Fu et al. (35) for enrichment evaluation.

Expression and Analysis in Xenopus Oocytes. Care and use of animals were performed in accordance to local and national guidelines in compliance with the European Directive on animal experiments (2010/63/EU). All animal procedures were approved by the Direction Départementale de la Protection des Populations de Paris. Oocytes were prepared and injected with 50 ng of PQLC2-EGFP, PQLC2-LC-REA43, or EGFP RNA as described (18). After 1 or 2 d, oocytes with high expression were selected under the epifluorescence microscope and analyzed for transport.

Radio tracer flux analysis was performed in 100 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2 with 5 mM Hepes, MES, or Bis-Tris propanoate adjusted to the required pH with NaOH or CsOH (ND100 solution). Groups of five oocytes per condition were incubated in ND100 with 0.5 μCi of [3H]Arg, [3H]Lys, or [3H]His and, unless stated otherwise, 100 μM of the same nonradiolabeled compound. Incubation time was fixed to 20 min, except for saturation kinetics, where it was reduced to 10 min to preserve linearity. High substrate concentrations were stopped by two ice-cold ND100 washes at pH 7.0. Intracellular radioactivity was counted individually for each oocyte, after lysis in 0.1 N of NaOH, using a Tri-Carb 2100 TR liquid scintillation analyzer (Packard).

Steady-state transport currents were recorded under a two-electrode voltage-clamp using an OopusXps 6000A workstation (Molecular Devices) and analyzed offline with Clampfit 10.5 software (Molecular Devices) as described (18).

Expression and Analysis in Mammalian Cells. HEK cells were electroporated with the PQLC2 plasmids and analyzed after 48 h by immunofluorescence as described (66). LAM1 was detected using the HA4A monoclonal antibody (Developmental Studies Hybrida Bank, University of Iowa, Ames, IA) at 0.75 μg/mL. Epifluorescence micrographs were acquired under a 100× objective lens with a Nikon Eclipse TE-2000 microscope equipped with a CCD camera (CoolSNAP). Deconvolution microscopy was performed as described (66). HEK-293 cells were transfected by lipofection and assayed for transport as described (38).

Quantitative RT-PCR. C57Bl/6 female mice (5–8 mo of age) were killed by cervical dislocation according to local and national guidelines. Tissues were immediately dissected and utilized for RNA extraction and RT using the iScript Max cDNA Synthesis Kit (Bio-Rad). Total RNA was isolated from liver, kidney, heart, and brain using TRIzol reagent (Invitrogen). cDNA was synthe-
sized from 1 μg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen) according to the method of Fu et al. (35) for enrichment evaluation. Expression levels were quantified under the epi-

Gene Silencing. Normal and cystinotic human skin fibroblasts [a kind gift from Corinne Antignac (Paris, France)] were cultured at 37 °C in 5% CO2 in MEM supplemented with 10% FBS. Cystinotic cells were derived from a heterozy-
gous patient with missense (G339R) and splice site (564 +1 G → A) mutations in the CTNS gene. Cells were transfected two or three times every 2–3 d with 25 nM ON-TARGETplus reagents no. J-020760-18 (5'-GGCAGGAAGUC-
CXAGGCUU; Dharmacon) or no. J-020760-19 (5'-CCAUACCAUCCGGUCGLUG; Dharmacon) or as a negative control with a luciferase-targeted siRNA (Eurofin MWG Operon) using DharmaFECT-1 (Dharmacon). To account for the possibility of a slow turnover of the PQLC2 protein, cells were plated at a density allowing growth (3–5 cell divisions) during the siRNA treatment.

Cystine and Cysteine-Cysteine Disulfide Measurement. Two or three days after the last siRNA transfection, cells were washed with Earle’s balanced salt solution (EBBS) and incubated for 2 h at 37 °C in 5% CO2 with or without cysteine (30 μM–1 mM) in EBBS. Cells were then washed in PBS, detached with trypsin, and centrifuged at 1,000 × g for 5 min. Cell pellets were extensively washed with PBS, resuspended in 75 μL of 5.2 mM N-ethyl-

In Situ [3H]Lys Efflux. Fibroblasts were washed with EBBS and incubated for 2 h at 37 °C in 5% CO2 with 0.2 mM [3H]LysOme (0.7 Ci/mmol). Cells were then quickly washed with chilled EBBS and further incubated for increasing times at 37 °C in EBBS. The reaction was stopped, and proteins were precipitated with 10% trichloroacetic acid. After ether extraction of the organic acid, water-soluble radioactivity was analyzed by TLC on silica gel 60 aluminum sheets (Merck Millipore) in dichloromethane/methanol/ammonia 50:50:15 (vol/vol/vol). [3H]LysOme and [3H]Lys were separated on adjacent lanes to provide external standards. Chromatograms were dried, cut into 1-mm strips, and counted by liquid scintillation.

Note Added in Proof During the review of our paper, a study by Liu et al. (68) reaching similar conclusions in Caenorhabditis elegans was published.

ACKNOWLEDGMENTS. We thank S. Brohée for the bioinformatic analysis of lysine-repressible genes in yeast; E. Lauwers for the initial characterization of Yoj1p and Yoj2p proteins; E. Dubois for yeast strains; O. Gribouval and C. Antignac for the gift of cystinotic fibroblasts; and E. I. Clous, M. W. Debono, S. Supplisson, and the Developmental Studies Hybrida Bank maintained by the University of Iowa for providing reagents or access to instruments. C.A. and C.S. are scientists from the Institut National de la Santé et de la Recherche Médicale. This study was supported by a grant from the Cystinosis Research Foundation (to B.G.), a grant from the Centre National de la Recherche Scien-
tifique (to B.G.), Grant 3.4.592.08.F from the Fonds de la Recherche Scientifique Médicale (to C.S.), and a grant from the Concertée GraAntigac (Paris, France)] were cultured at 37 °C in 5% CO2 with or without cysteine (30 μM–1 mM) in EBBS. Cells were then washed in PBS, detached with trypsin, and centrifuged at 1,000 × g for 5 min. Cell pellets were extensively washed with PBS, resuspended in 75 μL of 5.2 mM N-ethyl-


