Caenorhabditis elegans functional orthologue of human protein h-mucolipin-1 is required for lysosome biogenesis

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Mucolipidosis type IV (MLIV) is an autosomal recessive lysosomal storage disease characterized by severe psychomotor retardation, achlorhydria, and ophthalmological abnormalities. Cells from several tissues in MLIV patients accumulate large vacuoles that are presumed to be lysosomes, but whose exact nature remains to be determined. Other defects include the deterioration of neuronal integrity in the retina and the cerebellum. MCOLN1, the gene mutated in MLIV patients, encodes a protein called h-mucolipin-1 that has six predicted transmembrane domains and functions as a Ca\(^{2+}\)-permeable channel that is modulated by changes in Ca\(^{2+}\) concentration. CUP-5 is the Caenorhabditis elegans functional orthologue of h-mucolipin-1. Mutations in cup-5 result in the accumulation of large vacuoles in several cells, in increased cell death, and in embryonic lethality. We demonstrate here that CUP-5 functions in the biogenesis of lysosomes originating from hybrid organelles. We also show that at least two h-mucolipin family members rescue cup-5 mutant endocytic defects, indicating that there may be functional redundancy among the human proteins. Finally, we propose a model that relates the lysosome biogenesis defect in the absence of CUP-5/h-mucolipin-1 to cellular phenotypes in worms and in humans.

Materials and Methods

C. elegans Strains and Methods. Standard methods were used for genetic analysis (19). Extrachromosomal arrays were generated by coinjecting restriction-digested plasmids and marker DNA, each at 1–10 μg/ml, along with EcoRV-digested genomic DNA at 100 μg/ml into the germ line (20). Several transgenic lines were generated and analyzed from each injection. Markers used: cup-5(ar465) III (12); cup-5(cu223) III (13); and unc-36(e251) III results in the worm having an uncoordinated phenotype (19) and is closely linked to cup-5 (11, 12); qC1 is a balancer chromosome that suppresses recombination between cup-5 and unc-36 (21).

Abbreviations: BSA-Rhod, BSA conjugated to rhodamine; MLIV, mucolipidosis type IV; mRFP1, monomeric red fluorescent protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. NM_020933 (MCOLN1) and AF475085 (MCOLN4)].

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arls37[pmyo-3::ssGFP] I (11); hs34[RME-8::GFP] expresses a functional RME-8::GFP fusion protein in several tissues, including coelomocytes (12, 22).

Molecular Methods. Standard methods were used for the manipulation of recombinant DNA (23). All enzymes were from New England Biolabs (Beverly, MA), unless otherwise indicated.

Plasmids. To express GFP::CUP-5 in coelomocytes, we introduced GFP::CUP-5 coding sequences in front of a coelomocyte-specific promoter (12). The LMP-1::GFP plasmid expresses a translational fusion of the C. elegans LAMP/CD68 protein LMP-1 (24), including its own promoter but lacking a stop codon, to a flexible Gly-Ala linker and to GFP (25). The GFP::RAB-5 plasmid expresses a translational fusion of GFP to the C. elegans RAB-5 (worm ORF F26H9.6) (26). To express mannosidase II::GFP in coelomocytes, we subcloned a published C. elegans mannosidase II::GFP fusion construct (27) in front of the coelomocyte-specific promoter (12). To express TRAM::GFP in coelomocytes, we subcloned a published C. elegans TRAM::GFP fusion construct (27) in front of the coelomocyte-specific promoter (12). To make an RME-8::mRFP1 fusion construct, we replaced GFP with monomeric red fluorescent protein 1 (mRFP1) (28), a monomeric form of DsRed, in the published RME-8::GFP plasmid (22). MCOLN1 (human) and MCOLN3 (human) cDNAs were amplified by PCR and were subcloned in front of a coelomocyte-specific promoter (12). GFP::CUP-5 and RME-8::mRFP1 plasmids express functional fusion proteins because they rescue the coelomocyte endocytosis defect in worms bearing mutations in cup-5 and in rme-8, respectively. In the absence of corresponding mutations, we determined that the rest of the plasmids did not induce a coelomocyte endocytosis defect when introduced into pmyo-3::ssGFP worms. Details of plasmid constructions are available on request.

BSA Conjugated to Rhodamine (BSA-Rhod) Endocytosis Assay. As described (12, 22), BSA-Rhod (Sigma) dissolved at 1 mg/ml in water was injected into the body cavity of young adult hermaphrodites at 20°C. At various times, the worms were mounted in a drop of ice-cold 1% formaldehyde, and slides were kept on ice until viewing. cup-5(zu223) worms exhibit maternal effect lethality. To assay these worms, we injected the uncoordinated progeny of cup-5(zu223) unc-36(e251)/qC1; hs34[RME-8::GFP] worms.

Microscopy. Worms were fixed in 1% formaldehyde. Deconvolution images were recorded as digital images (with Z series containing 0.15-μm sections) by using a Series 300 charge-coupled device camera (Photometrics, Tucson, AZ) and were deconvolved by using DEFTAVISION Software (Applied Precision, Issaquah, WA). Confocal images were taken with a Nikon PCM 2000, by using HeNe 543 excitation for the red dye and argon 488 for the green dye.

Results

Endocytosis Is Normal Until Lysosome Biogenesis in cup-5 Mutant Coelomocytes. To detect the stage in endocytosis at which a defect is first observed, we injected BSA-Rhod into the body cavity of worms and monitored its uptake and trafficking through endocytic compartments in coelomocytes expressing the late endosomal marker RME-8::GFP (22). By using such an assay, we had previously determined that early stages of endocytosis were normal in coelomocytes of the hypomorphic allele, cup-5(ar465) (12). We describe here a more comprehensive analysis in the null allele, cup-5(zu223) (13). After injection of BSA-Rhod into the body cavity of WT rme-8::GFP worms, we observe three distinct and sequential morphological events: (i) the BSA-Rhod appears in RME-8::GFP-labeled late endosomes; (ii) BSA-Rhod is condensed in small compartments that are “budding” from the RME-8::GFP-labeled endosomes; which are presumed to be nascent lysosomes forming from late endosomal compartments (large arrows in Fig. 1); and (iii) by 30 min, the BSA-Rhod appears in distinct compartments, lysosomes, that are not labeled with RME-8::GFP but can be labeled with a lysosomal marker LMP-1::GFP (see Fig. 3). BSA-Rhod remains localized to these lysosomes for the duration of the experiment (arrowheads in Fig. 1).

Although the early stages of endocytosis are normal in cup-5(zu223) coelomocytes, we do not see a similar appearance of the dye in a distinct lysosomal compartment. Indeed, in cup-5 mutant coelomocytes, the BSA-Rhod remains concentrated in the budding regions of the late endosomal compartments after 60 min (large arrows in Fig. 1) and even after 120 min (data not shown), indicating that CUP-5 is required for the exit of endocytosed material from the late endosomal compartment. We observed similar results in the independently derived cup-5(ar465) mutant (12). Our results indicate that the coelomocyte defect in the viable allele ar465 is as pronounced as in the maternal-effect-lethal allele zu223 (H.F., unpublished results).

CUP-5 Localizes Strongly to Mature Lysosomes and also to Nascent Lysosomes Budding from RME-8-Labeled Organelles in WT Coelomocytes. Based on our functional observations indicating a requirement for CUP-5 in lysosome formation, we predicted that CUP-5 would normally localize to late endosomes, to lysosomes, or to
both compartments in WT cells. To identify the CUP-5-positive compartment(s), we repeated the time course of BSA-Rhod uptake in coelomocytes expressing a rescuing GFP::CUP-5 fusion protein. After 15 min of uptake, most of the BSA-Rhod is in compartments that mostly lacked GFP::CUP-5 (large arrow in Fig. 2A). These late endosomes are the ones that are normally labeled with RME-8::GFP (see Fig. 1). However, GFP::CUP-5 colocalized with 30% (n = 30) of the BSA-Rhod budding from these compartments (arrowhead in Fig. 2A); indeed, GFP::CUP-5 localized to the limiting membrane of these nascent lysosomes (Fig. 2A Inset). Almost all of the internalized BSA-Rhod colocalized with the GFP::CUP-5 on mature lysosomes 60 min (90%, n = 100; small arrow in Fig. 2A) or 24 h (100%; n = 100; H.F., unpublished results) after the start of the uptake assay. These results indicate that CUP-5 localizes to nascent and mature lysosomes.

We performed further experiments to determine whether the late endosomal marker RME-8, tagged with mRFP1, ever colocalized with CUP-5::GFP in WT coelomocytes. In agreement with the BSA-Rhod time courses in both RME-8::GFP and GFP::CUP-5 expressing coelomocytes, most of the RME-8 and CUP-5 proteins localize to different compartments. However, we consistently observed small discrete domains of CUP-5 localization on RME-8-labeled compartments (arrowheads in Fig. 2B).

These results indicate that CUP-5 is found on subdomains of the late endosomes. Experiments above indicated that this CUP-5-positive subdomain is where lysosomally-directed endocytic cargo accumulates in buds. Taken together, these experiments indicate that CUP-5 is normally located in a position to directly participate in the exit of lysosomally-directed cargo from late endosomes.

The Large Vacuoles in cup-5 Mutant Coelomocytes Display Properties of Aberrant Late Endosome/Lysosome Hybrid Organelles. One consequence of a defect in lysosome biogenesis in cup-5 mutant coelomocytes is that by 24 h, the BSA-Rhod-positive compartments become grossly enlarged, most likely because they continue to receive fluid and membrane from other compartments in the cell, but are defective in export from this compartment (12). The BSA-Rhod staining within the large vacuoles is not homogenous, indicating the presence of subendosomal structures (asterisk in Fig. 3).

We had previously reported (12) that the late endosomal marker RME-8::GFP does not localize to the large vacuoles in cup-5 mutant coelomocytes. After closer examination by using longer exposures, we now see that RME-8::GFP is present on the large abnormal vacuoles (large arrow in Fig. 3). Its staining is much less pronounced than on normal late endosomal compartments (arrowhead in Fig. 3), most likely due to the aberrant molecular composition of these large vacuoles. Note that the RME-8::GFP localization to large vacuoles is not apparent at standard exposures that reveal the staining around late endosomes (arrowhead in Fig. 3), and that we never see RME-8::GFP around lysosomes in WT cells at any exposure (H.F., unpublished data). The surprising localization of RME-8 to these abnormal vacuoles indicates that the vacuoles maintain late endosomal character even many hours after they receive endocytic cargo.

Further experiments indicated that the abnormal vacuoles contain molecular markers for lysosomes as well. We established a GFP-tagged marker for the C. elegans orthologue of LAMP/
CD68, LMP-1 (24), a standard marker for mammalian lysosomes, and showed that this protein is expressed in coelomocytes. BSA-Rhod pulse–chase analysis established that this marker normally resides in the limiting membrane of lysosomes, the terminal endocytic compartment in coelomocytes (small arrow in Fig. 3). Interestingly, we observed that LMP-1::GFP localizes to the limiting membrane of the large abnormal vacuoles of cup-5 mutant coelomocytes (large arrow in Fig. 3). We examined the localization of a Golgi marker in cup-5 mutant coelomocytes. We reasoned that Golgi resident proteins that are transiently transported to normal late endosomes, either directly or after transport to the plasma membrane and subsequent endocytosis, and are then recycled back to the Golgi or are sent to lysosomes for degradation might get trapped in the aberrant large vacuoles. Mannosidase II::GFP, a Golgi-resident protein, shows a punctate pattern of staining in coelomocytes (G in Fig. 3) and in muscle cells (27). In cup-5 mutant coelomocytes, the mannosidase II::GFP is partially mislocalized to the membrane of the large vacuoles (large arrow in Fig. 3).

Finally, we examined the localization of an early endosome marker (RAB-5) and of an endoplasmic reticulum marker (TRAM) (27, 29). Neither marker localized to the large vacuoles (Fig. 6, which is published as supporting information on the PNAS web site). These large vacuoles therefore constitute aberrant hybrid organelles that have characteristics of both late endosomes and lysosomes.

**Expression of Human MCOLN1 or MCOLN3 in Worms Rescues the cup-5 Coelomocyte Endocytosis Defect.** Expression of MCOLN1 in worms has been previously shown to partially rescue the maternal effect lethality of cup-5(zu223) worms (13). To assay rescue of the coelomocyte lysosome biogenesis defect, we subcloned the human MCOLN1 and MCOLN3 into a coelomocyte-specific expression vector and introduced these plasmids into cup-5(ar465); pmyo-3::ssGFP worms. All of the coelomocytes in this mutant strain accumulate abnormally large and bright GFP-positive vacuoles (12). Suppression of this mutant phenotype is seen as a reduction in the size and brightness of the vesicles in coelomocytes as they appear in WT worms. Expression of MCOLN1 and MCOLN3 almost completely suppressed (> 80%, n = 100) the cup-5 coelomocyte phenotype (Fig. 4). This finding indicates that CUP-5 is a functional orthologue of h-mucolipin-1 and h-mucolipin-3, and possibly other mucolipin proteins as well.

These data support the idea that CUP-5 and human mucolipin proteins perform the same biological function within cells.

**Discussion**

**CUP-5 Function in the Biogenesis of Lysosomes.** In all fixed worms, and by using the appropriate markers, we consistently observe condensation of lysosomal content in structures within late endosomes of coelomocytes. This observation suggests that lysosome biogenesis occurs at a very high rate in these cells, which makes them ideal for continued studies of this process.

In WT cells, BSA-Rhod first appears in RME-8-labeled late endosomes. Subsequently, the BSA-Rhod condenses in buds containing CUP-5 but not RME-8. Our observation that only 30% of these buds contain CUP-5 is misleading because the concentration of CUP-5 in these structures is very low, making it very hard to consistently detect the protein on these structures. Similarly, the relatively weak expression of LMP-1::GFP in coelomocytes precluded us from observing LMP-1 on these buds. This finding is in contrast to mature lysosomes where CUP-5 and LMP-1 accumulate and are easily detected.

Given that the size of these buds/vesicles is 0.3–0.4 μm (12, 22), they most likely represent nascent lysosomes rather than transport vesicles, which are typically 50–100 nm in diameter, destined for lysosomes. As such, the late endosomal compartments that contain these budding vesicles are reminiscent of hybrid organelles that originate from the fusion of late endosomes and lysosomes that have been described in mammalian cells (7, 30). It has been shown that members of the CUP-5/h-mucolipin-1 protein family can function as ion channels. Further...
thermore, it has been demonstrated that the resolution of hybrid organelles into lysosomes and late endosomes requires intracellular Ca\(^{2+}\) (10). Our studies implicate CUP-5/h-mucolipin-1 in this process and indicate them as excellent candidates for the essential Ca\(^{2+}\) channel. In addition to their channel function, CUP-5/h-mucolipin-1 may use other unidentified molecular mechanisms to regulate lysosome biogenesis, lysosome function, and other cellular processes.

In cup-5 mutants, the condensation of lysosomal content into nascent lysosomes proceeds normally. However, the complete maturation of these structures into discrete lysosomes is blocked/delayed, ultimately resulting in the accumulation of large vacuoles that display some of the characteristics of both late endosomes and of lysosomes (Fig. 5). The appearance of the large vacuoles indicates that the “arrested” hybrid organelles in cup-5 mutants are still competent to receive fluid and membrane and/or to fuse with other compartments. Indeed, we have previously shown that BSA-Rhod endocytosed by cup-5(ar465); pmyo-3::ssGFP mutant coelomocytes accumulates in the same large vacuoles containing previously endocytosed GFP (12).

Given the early defect in lysosome biogenesis, potential roles for CUP-5 in mature lysosomes cannot be assayed in cup-5 mutant cells. Cells in various tissues from MLIV patients display large heterogeneous vacuoles similar to those found in cup-5 mutant C. elegans coelomocytes; such structures do not have the characteristic morphology of dense core lysosomes (31–34). The large vacuoles in cells from MLIV patients contain functional lysosomal hydrolases (35) that could start to degrade the internalized membranes, thus contributing to the heterogeneity in content of the large vacuoles. The aberrant biochemical composition of the large vacuoles is consistent with a block or delay in sorting of molecules from these structures to other compartments. This kind of phenotype is also consistent with the observed delay in the metabolism of lipids that accumulate in aberrant vacuoles in MLIV cells (36–39).

**Other Phenotypes of cup-5 Mutants/MLIV Patients.** We had previously described preliminary evidence indicating that a second defect in cup-5 mutant coelomocytes is an increase in fluid phase uptake (12). The present study does not address this defect and concentrates on the lysosome biogenesis defect. It should be noted that the possible increased uptake makes our observation that the primary defect is in the scission of nascent lysosomes from late endosomes more striking, because it is based on morphological comparisons between WT and mutant cells at different times during the processing of endocytosed material. cup-5(zu223) completely eliminates CUP-5 activity resulting in a lysosome biogenesis defect in coelomocytes, in maternal effect embryonic lethality, and in increased cell death (13). cup-5(ar465) is a hypomorphic mutation that only exhibits the coelomocyte defect, most likely because reduced CUP-5 activity is only limiting in coelomocytes that exhibit very high rates of endocytosis (12). Analogous to the cup-5 phenotypes in worms, the absence of h-mucolipin-1 causes a severe MLIV developmental phenotype partly caused by the degeneration of some tissues (40), and the accumulation of large vacuoles in some tissues that are associated with defects in cellular functions, whereas a milder point mutation that does not eliminate the protein only affects certain cellular functions (41).

It is still not known whether the more severe phenotypes in worms and in humans are the response of specific cell types to perturbations in endocytosis or whether they are due to a function of CUP-5/h-mucolipin-1 in cells. It is clear that blocking apoptosis in worms does not rescue the endocytic defect of cup-5 null worms and only partially rescues the maternal effect lethality, indicating that the embryonic lethality is at least partly due to additional unspecified defects in cell function (13).

We propose the following model for the observed phenotypes. Loss of function of CUP-5 results in a lysosome biogenesis defect and the accumulation of large vacuoles in most cells. Either directly or indirectly, this results in a rise in Ca\(^{2+}\) concentration in the cytoplasm. It is relevant in this context that inducing swelling of lysosomes in Madin–Darby canine kidney cells results in a transient release of the Ca\(^{2+}\) stores from these compartments (42). Furthermore, it has been shown that elevating cytosolic Ca\(^{2+}\) concentration can trigger fusions of lysosomes with each other and with other compartments (43, 44). This finding establishes a positive feedback loop leading to the appearance of the extremely large vacuoles in cup-5 and MLIV mutant cells.

What are the effectors of the cell death/degeneration in cells lacking CUP-5 or h-mucolipin-1? Recent evidence (45) indicates that cellular Ca\(^{2+}\) overload, or perturbation of intracellular Ca\(^{2+}\) compartmentalization, can cause cytotoxicity, and trigger either apoptotic or necrotic cell death. Indeed, it has been determined that necrotic cell death in C. elegans is inhibited by reducing the function of regulators of Ca\(^{2+}\) release from the endoplasmic reticulum (46). Another likely possibility is that the large vacuoles in cup-5 mutant cells might be fragile or leaky and may release lysosomal hydrolases into the cytoplasm. Indeed, the aberrant localization of cathepsin D, a lysosomal aspartic protease, to the cytoplasm, either through ectopic expression or by permeabilization of lysosomal membranes, can induce cell death (47, 48).

**Functional Redundancy in the h-mucolipin Family.** Both h-mucolipin-1 and h-mucolipin-3 can substitute for CUP-5 function in lysosome biogenesis in coelomocytes, indicating functional redundancy among the human proteins. However, the symptoms seen in MLIV patients lacking MCOLN1 and in mice lacking MCOLN3 do not overlap. It remains to be determined whether this is due to tissue-specific expression of the two proteins, or whether, if ubiquitously expressed, they have some unique functions in cellular pathways. Based on the rescue of the...
cup-5 defect and the high sequence similarity between h-mucolipin-1 and h-mucolipin-3, we would predict that both proteins are involved in aspects of lysosome biogenesis and/or function. We speculate that at least in some tissues, h-mucolipin-3 can replace h-mucolipin-1 because, in contrast to their other severe defects, MLIV patients have no hearing problems despite the fact that h-mucolipin-1 is highly expressed in inner ears (E.G., unpublished results). Finally, given the functional conservation between many worm and human genes and pathways, insights into the functions of worm proteins that modulate CUP-5 activity or processes are immediately translatable to humans and can be easily verified.

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