**LAPTMs regulate lysosomal function and interact with mucolipin 1: new clues for understanding mucolipidosis type IV**

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**Summary**

Loss-of-function mutations in mucolipin 1 (MCOLN1) result in mucolipidosis type IV (MLIV), a lysosomal storage disorder characterized by severe mental and psychomotor retardation. MCOLN1 is a lysosomal ion channel that belongs to the transient receptor potential (TRP) superfamily. To better understand the cellular function of MCOLN1, a split-ubiquitin yeast two-hybrid screen was performed with the purpose of revealing new MCOLN1 interaction partners. The screen identified two members of the lysosome-associated protein transmembrane (LAPT) family as novel interaction partners of MCOLN1. The binding between MCOLN1 and LAPT members (LAPTMs) was confirmed by co-immunoprecipitation and yeast two-hybrid assays. In addition, MCOLN1 and LAPTMs extensively colocalize at late endosomes and lysosomes. Overexpression of LAPT4b caused enlargement of lysosomes and defective lysosomal degradation, indicating that LAPTMs are important for proper lysosomal function. Interestingly, lysosomal swelling induced by LAPT4b was rescued by expression of MCOLN1, suggesting a functional connection between the two proteins. Finally, depletion of endogenous LAPTMs by siRNA induced accumulation of concentric multi-lamellar structures and electron-dense inclusions that closely resemble the structures found in MLIV cells. Overall, our data provide new insight into the molecular mechanisms of MCOLN1 function and suggest a potential role for LAPTMs in MLIV pathogenesis.

**Key words:** Mucolipin, LAPT, TRPML, MLIV, Lysosomes

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**Introduction**

Mucolipidosis type IV (MLIV) is an autosomal recessive disorder characterized by severe neurologic and ophthalmologic abnormalities (Altarésçu et al., 2002; Amir et al., 1987; Bach, 2001). Symptoms appear during the first year of life and include intellectual disabilities, psychomotor delay, weak muscle tone (hypotonia), corneal clouding and progressive retinal degeneration (Berman et al., 1974; Riedel et al., 1985). In addition, MLIV patients show impaired secretion of gastric acid (achlorhydria) leading to defective iron absorption and anemia (Schiffmann et al., 1998). By their early teens, most affected individuals are unable to walk, present limited or no ability to speak and have severe vision loss or blindness.

MLIV is caused by loss-of-function mutations in MCOLN1 (also referred to as TRPML1), an ion channel that belongs to the TRP superfamily (Bargal et al., 2000; Bassi et al., 2000; Slaugenhaupt et al., 1999; Sun et al., 2000). MCOLN1 is a protein with six transmembrane-spanning domains with both amino- and C-terminal tails oriented toward the cytosol and the pore located between transmembrane segments 5 and 6. Electrophysiological studies indicate that MCOLN1 is an inwardly (from lumen to cytoplasm) rectifying channel permeable to Ca\(^{2+}\), Na\(^{+}\), K\(^{+}\) and Fe\(^{2+}\) or Mn\(^{2+}\), and whose activity is modulated by pH and Ca\(^{2+}\) (Cheng et al., 2010; Puertollano and Kiselyov, 2009). However, the selectivity and mechanisms of activation of MCOLN1 under physiological conditions remain unknown.

MCOLN1 is localized at late endosomes and lysosomes. Two acidic di-leucine consensus motifs located at the N- and C-terminal tails regulate trafficking of MCOLN1 to the late endosomal pathway through interactions with clathrin adaptors (Miedel et al., 2006; Pryor et al., 2006; Vergarajauregui and Puertollano, 2006). MCOLN1 undergoes several post-translational modifications that regulate its function. For example, palmitoylation and phosphorylation at the C-terminal tail modulate trafficking and channel activity, respectively, whereas cleavage at the first luminal loop inactivates the protein (Kiselyov et al., 2005; Vergarajauregui and Puertollano, 2008b; Vergarajauregui and Puertollano, 2006).

According to previous reports, analysis of fibroblasts from MLIV patients by electron microscopy revealed the accumulation of enlarged vacuolar structures that contain mucopolysaccharides and lipids forming characteristic monocentric lamellae (Bach et al., 1975; Bach et al., 1977; Crandall et al., 1982; Tellez-Nagel et al., 1976). These enlarged vacuoles are present not only in fibroblasts but in every tissue and organ of MLIV patients, suggesting a general impairment of lysosomal function. Accumulation of defective lysosomes appears to affect other cellular processes such as autophagy. Increased levels of insoluble p62, ubiquitinated aggregates and autophagosomes have been observed in MLIV fibroblasts, indicating that autophagy-mediated clearance of harmful cellular products is impaired in MLIV patients (Vergarajauregui et al., 2008a). Generation of two different MLIV animal models in Drosophila and mouse confirmed that absence of MCOLN1 results in defective autophagy (Micsenyi et al., 2009; Venkatachalam et al., 2008). However, these observations are based on studies characterizing cellular effects resulting from the loss of MCOLN1. Thus, it is unclear whether the observed phenomena directly result from the absence of MCOLN1 or whether they are a secondary consequence of lipid accumulation in lysosomes.
In order to gain insights into MCOLN1 function, a yeast two-hybrid screen was performed to identify proteins that interact with MCOLN1. Here, we report a novel interaction between MCOLN1 and the members of the LAPTM family. Although the cellular function of LAPTM proteins is not well understood, it has been suggested that LAPTM proteins participate in the transport of small molecules across intracellular membranes (Hogue et al., 1996; Hogue et al., 1999). We found that MCOLN1 and LAPTM proteins colocalize to late endosomes and lysosomes and confirmed the interaction by co-immunoprecipitation in human cells. Overexpression of LAPTM proteins caused enlargement of lysosomes and defective lysosomal degradation, whereas depletion of endogenous LAPTM proteins induced accumulation of concentric multi-lamellar structures and electron-dense inclusions that closely resemble the structures found in MLIV cells. Overall, our data provide new insights for understanding MCOLN1 function and reveal a novel role for LAPTM proteins in the regulation of lysosomal function.

**Results**

**Identification of LAPTM proteins as novel MCOLN1 binding partners**

In order to further understand the cellular function of MCOLN1, we searched for novel binding partners of MCOLN1. Given that MCOLN1 is a highly hydrophobic transmembrane protein that oligomerizes and undergoes post-translational modifications, we used the split-ubiquitin membrane-based yeast two-hybrid system. This system uses the split-ubiquitin approach, in which reconstitution of two ubiquitin halves (Nub and Cub) is mediated by a protein–protein interaction, resulting in the release of a transcription factor and expression of reporter genes (Johnsson and Varshavsky, 1994). The advantage of this approach is that it allows us to use full-length MCOLN1 as bait and reveals interactions that take place at the organelle where the protein typically localizes (in this case the vacuole).

To generate the MCOLN1 bait, we cloned the full-length human MCOLN1 protein into the pBTE-STE vector, thus generating MCOLN1–Cub. The bait was screened against a human adult brain library of cDNAs fused to the mutated form of N-ubiquitin in the pPR3-N vector (NubG-x) and was carried out by Dualsystems Biotech AG (Schlieren, Switzerland). Among 277 positive clones isolated, two independent clones encoded members of a family of endosomal and lysosomal transmembrane proteins named LAPTM proteins. The clones included the first 217 amino acids (aa) of LAPTM4a and the N-terminal sequence (aa 27–47) of LAPTM4b, respectively. Both clones were in-frame with the N-terminal half of ubiquitin. The function of LAPTM proteins is not completely understood but it has been suggested that they are transporters involved in the subcellular compartmentalization of different compounds (Hogue et al., 1996; Hogue et al., 1999). MCOLN1 protein binding to LAPTM proteins was confirmed by performing additional yeast two-hybrid experiments. As seen in Fig. 1, MCOLN1 interacted with the three members of the LAPTM family (LAPTM4a, LAPTM4b and LAPTM5). By contrast, MCOLN3, another member of the mucolipin family responsible for the varitint-waddler phenotype in mice, did not show any significant binding to LAPTM proteins (Fig. 1).

**Confirmation of the LAPTM–MCOLN1 interaction by in vivo binding assays**

To confirm the association between LAPTM proteins and MCOLN1, we coexpressed full-length MCOLN1-FLAG and GFP–LAPTM proteins in HeLa cells and performed immunoprecipitation using an anti-FLAG antibody directly coupled to protein-G–agarose beads. Co-immunoprecipitation of LAPTM proteins was detected by immunoblot using polyclonal anti-GFP antibodies. As shown in Fig. 2A, GFP–LAPTM4b was present in immunoprecipitates of cell lysates transfected with MCOLN1–FLAG cDNA but not in those collected after transfection with the FLAG vector alone, suggesting that both proteins interact in vivo. Co-immunoprecipitation experiments also confirmed the interaction of MCOLN1 with GFP–LAPTM4a and GFP–LAPTM5 (Fig. 2A,B). In support of our two-hybrid data, MCOLN3 did not interact with LAPTM proteins (Fig. 2B). Western blot profiles of LAPTM4a, LAPTM4b and LAPTM5 showed the presence of low molecular mass bands that probably correspond to proteolytic fragments (Fig. 2, arrows). In addition, MCOLN1 and LAPTM proteins did not interact with LAMP1, another lysosomal transmembrane protein used as a negative control (supplementary material Fig. S1). Overall, our data reveal that MCOLN1 interacts with the three members of the LAPTM family both in yeast and human cells.

**MCOLN1 and LAPTM proteins colocalize to late endosomes and lysosomes**

Next, we asked whether MCOLN1 and LAPTM proteins colocalize, which would indicate that the proteins have the ability to interact at the physiological level. To analyze the colocalization between LAPTM proteins and MCOLN1, ARPE-19 cells were transiently co-transfected with GFP–LAPTM and Cherry–MCOLN1 constructs and observed by confocal microscopy. As seen in Fig. 3, all the members of the LAPTM family (LAPTM4a, LAPTM4b and LAPTM5) showed a very high degree of colocalization with MCOLN1. In fact, nearly all LAPTM-containing vesicles were also labeled for MCOLN1. We have previously described that MCOLN1 localizes to late endosomes and lysosomes and identified two di-leucine-sorting consensus motifs that mediate the transport of MCOLN1 to late-endocytic compartments (VergaraJauregui and Puertollano, 2006). Therefore, these results suggest that the interaction between MCOLN1 and LAPTM proteins probably occurs at late endosomes and lysosomes. These data are also consistent with previous reports.
describing the distribution of LAPTM4a and LAPTM5 in late endosomes and/or lysosomes (Cabrita et al., 1999; Pak et al., 2006).

To examine the subcellular localization of LAPTM4b in more detail and to ensure that its distribution was not altered by coexpression of MCOLN1, we expressed GFP–LAPTM4b alone in ARPE-19 cells and performed co-staining with different endogenous markers. As shown in Fig. 4, most of the LAPTM4b-positive vesicles also contain the late-endosomal and -lysosomal marker CD63. By contrast, colocalization of LAPTM4b with early endosomal markers such as Hrs was very limited, thus confirming the distribution of LAPTM4b at the late-endocytic pathway (Fig. 4).
LAPTM4b undergoes proteolytic cleavage in late endosomes and lysosomes

As mentioned previously, expression of LAPTM4b in ARPE-19 cells resulted in two major protein products: the predicted full-length protein and a shorter form that we propose might correspond to a proteolytic fragment. To test this hypothesis, we followed the fate of newly synthesized LAPTM4b by performing 'pulse-chase' experiments upon treatment with brefeldin A (BFA). This approach has been previously used to monitor the transport of MCOLN1 to lysosomes (Vergarajauregui and Puertollano, 2006). ARPE-19 cells were transfected with GFP–LAPTM4b and, 6 hours after transfection, cells were incubated with BFA for 12 hours to accumulate the newly synthesized proteins in the endoplasmic reticulum (ER; pulse) (Lippincott-Schwartz et al., 1989). Proteins were then released from the ER by incubation in medium without BFA (chase) and cells were analyzed at different time-points by either immunofluorescence or western blot. As presented in Fig. 5A, LAPTM4b started showing colocalization with CD63 3 hours after BFA removal, whereas colocalization between LAPTM4b and CD63 was nearly 100% complete after 6 hours. As expected, delivery of LAPTM4b to late endosomes and lysosomes correlated with the appearance of the low molecular mass band, consistent with the idea that it is the result of proteolytic cleavage (Fig. 5B). The fragment was not observed when LAPTM4b was retained at the ER or shortly after release of the protein from the ER, but it was most abundant at 6 hours chase when most of the protein had reached the late endosomes and lysosomes. Appearance of the proteolytic fragment was inhibited by incubation with leupeptin, an inhibitor of lysosomal proteases, thus confirming that cleavage of LAPTM4b occurs at lysosomes. In addition, the amount of high molecular mass bands increased over time, suggesting that LAPTM4b might form homo-oligomers upon reaching the lysosomes.

LAPTM4b and MCOLN1 are transported to lysosomes independently of each other

Next, we addressed whether the binding of LAPTM4b to MCOLN1 is required for the proper transport of LAPTM4b to late endosomes and lysosomes. For that, we generated a stable cell line in which the levels of endogenous MCOLN1 were reduced by lentivirus-mediated shRNA interference. Quantitative RT-PCR showed that the lentivirus-mediated shRNA against MCOLN1 caused a greater than 80% reduction in MCOLN1 mRNA levels compared with a lentivirus-expressing control shRNA (data not shown). Delivery of LAPTM4b to late endosomes and lysosomes was not affected by depletion of MCOLN1, as assessed by the appearance of LAPTM4b proteolytic fragments (Fig. 6A) and by confocal microscopy (data not shown). These results indicate that the binding of LAPTM4b to MCOLN1 does not regulate sorting of LAPTM4b along the
endocytic pathway. Similarly, MCOLN1 efficiently reached late endosomes and lysosomes in cells depleted of LAPTM4a and LAPTM4b (LAPTM5 is not expressed in HeLa cells; Fig. 6B). The levels of LAPTM4a and LAPTM4b mRNAs were reduced by more than 90% in cells treated with specific siRNAs when compared with control cells (data not shown). Therefore, our results indicate that LAPTMs and MCOLN1 are sorted independently of each other.

Overexpression of LAPTM4b causes enlargement of late endosomes and lysosomes

Next, we sought to determine the effect of LAPTM4b overexpression on the late-endosomal pathway. In order to achieve high levels of protein expression, we generated recombinant adenoviruses expressing GFP–LAPTM4b (Ad-LAPTM4b) or an adenovirus expressing GFP alone (Ad-GFP) as a control. As shown in Fig. 7A, overexpression of GFP–LAPTM4b caused a significant change in the size and distribution of CD63-positive vesicles. Late endosomes and lysosomes appeared enlarged and, in some cases, clustered to the perinuclear region when compared with non-infected cells. Expression of Ad-GFP did not affect the extent of distribution of late endosomes or lysosomes. The average diameter of CD63-positive structures was 0.45 μm (±0.12 s.d., n=56) in mock-infected cells and 0.46 μm (±0.15 s.d., n=98) in Ad-GFP-infected cells. By contrast, the average diameter of late endosomes and lysosomes increased to 1.33 μm (±0.28 s.d., n=89) in LAPTM4b-expressing cells (supplementary material Fig. S2).

Interestingly, the enlargement of late endosomes and lysosomes caused by the overexpression of LAPTM4b was rescued by coexpression of MCOLN1. As seen in Fig. 7B, co-infected ARPE-19 cells with Ad-LAPTM4b and Ad-MCOLN1–FLAG prevented the accumulation of swelled late endosomes and lysosomes. The presence of MCOLN1 did not affect the localization of LAPTM4b with CD63 but induced a 60% reduction in the size of late endosomes and lysosomes, changing from 1.33 μm in LAPTM4b-expressing cells to 0.53 μm (±0.12 s.d., n=76) in cells co-expressing LAPTM4b and MCOLN1 (supplementary material Fig. S2). These results indicate that the interaction between MCOLN1 and LAPTM4b is functionally relevant in cells.

Autophagy is altered in LAPTM4b-expressing cells

To analyze whether the enlargement of late endosomes and lysosomes upon LAPTM4b overexpression correlates with defects
in lysosomal function, we monitored autophagic flux. Autophagy is a crucial clearance mechanism that prevents accumulation of protein aggregates and abnormal organelles (Cuervo, 2004; Martinez-Vicente and Cuervo, 2007). It has been described that alterations in lysosomal function result in defective autophagosome degradation. Interestingly, we found a remarkable accumulation of LC3-positive structures in cells expressing LAPTM4b, whereas accumulation of autophagosomes was not observed in mock-infected cells or in cells expressing Ad-GFP (Fig. 8A). LC3 is considered to be one of the most specific markers for autophagosomes (Kabeya et al., 2000); therefore, our data indicate that autophagosome maturation is severely diminished by overexpression of LAPTM4b. Quantification of the number of autophagosomes per cell revealed that mock-infected and Ad-GFP-infected cells show an average of 20 LC3-positive vesicles per cell (18.82±4.93, n=40 in mock-infected cells and 19.46±5.14, n=46 in cells expressing Ad-GFP, where n represents the number of cells). By contrast, there was a greater than 6-fold increase in the average number of autophagosomes in cells infected with Ad-LAPTM4b (123±28.39, n=36; Fig. 8B).

Autophagy can also be monitored by immunoblot. LC3 is initially synthesized in an unprocessed form that is rapidly cleaved in its C-terminal region to generate soluble LC3I. During autophagy induction, the LC3I isoform is converted into LC3II by conjugation of a phosphatidylethanolamine group. This post-translational modification allows LC3 to translocate to autophagosomal membranes. It is well established that the LC3II/LC3I ratio correlates with the extent of autophagosome formation (Klionsky et al., 2007). We prepared lysates from mock-infected cells and from cells infected with adenovirus expressing GFP or GFP–LAPTM4b. As seen in Fig. 8C, a robust increase in the level of LC3II was observed in cells expressing GFP–LAPTM4b, confirming disruption of the autophagic process. Quantification of three independent experiments revealed an approximately threefold increase (2.85±0.2) in the LC3II/LC3I ratio in LAPTM4b-expressing cells (Fig. 8D). To further confirm that basal autophagic flux is interrupted upon overexpression of LAPTM4b, we examined the levels of ubiquitinated proteins. As expected, a remarkable accumulation of ubiquitinated proteins was observed by western blot (Fig. 8E) and immunofluorescence (supplementary material Fig. S3). Therefore, our results indicate that appropriate levels of LAPTM4b are crucial for optimal lysosomal function because overexpression of the protein results in enlargement of late endosomes and lysosomes and defective autophagosome maturation.

Depletion of LAPTM4b induces accumulation of electron-dense and multi-laminar structures

In MLIV, absence of MCOLN1 leads to the buildup of membranous and electron-dense organelles containing undigested lipid products. This heterogeneous storage is readily identified by electron microscopy and suggests that proper MCOLN1 function is essential for the maintenance of lysosomal integrity. To further confirm the role of LAPTM4b in lysosomal function, we depleted endogenous LAPTM4b by treatment with specific siRNAs. Quantitative RT-PCR revealed that only LAPTM4a and LAPTM4b are expressed in HeLa cells. These results are in agreement with previous reports suggesting that LAPTM5 is preferentially expressed in hematopoietic cells (Adra et al., 1996; Scott et al., 1996). After two 72-hour consecutive transfections with a combination of LAPTM4a and LAPTM4b siRNAs, we were able to achieve a greater than 90% reduction in the levels of LAPTM4 (a+b) mRNA, whereas no variations were observed in cells transfected with control non-target siRNA. Ultrastructure analysis revealed a significant accumulation of lysosomal inclusions in LAPTM4 knockdown cells when compared with control cells (Fig. 9A,B). These storage bodies, composed of electron-dense materials and concentric multi-laminar (fingerprint) organelles (Fig. 9E; supplementary material Fig. S4), closely resemble those found in MLIV patients (Fig. 9D,G; supplementary material Fig. S4). Quantification experiments confirmed that accumulation of lysosomal inclusions in LAPTM4 knockdown cells is highly significant when compared with control cells (Fig. 10). For example, only 19% (n=54) of the cells treated with control siRNA showed more than 10 electron-dense structures, whereas 78% (n=50) of the LAPTM4-depleted cells have over 10
inclusions (Fig. 10A). Interestingly, simultaneous depletion of LAPTM4 (a+b) and MCOLN1 caused a greater than twofold increase in the size of the storage bodies when compared with cells lacking just LAPTM4 (Fig. 9C,F; Fig. 10B). Overall, our results confirm that endogenous LAPTMs are important for the maintenance of lysosomal integrity and indicate that defects in LAPTM function lead to accumulation of storage material in lysosomes that mimics the phenotype found in MLIV patients.

Discussion

Mutations in MCOLN1 are the cause of MLIV, a devastating lysosomal storage disorder that affects children in their first year of life. To better understand the cellular function of MCOLN1, we performed a split-ubiquitin yeast two-hybrid screen with the goal of revealing novel interaction partners of MCOLN1. As a result of the screen, we found that MCOLN1 physically interacts with the three members of the LAPTM family, and we observed extensive colocalization between MCOLN1 and LAPTMs in late-endosomes and lysosomes.

Initial studies identified LAPTM4a as a transporter that transfers nucleosides (and/or nucleoside metabolites) between the cytosol and intracellular organelles (Hogue et al., 1996). Further work suggested that LAPTMs might have a more general role in the transport of structurally unrelated amphiphilic molecules (Hogue et al., 1999). Thus, drug-sensitive yeast expressing mouse LAPTM4a were found to have increased resistance to several compounds such as daunorubicin, doxorubicin, erythromycin, progesterone and rhodamine 123, whereas they showed increased sensitivity to 5-fluorouracil, 5-fluorouridine and trifluoperazine. Moreover, the finding that LAPTM4a and LAPTM5 located to lysosomes (Cabrita et al., 1999; Pak et al., 2006) suggested that LAPTMs mediate transport of one or more substrates across lysosomal membranes.

In this study, we described for the first time that LAPTM4b also localizes to late endosomes and lysosomes. LAPTM4b is a candidate oncogene whose expression is increased in hepatocellular carcinoma, ovarian cancer and gastric cancer (Kasper et al., 2005; Liu et al., 2007; Yang et al., 2010). Furthermore, expression of LAPTM4b contributes to chemotherapy resistance in breast cancer by preventing transport of the anthracycline doxorubicin to the nucleus (Li et al., 2010). Therefore, this could suggest that sequestration of doxorubicin in the lysosomal lumen mediated by LAPTM4b is the cause of anthracycline chemoresistance.

We are also the first to describe that LAPTMs undergo proteolytic cleavage upon reaching lysosomes. The approximate molecular masses of the proteolytic products were 8.5 kDa for LAPTM4a, 17 kDa for LAPTM4b and 7 kDa for LAPTM5, indicating that the cleavage probably occurs at the first intraluminal loop. Interestingly, injection of a truncated version of mouse LAPTMs in lysosomal function
LAPTM4a (lacking the first 83 residues) into Xenopus oocytes stimulated uptake of thymidine, whereas full-length mouse LAPTM4a did not show transport activity (Hogue et al., 1996). These data suggest that LAPMTs might be activated by proteolytic cleavage only after being delivered to lysosomes. The first luminal loop of MCOLN1 is also proteolytically cleaved in lysosomes. However, in the case of MCOLN1, it has been suggested that proteolytic cleavage might induce inactivation of the protein (Kiselyov et al., 2005). In addition, we did not observe significant homology between the loops of MCOLN1 and LAPMTs, thus suggesting that the cleavage might be mediated by different lysosomal hydrolases.

Overexpression of LAPTM4b has a profound effect on the morphology and functionality of lysosomes. Infection of ARPE-19 cells with Ad-LAPTM4b caused enlargement of CD63-positive vesicles, accumulation of ubiquitinated proteins and defective autophagosome maturation. These data are consistent with the proposed role for LAPMTs in the transport of molecules across the lysosomal membrane, as the accumulation of certain substrates is likely to impair lysosomal function. The data are also in agreement with recent evidence showing that overexpression of LAPTM5 associates with spontaneous regression of neuroblastomas by inducing lysosomal cell death with impaired autophagy (Inoue et al., 2009). We and others have previously described that overexpression of MCOLN1 also causes accumulation of enlarged aberrant lysosomes and impaired autophagy, thus indicating that LAPMTs and MCOLN1 are required to maintain adequate lysosomal function (Vergarajauregi et al., 2008; Manzoni et al., 2004).

The importance of LAPMTs in lysosomal function was further corroborated by the accumulation of storage bodies in cells depleted of endogenous LAPMTs. Storage material included fine lamellated membrane structures (fingerprints), as well as electron-dense bodies. Interestingly, the same types of lysosomal inclusions are found in MLIV cells. It has been suggested that the ultrastructural appearance of storage material might be specific to the type of lipid accumulated in each lysosomal storage disorder (Alroy and Ucci, 2006). For example, fingerprint structures often result from the accumulation of gangliosides and other glycolipids, and have been described in MLIV, Fabry disease, fucosidosis, GM1 and GM2 gangliosidosis, galactosialidosis and sialidosis. Therefore, the appearance of concentric membranes in the absence of either MCOLN1 or LAPMTs could indicate accumulation of the same type of storage material.

The present evidence suggests that MCOLN1 and LAPMTs are functionally connected. The fact that MCOLN1 and LAPMTs interact and colocalize to late endosomes and lysosomes, the ability of MCOLN1 to rescue the enlargement of lysosomes caused by LAPTM4b overexpression, the accumulation of the same type of storage material upon depletion of either LAPMTs or MCOLN1 and the increase in the size of lysosomal inclusions by simultaneous depletion of both MCOLN1 and LAPMTs suggest that MCOLN1 might regulate LAPTM function. We speculate that the release of ions by MCOLN1 might provide energy for the LAPTM-dependent transport of specific molecules across lysosomal membranes. Alternatively, binding of LAPMTs to MCOLN1 might induce changes in the conformation and/or activity of the transporters.

Our model has important implications for the understanding of MLIV. Alterations in the transport activity of LAPMTs caused by the absence of MCOLN1 might lead to the storage disorder observed in MLIV cells. Studies have shown that most lysosomal storage diseases are caused by deficiencies of soluble lysosomal hydrolases or lysosomal transporters that cause accumulation of specific molecules in the lumen of lysosomes. In support of this, expression of LAPTM4a and LAPTM5 in MLIV cells is increased 3-fold and 7-fold, respectively (Bozzato et al., 2008). This increase might represent an attempt by MLIV cells to compensate for the lack of LAPMT activity.

Further studies will try to identify the physiological ligands transported by LAPMTs as well as discover compounds that modulate LAPTM activity. This information will further improve our understanding of MLIV and might open new and exciting avenues for the development of a treatment for the disease.

Materials and Methods

Antibodies and reagents

The primary antibodies used were: mouse monoclonal anti-CD63 (HSC6; BD PharImingen, San Jose, CA), mouse monoclonal anti-actin (clone Ab-5; BD Biosciences, San Jose, CA), mouse monoclonal anti-FLAG (Covance, Princeton, NJ), mouse monoclonal anti-Lamp1 (clone HA43; Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit polyclonal anti-LC3 (Sigma, St Louis, MO), rabbit anti-GFP and mouse monoclonal anti-ubiquitin (clone FK2) (MBL International, Woburn, MA), rabbit polyclonal anti-Hrs (Novus Biologicals, Littleton, CO) and mouse monoclonal anti-FLAG M2 (Sigma). The secondary antibodies used were: goat anti-mouse or goat anti-rabbit conjugated to Alexa Fluor 488 or 555, goat anti-mouse conjugated to Alexa Fluor 633 (Molecular Probes, Eugene, OR) and HRP-conjugated anti-mouse or anti-rabbit IgG (Amersham Bioscience, Piscataway, NJ). FuGEn-6 reagent and protease inhibitor cocktail tablets were obtained from Roche Applied Science (Indianapolis, IN). BFA and leupeptin were from Sigma and were used according to the manufacturer’s instructions.

Split-ubiquitin yeast two-hybrid assays

Screening of an adult human brain cDNA library with full-length MCOLN1 was performed by Dualsystems Biotech AG (Schlieren, Switzerland). Full-length MCOLN1 was inserted into the bait expression vector pBT3-STE (Dualsystems Biotech, Switzerland) with Cub-LexA-PV16 downstream of MCOLN1. The bait vector carries the LEU2 gene for auxotrophic selection. A human adult brain cDNA library of 50.3×106 clones fused to a mutated version of the amino-terminal portion of ubiquitin (NubG-x) in their amino-terminal sequence in the pPR3-N (prey vector) was used to screen for potential positive interactions. The prey vector carries the TRPI (Triap1) gene for auxotrophic selection. The screening was carried out by the mating of NMT32 yeast transformed with the bait and prey constructs. A total of 277 positive clones representing 23 distinct human proteins were isolated. One clone was found to encode the first 217 amino acids of LAPTM4a and a second one encodes amino acids 27–47 of LAPTM4b. Selection of positive interactions was performed by the ability of yeast to grow on selective synthetic dropout (SD) agar plates [SD – tryptophan – leucine – histidine (Clontech, Palo Alto, CA)] containing 10 mM 3-amino-1,2,4-triazole (3AT). The selected positive clones were sequenced and the resulting nucleotide sequences were analyzed using the NCBI BLAST algorithm.

Plasmids and adenovirus preparation

The complete open reading frames (ORFs) of human LAPTM4a and LAPTM5 were PCR-amplified from a placenta cDNA library using specific primers and cloned into the EcoRI and Sall sites of the pEGFP-C2 vector (Clontech). The ORF of human LAPTM4b was PCR-amplified from a cDNA clone from OriGene (NM_018407) using specific primers to introduce an EcoRI site at the 5’ end and an Xhol site at the 3’ end and cloned into the EcoRI and Sall sites of pEGFP-C2. To generate Cherry-MCOLN1, full-length MCOLN1 was cloned into the EcoRI and Sall sites of the pCherry vector (Clontech). MCOLN1–FLAG was cloned into the EcoRI–Sall sites of the pCMV-TAG2B vector. Cloning of MCOLN1–FLAG has been described previously (Vergarajauregi et al., 2008b). MCOLN1, MCOLN3, LAPTM4a, LAPTM4b and LAPTM5 were cloned into the XhoI sites of the pB anticipate–STE, pPR3-N and pPR3-STE vectors (Dualsystems Biotech AG, Switzerland) according to the manufacturer’s directions. We used PD2.1-Al5g as a negative control for the split-ubiquitin two-hybrid system. To check if the bait was properly expressed and functional, we used the control prey pAl-Al5g. All constructs were confirmed by DNA sequencing. Adenoviruses expressing GFP–LAPTM4b and MCOLN1–FLAG were prepared, amplified and purified by Welgen (Worcester, MA).

shMCOLN1 cell line

Control and MCOLN1 lentiviral shRNAs were obtained from Sigma (MISSION shRNA Lentiviral Transduction Particles). HeLa cells were infected with either the MCOLN1-shlentivirus (MISSION shRNA TRCN0000083297) or the control lentivirus (MISSION Non-Target shRNA Control SHC002) together with 8 µg/ml of Polybrene. Clones were selected using 1 µg/ml puromycin. Q-RT-PCR was used to assay for knockdown of MCOLN1.
Role of LAPTMs in lysosomal function

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Supplementary material available online at http://jcs.biologists.org/cgi/content/full/124/3/459/DC1

References


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WB: anti-GFP

WB: anti-Lamp1

B

<table>
<thead>
<tr>
<th></th>
<th>IP: anti-FLAG</th>
<th>Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCOLN1-FLAG</td>
<td>+  +</td>
<td>+  +</td>
</tr>
<tr>
<td>GFP-LAPTM4b</td>
<td>+  -</td>
<td>+  -</td>
</tr>
<tr>
<td>GFP-LAMP1</td>
<td>-  +</td>
<td>-  +</td>
</tr>
</tbody>
</table>

WB: anti-FLAG

WB: anti-GFP
**A**

LAPTM4b (+ MCOLN1)

merge

CD63

LAPTM4b

merge

CD63

**B**

Size of CD63-positive vesicles (µm)

- Control
- Ad-GFP
- Ad-LAPTM4b
- Ad-LAPTM4b + Ad-MCOLN1

Bar chart showing the size of CD63-positive vesicles for different conditions.