Overexpression of wild-type and mutant mucolipin proteins in mammalian cells: effects on the late endocytic compartment organization

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Abstract Mucolipin-1 is a 65-kDa membrane protein encoded by the *MCOLN1* gene, which is mutated in patients with mucolipidosis type IV (MLIV), a rare neurodegenerative lysosomal storage disorder. We studied the subcellular localization of wild-type and three different mutant forms (T232P, F408del and F465L) of mucolipin by expressing Myc-tagged proteins in HeLa cells. The overexpressed wild-type mucolipin colocalizes to late endocytic structures and induces an aberrant distribution of these compartments. F408del and F465L MLIV mutant proteins show a distribution similar to the wild-type protein, whereas T232P is retained in the endoplasmic reticulum. Among the mutants, only F408del induces a redistribution of the late endocytic compartment. These findings suggest that the overexpression of the mucolipin cation channel influences the dynamic equilibrium of late endocytic compartments.

Keywords: Mucolipin; Mucolipidosis type IV; Lysosome; Late endosome

1. Introduction

First described in 1974 [1], mucolipidosis type IV (MLIV, MIM 252650) is an autosomal recessive lysosomal storage disorder. To date approximately 100 patients have been reported all over the world, the majority of whom are of Ashkenazi Jewish (AJ) descent [2,3]. The frequency in the AJ population has recently been estimated to be 1/40 000, with heterozygote frequency of 1/100 [4,5]. The disease presents itself during the first years of life and patients show little deterioration for the first three decades [6], with life expectancy and prognosis beyond the third decade still unknown. Clinically, MLIV is characterized by profound psychomotor retardation, ophthalmologic abnormalities including corneal opacity, retinal degeneration, strabismus [7] and markedly elevated blood gastrin levels. This last finding is apparently caused by constitutive achlorhydria and provides a simple biochemical marker to assist in diagnosis [2]. Considerable heterogeneity in the clinical symptoms and severity of the disease has been reported even among siblings. At the cellular level, sphingolipids, phospholipids and acid mucopolysaccharides accumulate in the lysosomes from every tissue and organ of MLIV patients as single-membrane bound granular inclusions and lamellar concentric bodies [1,6,8–13]. The lysosomal storage results from an abnormal sorting and/or transport along the late endocytic pathway [6,8], while the lysosomal hydrolases involved in the catabolism of the stored products have normal activity and the stored materials are normally catabolized. The loss of balance between transport of components into lysosomes and their catabolism [8,14] offers an explanation for the heterogeneous nature of the stored molecules and the absence of organomegaly or skeletal deformations seen in many other lysosomal storage diseases [6]. MLIV is caused by mutations in *MCOLN1*, a gene mapping to 19p13.3 [15–17]. Two founder *MCOLN1* mutations were identified to comprise 95% of the mutant alleles among Ashkenazi MLIV families in correlations with two haplotypes [18]. The major AJ mutation, present on the 72% of the AJ MLIV alleles, is an A–G transition at the 3′ acceptor site of intron 3. The minor mutation is a 6432-bp genomic deletion spanning a region from the 5′ end of the gene to the first 12 bp of exon 7 and is found in the 23% of the AJ MLIV alleles. *MCOLN1* encodes a 580-amino acid protein with a predicted molecular weight of 65 kDa that has been named mucolipin-1 (MLN1). Structural analysis of the amino acid sequence predicts that the protein has six transmembrane (TM) domains, with both the N- and C-termini residing in the cytoplasm (Cyt), and a putative di-leucine motif (L–L–X–X) located at the C-terminus as a late endosomal–lysosomal-targeting motif (Fig. 1A). MLN1 shows 49% amino acid identity to MLN2 and 57.2% to MLN3 [19], two yet uncharacterized polypeptides belonging to the same transient receptor potential (TRP) family [20]. Mucolipin has indeed been recently demonstrated to represent a novel Ca^{2+}-permeable channel transiently modulated by changes in Ca^{2+} concentration whose function is inhibited by reduction of pH [21,22]. It also has significant structural similarity to polycystin-2 (PC2), another 6 TM Ca^{2+}-permeant channel, which is mutated in some cases

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Abbreviations: MLIV, mucolipidosis type IV; *MCOLN1*, mucolipin-1 gene; MLN1, mucolipin-1 protein; MLN2, mucolipin 2 protein; MLN3, mucolipin 3 protein; AJ, Ashkenazi Jews; TRP, transient receptor potential; PC2, polycystin-2

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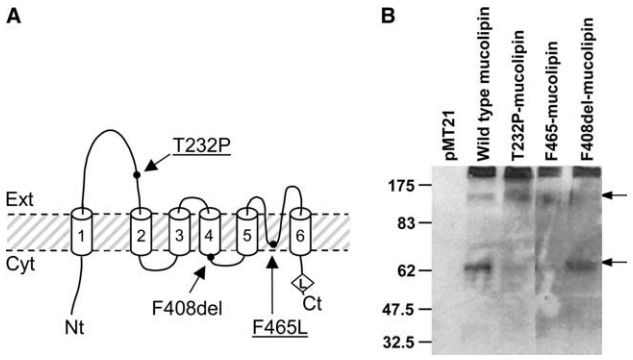

Fig. 1. Schematic illustration of the putative structure of mucolipin and Western blot analysis of wild-type and mutant mucolipin. (A) Mucolipin has six predicted transmembrane domains (1–6), with the N- and C-termini in the cytoplasm (Cyt). The approximate position of the mutations analyzed is shown. They are two missense mutations (T232P and F465L) and a single codon deletion (F408del). Mutations described in patients with a severe phenotype are underlined. Ext, external; Cyt, cytoplasmic; L, lysosomal targeting signal (L–L–X–X). (B) HeLa cells were transfected with wild-type, T232P, F465L and F408del Myc-tagged mucolipins or control vector (pMT21). Cells were harvested 24 h after transfection and subjected to SDS–8 M urea PAGE, followed by immunoblot analysis with rabbit anti-Myc antibody. The overexpressed protein isoforms are indicated at the top of each lane. Arrows indicate the position of the mucolipin monomer and putative dimer. No signal is detectable in the mock (pMT21) transfected lane.

of autosomal dominant polycystic kidney disease [15–17,20]. Interestingly, PC2 is involved in basolateral trafficking of proteins and lipids in polarized cells.

This study represents the first comparative analysis of the cellular distribution of wild-type and mutant mucolipin forms.

2. Materials and methods

2.1. Expression constructs

Mucolipin-Myc construct. Primers MYML129E (5′-AGGAATTCATGACAGCCCCGGCGGGTC-3′) and MY129S (5′-AAGTCGACAATTCACCAGCAGCGAATG-3′) were used to amplify the entire *MCOLN1* ORF by PCR using cloned *Pfu* polymerase (Stratagene) and 2517653 IMAGE cDNA clone as template. The PCR product was digested with *Eco*RI and *Sal*I and was cloned into the pMT21 expression vector (which contains a CMV promoter and a SV40 polyadenylation signal) to generate an open reading frame encoding mucolipin with a COOH-terminal Myc-tag (mucolipin-Myc construct).

Mucolipin-HA construct. ML129BGL (5′-ATAGATCTATGACAGCCCCGGCGGGTC-3′) and 129/ECO (5′-GGAATTCTCAATTCACCAGCAGCGAATG-3′) primers containing suitable restriction sites were used to amplify and clone the *MCOLN1* coding region in pMHA vector. Mucolipin-hemagglutinin (HA) contains the amplified insert 5′ in-frame with the HA epitope within plasmid pMHA. The pMHA vector contains a CMV promoter and a SV40 polyadenylation signal.

All mutant mucolipin constructs (F408del-, T232P- and F465L-mucolipin) [5,16,17] were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol and the mucolipin-Myc construct previously described. Mutations were confirmed by automated fluorescent sequencing. The ORF encoding each mucolipin mutant is 3′ in-frame with the Myc epitope into the plasmid pMT21.

2.2. Cell culture and transient transfections

Human cervical cancer cell line HeLa and African green monkey kidney cells Cos7 were maintained in Dulbecco's modified Eagle's medium (Euroclone Life Sciences), supplemented with 5% (v/v) fetal bovine serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Gibco, Invitrogen Life Technologies) at 37 °C in a 5% CO_2 humidified incubator. For immunofluorescence studies, HeLa and Cos7 cells were seeded on sterile glass coverslips (BDH) in six-wells plates (Corning Life Sciences) or in 60-mm culture dishes (Corning Life Sciences). Transfections were carried out using FuGene 6 (Roche) according to the manufacturer's guidelines. Briefly, cells were incubated with DNA/FuGene 6 reagent mixture (ratio DNA/Fugene = 1/3) in serum-free medium (Optimem, Invitrogen Life Technologies) for 5 h, followed by culturing in 5% FBS-containing medium. Mock transfectants were obtained with the same amount of pMT21 or pHA expression vector. Cells were either collected for protein analysis or fixed for immunofluorescence at different times after transfection.

2.3. Protein extraction and immunoblot analyses

HeLa cells were transfected with Myc-tagged mucolipin or one of the Myc-tagged mucolipin mutants for 24 h; 10 μg of the cell lysate were subjected to 8 M urea–SDS–10% (w/v) polyacrylamide gel electrophoresis and subsequently transferred by electroblotting onto Immobilon-P blotting membrane (Amersham Biosciences). The membrane was incubated for 30 min in PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) dried milk (blocking buffer) and subsequently incubated with rabbit anti-Myc antibody (Sigma) followed by incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulins (Amersham Biosciences). Proteins were visualized using enhanced chemiluminescence immunoblotting detection reagent (Amersham Biosciences).

2.4. Lysosomal enzyme assays

Control and transfected HeLa cells were cultured for 12 h in the presence or absence of 5 mM mannose 6-phosphate sodium salt (Sigma) to inhibit receptor-mediated endocytosis of secreted lysosomal hydrolases. Lysosomal enzyme activities including β-hexosaminidase and β-galactosidase were determined both in cell extracts and culture media by using appropriate colorimetric substrates by commonly used methods [23]. The assay was carried out in two separate experiments, each point in triplicate.

2.5. Immunofluorescence and microscopy

Transfected cells grown on coverslips were rinsed with PBS, immediately fixed with 3% (w/v) PBS-buffered paraformaldehyde for 15 min, washed in PBS, quenched in 50 mM NH_4Cl for 10 min and permeabilized in PBS containing 0.5% saponin. Cells were subsequently immunolabeled using an indirect procedure in which all incubations (primary, secondary antibodies and washes) were performed in solution containing 0.5% saponin.

Primary antibodies used were: rabbit anti-Myc antibody (Sigma), mouse anti-Myc (Sigma), mouse anti-LAMP1 (BD Biosciences), mouse anti-early endosome antigen 1 (EEA1) (BD Biosciences Pharmingen), rabbit anti-calnexin (Stressgen), mouse anti-GM130 (BD Biosciences Pharmingen), anti-cytochrome *c* (Promega) and rabbit anti-catalase (Calbiochem). Anti-lysobisphosphatidic acid (LBPA) and anti-46-kDa mannose 6-phosphate receptor (MPR46) were generously provided by J. Gruenberg (University of Geneve) and S. Hoening (University of Gottingen), respectively. For lysosomes labeling, Lysotracker Red (Molecular Probes) was used. After 24 h transfection, cells were incubated with 100 nM Lysotracker for 30 min following the manufacturer's instructions. Staining was obtained after incubation with Cy2- and/or Cy3-conjugated isotype-specific antibodies (Jackson ImmunoResearch Laboratories). Controls included staining with each secondary antibody separately. Coverslips were then washed in PBS, mounted on a glass microscopic slide (BDH) with Fluorescent Mounting Medium (Dako) and examined using a confocal microscope (MRC-1024 BioRad).

Images were processed with Image ProPlus (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop software. For quantitation of overlap between mucolipin (wild-type and mutants) and LAMP1 computer-assisted image quantification with the Image ProPlus software was used. Briefly, binary images for both markers to be compared were combined using a logical *and*, so as to yield an image of only those pixels which were positive in both images. The total number of pixels in each of the binary images and the combined image were compared to give the percentage of colocalization [24]. Values given represent the mean from five separate cells, in at least two different experiments.

3. Results and discussion

3.1. Generation and expression of wild-type and mutant mucolipin constructs

To get more insight into the molecular mechanism and the function of mucolipin, we localized the wild-type protein in cultured mammalian cells and analyzed whether mutations in mucolipin affect proper stability and subcellular distribution of the protein. A schematic illustration of the putative structure of mucolipin together with the position of the mutations studied is shown in Fig. 1A. The in-frame deletion F408del and the missense mutation F465L are within the transient receptor potential (TRP) domain of the protein [17], located near the intracellular border of the fourth transmembrane domain and in the pore-forming loop, respectively. The position of these mutated residues suggests an alteration of the channel functionality. A mutation in the same region of the protein has been recently described (V446L) that does not impair mucolipin channel activity but affects its inhibition by reduction of pH [22]. Conversely, the T232P mutation, located in the first putative extracellular loop outside the TRP domain [25], is probably not directly involved in channel activity.

Due to the lack of antibodies that allow the detection of the endogenous mucolipin, the expression and intracellular localization of the wild-type and mutant mucolipin isoforms were studied by epitope-tagged protein transient expression.

We first constructed Myc- and HA-tagged wild-type mucolipin and three Myc-tagged mucolipin mutants. The T232P-, F465L-, F408del-mucolipin mutations were generated individually in *MCOLN1* cDNA by PCR-mediated site-directed mutagenesis. We subsequently transfected HeLa cells with the different Myc-tagged mucolipin constructs.

The expression of wild-type mucolipin and of mucolipin mutant proteins was confirmed by Western blot analysis. We were not able to separate mucolipin as a discrete protein band using classical SDS–PAGE under reducing conditions (data not shown). On the contrary, using SDS–8 M urea PAGE, wild-type and mutant mucolipin appear as a band of about 130 kDa (Fig. 1B). In addition, a minor band of approximately 65 kDa, corresponding to the calculated molecular weight of tagged mucolipin, is detected by anti-Myc antibody. No significant degradation phenomena are observed, as demonstrated by the absence of low molecular weight bands. Overall, this electrophoretic behavior suggests that mucolipin tends to form aggregates and/or to interact with other proteins to create complexes characterized by strong intra-molecular interactions. This hypothesis is supported by the finding that TRP channels are composed by polypeptide subunits that assemble as multimers to form cation permeable pores [20,26]. The composition of these mucolipin complexes is one of the issues that might be clarified to better understand the biochemical and functional characteristics of this integral membrane protein.

3.2. Subcellular localization of wild-type mucolipin

Subcellular localization experiments were initially carried out using both Myc- and HA-tagged proteins (data not shown). The Myc-tag construct was then routinely used because we obtained the brightest staining under our experimental conditions. Wild-type Myc-tagged mucolipin has a similar distribution both in HeLa and Cos7 cells (data not shown) and localizes in punctuated structures dispersed in the Cyt (Fig. 2A). To determine if the protein distribution varies with expression time, we monitored the subcellular localization of the Myc-tagged mucolipin in HeLa cells at 6, 9, 12, 24 and 48 h post-transfection and no significant time-dependent changes were observed (data not shown). To further characterize the cellular structures containing mucolipin, we compared its distribution to markers of different cellular compartments. Mucolipin failed to localize to either mitochondria, peroxisomes, the Golgi apparatus, or to the endoplasmic reticulum (data not shown). Mucolipin partially colocalizes with the late endosomes/lysosomes marker LAMP1 (Fig. 2). This colocalization is in agreement with: (i) the presence of a di-leucine motif (L–L–X–X) as a late endosomal–lysosomal targeting signal located at the C-terminus of the protein [17]; (ii) the lysosomal nature of the disease and (iii) the analogous results obtained in *Xenopus* oocytes [21]. As shown in Fig. 2, despite a 52% of colocalization, a subset of mucolipin appears to be strictly adjacent to some LAMP1-positive vesicles, but it does not exactly colocalize with them (see blue arrows, Fig. 2D). These mucolipin-positive LAMP1-negative structures are difficult to identify unambiguously but may reasonably represent trafficking intermediates in these fluid cellular compartments.

Interestingly, HeLa cells overexpressing mucolipin (indicated with an asterisk in Fig. 2B) show an anomalous distribution and morphology of the compartment identified by LAMP1 compared to non-transfected cells. In particular, lysosomes appear more dispersed within Cyt with respect to

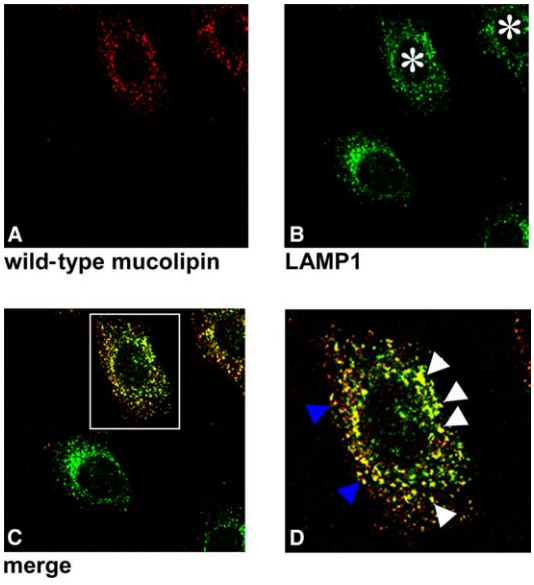

Fig. 2. Confocal microscopy analysis of the intracellular distribution of wild-type mucolipin and colocalization with LAMP1. HeLa cells were transiently transfected with wild-type mucolipin-Myc construct. 24 h after transfection, cells were stained with rabbit anti-Myc, mouse anti-LAMP1 antibodies and subsequently Cy2- and Cy3-conjugated secondary antibodies. (A) Intracellular distribution of wild-type mucolipin. (B) Endogenous LAMP1 subcellular distribution. Cells overexpressing mucolipin, indicated by asterisk, show an altered distribution of LAMP1-positive compartments. (C) Colocalization between wild-type mucolipin and LAMP1. (D) Higher magnification of the inset in C. Computer-assisted image quantification indicates that about 52% of mucolipin colocalizes with LAMP1-positive compartments (see white arrows). In addition, blue arrows show mucolipin-positive structures that do not completely colocalize with LAMP1.

non-transfected cells. The meaning of this redistribution is not yet understood and could be due to the overexpression of a functional mucolipin cation channel, since we cannot observe any similar redistribution of lysosomes in cells overexpressing the mutant F465L and T232P forms (see below).

To further investigate this interesting phenomenon, additional markers for the endocytic pathway have been used. These include a marker for the early endocytic compartment, such as EEA1, and the more widely distributed MPR46, a protein involved in the delivery of lysosomal enzymes [27]. No evident colocalization with EEA1 was observed and no redistribution of the early endocytic compartment was promoted by the overexpression of mucolipin (data not shown). Conversely, mucolipin is present, to a limited extent, in compartments labeled by MPR46 that might correspond to late endosomal structures (Fig. 3A). It is not obvious whether the overexpression of mucolipin promotes also a redistribution of MPR46-positive structures.

For late endosomal–lysosomal compartment we used Lysotracker, a fluorescent acidotropic probe for labeling and tracking acidic organelles, and LBPA, a marker of late endosomes. Similarly to what we observed for LAMP1, also Lysotracker and LBPA-positive compartments appear to be redistributed in mucolipin overexpressing cells (Figs. 3B and C, respectively). The merge of the confocal images indicates that mucolipin colocalizes with Lysotracker and LBPA although with a lower degree of colocalization compared with the one observed for LAMP1. This may indicate that overexpressed mucolipin behaves like a dynamic protein that moves through the late endocytic compartment in a way similar to LAMP1. On the contrary, LBPA is a resident late endosomal marker and Lysotracker is a unidirectional endocytic fluid phase marker that accumulates in mature lysosomes. The lysosomal–late endosomal localization of mucolipin is consistent with recent data on cup-5, a *Caenorhabditis elegans* functional ortholog of *MCOLN1*. Mutations in cup-5 result in the accumulation of large vacuoles in several cells, in increased cell death and in embryonic lethality [28,29]. CUP-5 protein localizes to nascent and mature lysosomes, in particular to subdomains of the late endosomes where lysosomally destined endocytic cargo accumulates in buds [30]. Taken together, these data indicate that CUP-5 and human mucolipin are required for lysosome reformation/biogenesis.

The delivery of lysosomal enzymes from the Golgi to the endosomal compartment is mediated by MPR46, while the delivery from endosomes to lysosomes is MPR46-independent. Although we did not observe a significant redistribution of MPR46 in mucolipin overexpressing cells, we cannot exclude that the final targeting of lysosomal enzymes is somehow impaired, considering the redistribution of the late endocytic

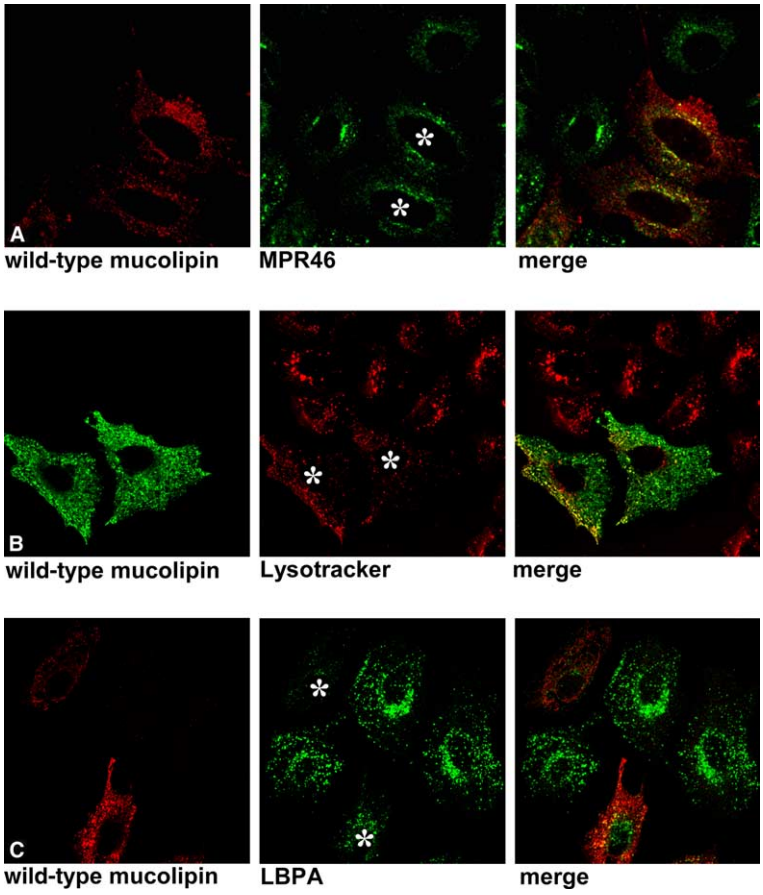

Fig. 3. Confocal microscopy analysis of wild-type mucolipin and colocalization with different markers for the endocytic pathway. HeLa cells were transiently transfected with mucolipin-Myc construct for 24 h and then stained alternatively with anti-MPR46, Lysotracker Red or anti-LBPA antibodies together with rabbit anti-Myc. Cells were subsequently stained with Cy2- and Cy3-conjugated secondary antibodies. Asterisks indicate mucolipin transfected cells. Mucolipin partially colocalizes with MPR46 (A), Lysotracker (B) and LBPA (C). Lysotracker (B, middle row) and LBPA (C, middle row) are redistributed in mucolipin expressing cells (asterisks).

structures we observed in transfected cells. To address this point, we evaluated the enzymatic activities of two lysosomal hydrolases (β-hexosaminidase and β-galactosidase) both in cells and media from mucolipin transfected cells. In order to inhibit receptor-mediated endocytosis of secreted lysosomal enzymes, cells were grown both in the presence or absence of mannose 6-phosphate. Under these experimental conditions, no significant differences were found neither in the media nor in cell extracts of transfected and untransfected cells (data not shown), indicating that the lysosomal sorting machinery does not appear to be grossly affected by mucolipin overexpression.

3.3. Subcellular localization of mucolipin mutants

We then analyzed the subcellular distribution of three previously described naturally occurring mutations located in distinct regions of the protein. The mutations studied were: a single amino acid deletion (F408del) [16,17] and two missense mutations, T232P and F465L [5] (Fig. 1A). Analysis of the mutated amino acid residues demonstrates a variable amount of sequence conservation amongst members of the mucolipin family. The residues corresponding to F408 and F465 in human mucolipin are conserved in the mouse, *Drosophila* and *C. elegans* [28,29] putative orthologs and in the MLN3 [19], while the T232 residue appears to be conserved only in mouse Mcoln1 [31].

The T232P mutant fails to localize to late endocytic compartments and clearly accumulates in the ER, as shown by its restricted distribution to calnexin-positive structures (Fig. 4A). As a possible explanation, the T232 residue might be somehow involved in the proper transport of mucolipin through the

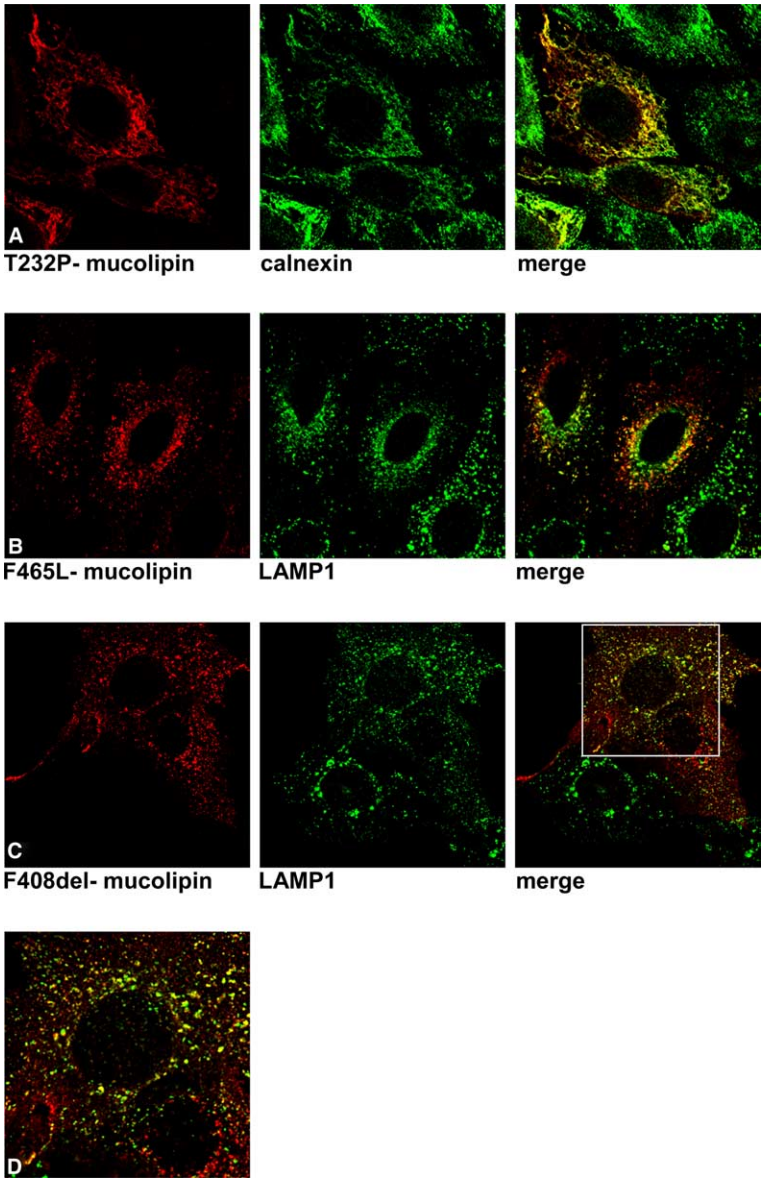

Fig. 4. Confocal microscopy analysis showing subcellular localization of tagged mutant forms of mucolipin. HeLa cells were transiently transfected with mucolipin mutants cDNAs (A–D) as indicated in the left row. Cells were fixed after 24 h of expression and stained with anti-Myc antibody and the respective cellular marker as indicated in the middle row. Merged images are shown in the right row. The T232P mutant protein colocalizes with the ER protein calnexin (A). F465L-mucolipin partially localizes (28%) to LAMP1-positive vesicles (B). F408del mutant protein shows a higher degree of colocalization (37%) with LAMP1-positive compartment (C). Only F408del mutant induces redistribution of LAMP1-positive vesicles. (D) A higher magnification of the inset in C.

biosynthetic pathway and/or the T232P substitution might result in the misfolding of the protein leading to a diverted route of maturation and delivery by chaperons. Although the functional role of the loop where this mutation occurs is still unknown, it is interesting to note that mucolipin and PC2 (both involved in diseases characterized by an intracellular trafficking defect) are the only members of the TRP superfamily having such a large extracellular loop between the first and the second transmembrane domains. Although these data have been obtained by overexpression techniques, the complete mislocalization of T232P mutant we observed is in agreement with the severe clinical phenotype described in the MLIV patient carrying this mutation [5].

Both F465L and F408del mutants show a punctuated cytoplasmic distribution similar to the wild-type protein. As shown in Fig. 4B–D, the mucolipin mutant proteins are either colocalizing or adjacent to LAMP1-positive compartments. To date the precise nature of the LAMP1-negative structures where the wild-type and F465L and F408del proteins distribute is not clear. A more detailed analysis performed using computer-assisted image quantification reveals that the degree of colocalization with LAMP1 is higher for the F408del than for the F465L protein (see also legend of Fig. 4). Interestingly, only overexpression of F408del induces an altered distribution of the late endosomal–lysosomal marker LAMP1, as already observed for the wild-type protein.

The latter observation suggests that, despite a similar subcellular localization of F465L and F408del mutants, only the latter seems to maintain at least part of the capability of the wild-type protein to promote a redistribution of the late endocytic compartments when overexpressed. These findings are supported by the clinical observations: these mutations have been described in two patients who are both compound heterozygotes for the major AJ splice mutation [5]. The patient carrying the in-frame deletion F408del is characterized by unusual mild psychomotor retardation [32], while the patient carrying F465L amino acid substitution mutation has a severe clinical phenotype similar to those patients completely lacking mucolipin protein.

Although our studies, based on protein overexpression, cannot unambiguously establish the subcellular localization of the endogenous wild-type and mutant mucolipin isoforms, they provide precious hints about the importance of the correct gene dosage in MLIV therapeutic approaches based on gene therapy.

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