TRP-ML1 Is a Lysosomal Monovalent Cation Channel That Undergoes Proteolytic Cleavage*

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Mutations in the gene MCOLN1 coding for the TRP (transient receptor potential) family ion channel TRP-ML1 lead to the lipid storage disorder mucolipidosis type IV (MLIV). The function and role of TRP-ML1 are not well understood. We report here that TRP-ML1 is a lysosomal monovalent cation channel. Both native and recombinant TRP-ML1 are cleaved resulting in two products. Recombinant TRP-ML1 is detected as the full-length form and as short N- and C-terminal forms, whereas in native cells mainly the cleaved N and C termini are detected. The N- and C-terminal fragments of TRP-ML1 were co-immunoprecipitated from cell lysates and co-eluted from a Ni2+ column. TRP-ML1 undergoes proteolytic cleavage that is inhibited by inhibitors of cathepsin B (CatB) and is altered when TRP-ML1 is expressed in CatB−/− cells. N-terminal sequencing of purified C-terminal fragment of TRP-ML1 expressed in Sf9 cells indicates a cleavage site at Arg200. Consequently, the conserved R200H mutation changed the cleavage pattern of TRP-ML1. The cleavage inhibited TRP-ML1 channel activity. This work provides the first example of inactivation by cleavage of a TRP channel. The significance of the cleavage to the function of TRP-ML1 is under investigation.

Mucolipidosis type IV (MLIV) is a lipid storage disorder characterized by an abnormal accumulation of membranous lipids in patients’ cells (reviewed in Refs. 1 and 2). Clinically, the disease manifests as corneal clouding, degeneration of the retina, and severe psychomotor retardation (1–6). MLIV is associated with mutations in MCOLN1 (TRP-ML1), a member of the TRP (transient receptor potential) family of ion channels (7–9). The TRP family includes several members that are implicated in human diseases, such as TRPP2 (10), TRPM1 (11), and TRPV6 (12). A critical question in MLIV pathogenesis is why do mutations in TRP-ML1 lead to the cellular phenotype of MLIV?

Previous work on the ion selectivity and permeation of TRP-ML1 produced conflicting results. Thus, transient expression in X. oocytes and in fibroblasts suggests that TRP-ML1 is targeted to the lysosomes and functions as a Ca2+-permeable channel that may regulate lysosomal Ca2+ release and consequently agonist-evoked Ca2+ signals (13, 14). On the other hand, TRP-ML1 synthesized in cell-free system and reconstituted into planar lipid bilayers behaves as a monovalent cationic permeable, outwardly rectifying channel (15). The outward rectification indicates that when present in lysosomes, TRP-ML1 primarily moves ions into the lysosomal lumen. The outward rectification makes it unlikely that in vivo TRP-ML1 would function as a lysosomal Ca2+ release channel, which suggested an alternative role of TRP-ML1 in lysosomal and cellular functions.

In the present report we analyzed the expression pattern and channel properties of TRP-ML1 and several disease-associated mutants. We report that TRP-ML1 is an outwardly rectifying monovalent cation-permeable channel that is primarily expressed in the lysosomes. In the lysosomes, TRP-ML1 is inactivated by proteolytic cleavage. These findings suggest a novel mechanism of regulating TRP-ML1 function.

EXPERIMENTAL PROCEDURES

Materials—The DNA-modifying enzymes N-glycosidase F (Endo F), and β-endo-N-acetylglucosaminidase H (Endo H) were from New England Biolabs. QuikChange site-directed mutagenesis kit was from Stratagene. Cathepsin B inhibitors were from Calbiochem, and cathepsin B was from Sigma. CatB−/− cells were generously provided by Dr. Terence S. Dermod (Vanderbilt University, Nashville, TN).

TRP-ML1−/− Cells—Human skin fibroblasts (HSF), clone WG9099, that is TRP-ML1−/−, and the WG9098 clone, a heterozygous relative, were obtained from the Repository for Mutant Human Cell Strains, Montreal Children’s Hospital. Fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1-glutamine, and non-essential amino acids.

TRP-ML1 Expression Constructs—The full-length sequence corresponding to the human TRP-ML1 coding region was amplified by PCR using IMAGE clone BF 529860 as template. The 1.7-kb amplified product was subcloned into the pCMV vectors either with no tag, an N-terminal HA tag, or a C-terminal Myc tag. Insert orientation and polymerase fidelity were verified by restriction enzyme mapping and sequencing.

Site-directed Mutagenesis, Cell Transfection, and Immunoblotting—The plasmid pCMV-HA-TRP-ML1 was used as a template to construct mutants using a mutagenesis kit (QuickChange, Stratagene). All mutations were confirmed by sequencing the entire DNA insert to verify the presence of the desired mutation and the absence of extraneous mutations. HEK293 cells were transfected in 60-mm dishes with 5 μg of plasmid DNA and 10 μl of Lipofectamine 2000 (Invitrogen).

Cell extracts were prepared by sonication in homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 5 mM MgCl2, and Complete protease inhibitor mixture tablet (Roche Applied Science). Microsomal pellets were extracted with 1% CHAPS or 1% Triton X-100 and subjected to SDS-PAGE and immunoblotting with

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FIGURE 1. Intracellular localization of WT and mutant TRP-ML1. A, co-localization of WT TRP-ML1 with LAMP1. HA-tagged TRP-ML1 was expressed in WT HSF, and the cells were co-stained with anti-HA and anti-LAMP1 antibodies. The overlap between TRP-ML1 and LAMP1 was analyzed using an RGID co-localization add-on to ImageJ software. B, limited co-localization of TRP-ML1 with the early and late endosomal markers mannose 6-phosphate receptor (MPR) and EEA1. C, localization of the HA-tagged T232P, D362Y, and F465L mutants. D and E, co-localization of the F465L mutant with LAMP1 (D) and WT TRP-ML1 tagged with Myc (E).

RESULTS AND DISCUSSION

Localization of WT TRP-ML1 and Mutants—Expression in HeLa cells suggested primarily lysosomal localization of TRP-ML1 (16). To verify TRP-ML1 localization, HA-tagged human TRP-ML1 (HA-TRP-ML1) was expressed in HEK293 cells, HeLa cells, and HSF. Fig. 1 shows that WT TRP-ML1 is primarily present in intracellular compartments. To identify the compartment in which TRP-ML1 is localized, cells transfected with HA-TRP-ML1 were co-stained with anti-HA antibodies and antibodies against EEA1, the mannose 6-phosphate receptors, or LAMP1 as markers for early endosomes, late endosomes/Golgi, and lysosomes, respectively. Fig. 1, A and B, shows that TRP-ML1 co-localized with mannose 6-phosphate receptors (MPR) only in the Golgi, probably because of TRP-ML1 over-expression. Significant overlap of TRP-ML1 was found only with LAMP1, confirming its lysosomal localization.

Several disease-associated TRP-ML1 mutants have been identified (7, 8, 17, 18). The expression pattern of some of these mutants is shown in Fig. 1C. The T232P and D362Y mutations resulted in retention of TRP-ML1 in the endoplasmic reticulum (Fig. 1C). Hence, these mutations cause MLIV probably because they are not targeted to the lysosomes. On the other hand the expression pattern of the F465L mutant was identical to that of WT TRP-ML1 (Fig. 1, D and E). As will be shown below, this mutation inhibits the channel activity of TRP-ML1, which explains why this mutation also results in MLIV.

TRP-ML1 Is Cleaved at a Post-Golgi Compartment—Expression of TRP-ML1 tagged with HA in its N terminus (HA-TRP-ML1) in HEK293 cells resulted in two major protein products: the predicted full-length (FL) TRP-ML1 (65–75 kDa) and a short form of 36 ± 1 kDa (Fig. 2A, left panel). Likewise, expression of TRP-ML1 tagged with Myc in its C terminus (TRP-ML1-Myc) also resulted in two glycosylated protein products (Fig. 2A, right panel). Hence, when over-expressed, a significant portion of TRP-ML1 is cleaved to result in N- and C-terminal fragments.

The large loop between the first and second transmembrane domains (1–2 loop) of TRP-ML1 contains four putative N-glycosylation sites (Fig. 2B). To test whether TRP-ML1 is cleaved at or after exit from the endoplasmic reticulum/Golgi compartments, lysates from cells expressing HA-TRP-ML1 and TRP-ML1-Myc were treated either with Endo F, which cleaves all N-linked sugars, or with Endo H, which cleaves only high mannose-type or hybrid-type sugar groups that have not been
digested by Golgi mannosidase II. Fig. 2A shows that the FL TRP-ML1 consolidated into a single lower molecular weight band following treatment with Endo F, but only a small fraction of the FL was sensitive to digestion with Endo H. Digestion with Endo F, but not Endo H, changed the migration of the N-terminal fragment of TRP-ML1. Therefore, both the FL and the N-terminal fragment of TRP-ML1 are sensitive to Endo F but are partially or completely resistant to Endo H.

The C-terminal fragment was sensitive to Endo F. The migration of the entire fragment was only slightly changed by Endo H (Fig. 2A, right panel). This may be because the C-terminal fragment was not modified in the Golgi. Alternatively, because the cleavage of TRP-ML1 is predicted to be between the four putative glycosylation sites (Fig. 2B), it is possible that only one of the glycosylation sites on the C-terminal fragment was modified in the Golgi. To distinguish between these possibilities we treated transfected cells with Swainsonine, a specific inhibitor of the plasma membrane or lysosomal lumen for a lysosome-resident channel. C, extracts prepared from WT and TRP-ML1 expressing cells were treated with Endo F and Endo H, the blots were probed with αN1.

Mutual co-IP of the N and C termini of TRP-ML1 (Fig. 3A) suggests that they remain associated after the proteolytic cleavage. Furthermore, IP of the C terminus depleted the N terminus and IP of the N terminus depleted the C-terminal fragments in cell extracts (Fig. 3B). The interaction between the C- and N-terminal fragments is most likely direct because elution of TRP-ML1-V5-His expressed in S9 cells after extensive washing of nonrelevant proteins co-eluted both fragments from the Ni2+–NTA column with buffer containing 1% CHAPS (Fig. 4A).

**Iden**tification of the Cleavage Site—To determine the cleavage site, TRP-ML1-V5-His was expressed in S9 cells and purified on a Ni2+–NTA column. The C-terminal fragment was subjected to N-terminal sequence analysis. In two experiments N-terminal sequencing yielded the sequence PPPP, corresponding to residues 201–204 of TRP-ML1, suggesting that the cleavage was between Arg200 and Pro201. Because the peaks were not very sharp, probably due to the glycosylation of the C-terminal, we verified the cleavage site by replacing the sequence 197PPERPPPPP205 with alanines. Although these mutations did not prevent the cleavage, they did alter the cleavage pattern of TRP-ML1 (Fig. 4A). Furthermore, the conserved substitution R200H also altered the cleavage pattern (Fig. 4B). All mutations tested around the potential cleavage site altered but failed to prevent cleavage. This is likely because in the lysosomes TRP-ML1 is cleaved by multiple proteases. TRP-ML1 has a classical dileucine lysosomal targeting motif at the C terminus, EEHSLLVN. In an attempt to prevent targeting of TRP-ML1 to the lysosomes, we deleted the LLVN sequence, which includes the critical dileucine motif (20). Unfortunately, deletion of this sequence did not affect TRP-ML1 localization (Fig. 4C), indicating that another sequence, yet to be identified, determines the lysosomal targeting of TRP-ML1. Nevertheless, the combination of sequencing and the mutation analysis point to Arg200 as a potential cleavage site. After this cleavage, the C- and N-terminal fragments remain resistant to further cleavages and accumulate in the cells.

**Potential Role of CatB in TRP-ML1 Cleavage**—Lysosomal localization of TRP-ML1 and the resistance of N- and C-terminal fragments to Endo H raised the possibility that TRP-ML1 is cleaved in the lysosome. To identify the lysosomal protease that cleaves TRP-ML1, HEK293 cells transfected with TRP-ML1 were treated with 1 μM E64d, a membrane-permeable cysteine protease inhibitor, and with 0.2 μM CA-074-Me, a membrane-permeable selective cathepsin B (CatB) inhibitor (21). The two inhibitors largely prevented cleavage of TRP-ML1 (Fig. 4D).
control, the cathepsin K inhibitor, CatK inhibitor II, did not affect TRP-ML1 cleavage at concentrations as high as 10 μM.

Inhibition of cleavage by CA-074-Me suggested that CatB is involved in the cleavage of TRP-ML1. A more direct role for CatB was obtained by expressing human TRP-ML1 in mouse CatB−/− fibroblasts (Fig. 4E). We were not able to analyze the fate of the native mouse TRP-ML1 in the CatB−/− fibroblasts because αN1 only recognized the human isoform. Human TRP-ML1 was cleaved in CatB−/− fibroblasts; however, the cleavage was at a site different from that observed in WT mouse fibroblasts as evident from the generation of an ~3-kDa longer N-terminal fragment in TRP-ML1−/− cells.

In aggregate, the findings shown in Fig. 4 indicate that TRP-ML1 is targeted to the lysosomes where it is cleaved by CatB or a CatB-dependent pathway. Another possibility is that TRP-ML1 is cleaved by multiple lysosomal proteases and the final cleavage, mediated by CatB, occurs at the Arg200↓Pro201 site. Inhibition of CatB and expression of TRP-ML1 in CatB−/− cells prevent the final cleavage by CatB and unmask the cleavage by other lysosomal proteases, resulting in the different cleavage patterns illustrated in Fig. 4. In either case, cleavage by CatB plays a prominent role in the cleavage of TRP-ML1 in the lysosomes.

That the cleavage occurs after the exit of TRP-ML1 from the endoplasmic reticulum is further confirmed by the differential processing of the mutants shown in Fig. 5A. Full-length T232P and D362Y were completely sensitive to digestion by both Endo F and Endo H, confirming that these mutants are retained in the endoplasmic reticulum or cis-Golgi (Fig. 1). Consequently, these mutants were also not cleaved. By contrast, the F465L mutant was cleaved and the cleaved product was resistant to Endo H, consistent with normal targeting and processing of this mutant. The finding that targeting and processing of the F465L mutant was normal suggests that the channel function of TRP-ML1 is not required for its targeting.

The Channel Function of WT TRP-ML1 and Mutants—Although the majority of TRP-ML1 is expressed in intracellular compartments, surface biotinylation showed that some of the over-expressed WT and F465L-TRP-ML1 was targeted to the plasma membrane (Fig. 5B). Under this moderate over-expression condition, no T232P and minimal amounts of the D362Y mutants were found at the plasma membrane. Saturation of the protein trafficking pathway by marked over-expression forced expression of significant amounts of the FL and short forms of TRP-ML1 and only the FL form of the T232P mutant at the plasma membrane (PM) (Fig. 5C). Such PM mistargeting was necessary to study TRP-ML1 channel properties using the whole cell configuration.

TRP-ML1 conductance was measured by whole cell current recording with intracellular solution containing cesium aspartate and extracellular solution containing sodium aspartate or NaCl. Under these conditions, TRP-ML1 showed characteristic strongly outwardly rectifying current (Fig. 6, A and B). The strong outward rectification indicates preferential transport of ions from the cytoplasm into the lysosomal...
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TRP-ML1 Is Inactivated by Proteolytic Cleavage—The finding that the two fragments of TRP-ML1 remained associated (Fig. 4) and that FL and short TRP-ML1 were present at the PM (Fig. 5, B and C) raised the question of which form of TRP-ML1 mediates the current and what is the role of the channel cleavage. The first clue that the cleavage inactivates TRP-ML1 was obtained by measuring the current in cells expressing the T232P mutant. Cells over-expressing the T232P mutant showed small TRP-ML1-specific current (Fig. 6, A and C), although not as often as cells over-expressing WT TRP-ML1. Because only the FL T232P was present at the PM (Fig. 5C), the TRP-ML1 current must be mediated by the FL protein.

Further evidence that CatB-mediated cleavage inactivates TRP-ML1 is presented in Fig. 7. In the first series of experiments, TRP-ML1 expressed in HEK293 cells was treated with CatB while recording the whole cell current. Fig. 7, A and B, shows that within 50–60 s of treatment with 0.2 or 0.5 units/ml recombinant CatB at pH 5, the current decreased by 30–60%. The cells were incubated at pH 5 for several minutes before CatB application. No run-down of TRP-ML1 channel activity was detected during this treatment. Another TRP channel, TRPC3, was used as a control for the specificity of the effect of CatB. Cells expressing TRPC3 were pretreated with CatB at pH 5, and the amplitude of the current activated by stimulation of the P2Y2 receptors with UTP was compared in control cells and cells treated with CatB. Treatment with CatB did not affect the amplitude of the UTP-stimulated TRPC3-dependent current (Fig. 7C).

In the second set of experiments HEK293 cells expressing TRP-ML1 were treated with the CatB inhibitor E64d. Biotinylation showed that inhibition of CatB, which reduces the cleavage to increase the level of FL TRP-ML1 (Fig. 4D), increased the level of FL TRP-ML1 at the plasma membrane (Fig. 7B). This correlated with an increased TRP-ML1-mediated current (Fig. 7B). Hence, the combined results with the T232P mutant, treatment with CatB, and the effect of the CatB inhibitor suggest that the cleavage served to inactivate TRP-ML1 channel function.

Conclusions—We report here that TRP-ML1 is a lysosomal, monovalent, cation channel that is inactivated by cleavage. The cleavage may be mediated by multiple lysosomal proteases, one of which is CatB, which appears to mediate the critical or final cleavage. Importantly, native TRP-ML1 is also cleaved. Furthermore, the cleaved form is the predominant form of the channel found in native cells. The cleavage serves to inactivate the channel function of TRP-ML1.

The question that arises is why do cells need to inactivate TRP-ML1? TRP-ML1 has a central role in lipid hydrolysis and processing, as evident from the disease phenotype and as will be shown in subsequent publications. It is possible that inactivation by cleavage constitutes a regulatory mechanism to limit the duration of TRP-ML1 channel activity. Unregulated TRP-ML1 activity may be detrimental to lipid processing. TRP-ML1 is probably targeted en masse to the lysosomes, where its function is rapidly completed and it is inactivated by cleavage to prevent disruption of lysosomal ionic homeostasis, which results in the accumulation of the cleaved form of the channel.
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