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Purification, cellular levels, and functional domains of lipase maturation factor 1

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ABSTRACT

Over a third of the US adult population has hypertriglyceridemia, resulting in an increased risk of atherosclerosis, pancreatitis, and metabolic syndrome. Lipoprotein lipase (LPL), a dimeric enzyme, is the main lipase responsible for TG clearance from the blood after food intake. LPL requires an endoplasmic reticulum (ER)-resident, transmembrane protein known as lipase maturation factor 1 (LMF1) for secretion and enzymatic activity. LMF1 is believed to act as a client specific chaperone for dimeric lipases, but the precise mechanism by which LMF1 functions is not understood. Here, we examine which domains of LMF1 contribute to dimeric lipase maturation by assessing the function of truncation variants. N-terminal truncations of LMF1 show that all the domains are necessary for LPL maturation. Fluorescence microscopy and protease protection assays confirmed that these variants were properly oriented in the ER. We measured cellular levels of LMF1 and found that it is expressed at low levels and each molecule of LMF1 promotes the maturation of 50 or more molecules of LPL. Thus we provide evidence for the critical role of the N-terminus of LMF1 for the maturation of LPL and relevant ratio of chaperone to substrate.

1. Introduction

LPL plays a critical and complex role in lipid metabolism. LPL hydrolyzes triglycerides from two classes of circulating lipoproteins, very low-density lipoproteins and chylomicrons, in order to distribute free fatty acids to peripheral tissues. Biochemical deficiency of LPL activity is one well-established cause of hypertriglyceridemia, which is associated with increased risk of atherosclerosis, acute pancreatitis, and presence of metabolic syndrome [1]. Mutations in both LPL and its interacting partners can result in biochemical deficiency of LPL activity. Here we investigate how one of these interacting partners, LMF1, promotes LPL activity.

Deleterious mutations in the gene for *LMF1* result in severe hypertriglyceridemia [2,3]. *LMF1*'s precise genetic location was recently discovered [2], but its role in promoting LPL activity is well established. Mice with a recessive mutation on chromosome 17 were severely deficient for LPL activity and hepatic lipase activity [4]. This mutation was termed *cld*, for combined lipase deficiency [4]. Mice with homozygous disruptions in the *LPL* gene had phenotypes that were indistinguishable from *cld*/*cld* mice (death within 48 h of birth with extreme hypertriglyceridemia [5]). However, the *cld* mutation did not affect the LPL and hepatic lipase structural genes, as these genes mapped to different chromosomes [6]. Furthermore, the amount of LPL protein present in tissues was not reduced, but its activity was [7]. LPL activity was reduced because the majority of the LPL was retained in the ER as inactive aggregates in *cld*/*cld* cells [8]. Recently, the *cld* mutation was mapped to a gene coding for an ER-resident, transmembrane protein, and renamed *LMF1* [2]. Subsequently, LMF1 was found to be important for the activity of a third dimeric lipase, endothelial lipase [9].

Although LMF1 is vital for secretion of active, dimeric lipases, it is not clear how it promotes the exit of dimeric lipases from the ER. It is therefore important to determine which domains of LMF1 contribute to dimeric lipase maturation. Mapping studies of LMF1's domain architecture reveal that it has a total of five transmembrane domains with its N-terminus in the cytosol and its C-terminus in the ER lumen [10]. The loops connecting these transmembrane domains are labeled A–D and are diagramed in Fig. 1A. Recent data suggest that loop C and the C-terminus of LMF1 are important for dimeric lipase maturation [10]. The importance of LMF1's C-terminal, ER resident domain was established in studies of the original *cld* mutation and in patients with *LMF1* mutations.

Abbreviations: LPL, lipoprotein lipase; LMF1, lipase maturation factor 1; ER, endoplasmic reticulum; *cld*, combined lipase deficiency.

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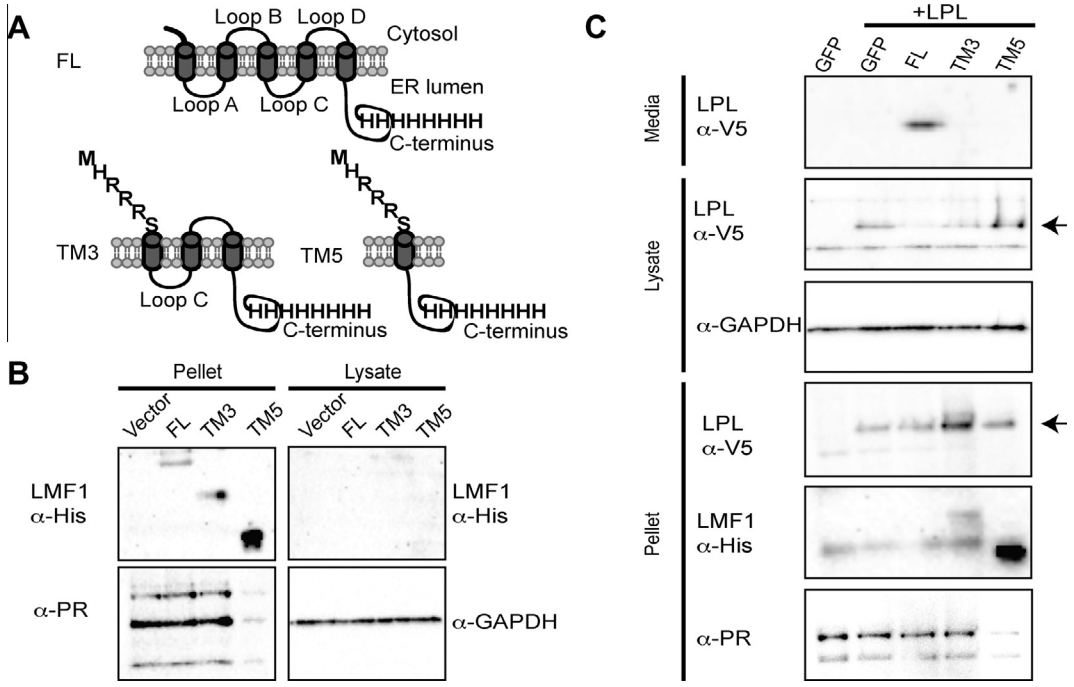

Fig. 1. The N-terminal domain of LMF1 contributes to LPL maturation. (A) A schematic of LMF1's topology. (B) Western blots against the C-terminal His tag show that FL, TM3 and TM5 truncations are associated with the pellet fraction. Loading controls include GAPDH for soluble proteins in the lysate fraction and PR for membrane proteins. (C) Western blots of the media fraction show that LPL-V5 is secreted in *cld*/*cld* cells co-expressing the FL, but not the TM3 or TM5, LMF1 constructs. Arrows indicate expression of LPL-V5. Although we loaded 1/5 as much of the highly expressed TM5 construct in all panels to allow detection of other constructs, saturated pixels were unavoidable in panel 5.

Two nonsense mutations in the C-terminal ER domain of LMF1 (Y439X and W464X) resulted in truncated variants that were unable to assist dimeric lipases in the maturation process [2,3]. Additionally, co-immunoprecipitation studies performed on C-terminal LMF1 truncations showed that loop C is important for interaction with dimeric lipases [10]. Before this current study, nothing was known about the role, if any, of the N-terminal portions of LMF1.

Here, we determine which of LMF1's domains are essential for its function and how LMF1 interacts with LPL by measuring the cellular levels of both proteins. To determine if the C-terminal portions of LMF1 were sufficient to promote dimeric lipase maturation, we made N-terminal LMF1 truncation variants. These LMF1 truncations are properly localized and oriented in the ER membrane. However, expression of these constructs in *cld*/*cld* cells show that the entire LMF1 protein is required for maturation of LPL. We generated a high-affinity, polyclonal antibody using purified LMF1. We found that endogenous LMF1 levels are low, and each LMF1 molecule promotes the maturation of at least 50 molecules of LPL.

2. Experimental procedures

2.1. Expression constructs

Constructs for the expression of CD3δ-YFP and CFP-CD3δ [11] and mCherry-KDEL (mCh-KDEL) [12] have been described. The coding sequence of human LPL was amplified from pCMV-SPORT6-LPL (Open Biosystems) with a C-terminal V5 epitope tag and inserted into the NheI and XbaI sites of pIRES-EGFP (Addgene). For LMF1 variants, the cDNA for human LMF1 was obtained from Open Biosystems (ID 100062174) and inserted into the BamHI/Xho1 sites of pcDNA5/FRT/TO with an 8× C-terminal polyhistidine tag. The forward primer for TM3 (5′-tcccggattgtcctgtggggc-3′) and TM5 (5′-tcccggattgtcctgtggggc-3′) were selected based on computational models for the topology of human LMF1 [10]. Both LMF1 truncations included an N-terminal MHRRRS ER retention signal from human invariant chain isoform Iip33 [13] and an 8× C-terminal polyhistidine tag. To generate pFastbacLMF1, the human *LMF1* coding sequence was amplified with a C-terminal His-tag and inserted into the Spe1 and Xba1 sites of pFastbac1 (Invitrogen).

2.2. Cell lines, transfection, and media collection

COS-7, *cld*/*cld* and *cld*/*wt* [14] cell lines were maintained in Dubelcco's modified Eagle's medium, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine (complete medium). Transfections were performed with 2 μg of DNA and X-treme gene (Roche) according to manufacturer's instructions. COS-7 cells were transfected using Fugene 6 (Promega) with 0.3 μg of mCh-KDEL, 1.7 μg of LMF1 constructs, and 1 μg of CD3δ-YFP and CFP-CD3δ. Cells were transfected at 80% confluency and harvested 24 h post-transfection. For secretion experiments, 24 h after transfection, the media of *cld*/*cld* or *cld*/*wt* cells was changed to complete media but with 1% FBS and 15 units/mL of heparin 3 h prior collection, 600 μL media was used per 9.5 cm^2 well.

2.3. Western blot analysis

Cells were lysed 24 h after transfection using lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris pH 8). The pellets were re-suspended with 3X SDS loading dye diluted with 8 M urea. Media, lysate, and pellet samples were separated using 12% SDS–PAGE. Proteins were transferred to PVDF (Millipore) and blocked with 5% nonfat milk in TBS-T. For LMF1 Westerns, chicken anti-LMF1 antibody was used at 1:10,000, AP-conjugated anti-chicken (Thermo Scientific) was used at 1:5000, and Westerns were developed using ECF reagent (GE Healthcare). Epitope tags were detected with anti-His and anti-V5 antibodies diluted 1:5000 (both mouse, Thermo Scientific). Mouse anti-GAPDH (Millipore) was

used at 1:20,000, and HRP-conjugated anti-mouse (Southern Biotech) was used at 1:20,000. Rabbit anti-progesterone receptor (PR, Santa Cruz) was used at a 1:100 dilution. HRP-conjugated secondary antibodies were used at 1:5000. Westerns were developed using ADVANSTA WesternBright reagent (Bioexpress).

2.4. Indirect Immunofluorescence

COS-7 cells were plated to 70% confluency on glass coverslips. Twenty-four hours post-transfection, the cells were fixed with 4% paraformaldehyde in PBS for 15 min. Cells were washed three times with PBS then incubated for 10 min with 0.1% triton X-100 and 100 mM glycine. Coverslips were washed three times with PBS, then blocked with 2% BSA for 30 min, and incubate with anti-His antibody (1:200 in 2% BSA) for 1 h at room temperature. Secondary antibody (Alexa Fluor 488-conjugated anti-mouse, Molecular Probe) was diluted 1:800 in 2% BSA and incubated for 1 h at room temperature in darkness. DAPI (Sigma–Aldrich) was used at 0.8 μg/mL for 10 min. After further washing in PBS, coverslips with cells were mounted facedown onto glass slides (Fisher) using ProLong Gold Antifade (Molecular Probes). Cells were examined at room temperature under a Zeiss LSM 710 confocal microscope with a 63X oil/1.4 Plan Apo.

2.5. Protease protection assay (PPA)

The PPA was performed as described [15] with the following modifications. COS-7 cells were permeabilized with 120 μM digitonin (Sigma Aldrich) for 1 min followed by 20 μM trypsin (Sigma Aldrich) incubation for 2 min. Complete medium was added to stop trypsin cleavage. The cells were spun at 9000 RPM for 5 min, washed with PBS, and lysed with lysis buffer for 30 min. Samples were analyzed by Western blotting as above. LMF1 and GFP were detected with anti-His and anti-GFP (rabbit, Abcam) antibodies, respectively, at 1:5000. HRP-conjugated secondary antibodies were used at 1:20,000.

2.6. Protein purification

LPL-V5 was purified essentially as previously described [16]. LMF1 was expressed in SF9 cells. Bacmids generated as per manufacturer's instructions (Invitrogen) were transfected into SF9 cells using Xtreme gene (Roche). Baculovirus was amplified for three passages and used to infect SF9 cells. Infected cells were harvested after 72 h, resuspended in Buffer 1 (20 mM Tris pH 7.5, 100 mM NaCl, and 5 mM β-mercaptoethanol) with complete protease inhibitor (Roche). Cells were lysed with 2 passes through an EmulsiFlex (Avestin). Lysate was spun at 100,000 × g for 1 h. The pellet was resuspended in Buffer 1 and dounced until in solution. Fos-Choline 12 was added to 20 mM, the solution was rocked overnight at 4°C and then spun at 100,000 × g for 1 h. The supernatant was load onto Ni–NTA (Qiagen), washed with Buffer 1 plus 3 mM Fos-Choline 12, and eluted in the same buffer plus 400 mM imidazole. Protein-containing fractions were loaded onto a MonoS column in Buffer 1 plus 3 mM Fos-Choline 12 and eluted over 15 column volumes to Buffer 2 (20 mM Tris pH 7.5, 1 M NaCl, 1 mM DTT, 3 mM Fos-Choline 12). Select fractions were further separated on S200 in Buffer 3 (20 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT, 3 mM Fos-Choline 12, 10% glycerol). Protein was flash frozen.

2.7. Antibody production

Antibodies against purified LMF1 were raised in chickens according to standard protocol (Covance) and purified as described [17].

2.8. Genomic PCR

DNA was isolated from *cld* cell lines with DNeasy kit (Qiagen) according to manufacturer's directions. The primer sequences used to distinguish between WT LMF1 and *cld* LMF1 have been described [2].

3. Results

3.1. The C-terminus of LMF1 is not sufficient for dimeric lipase maturation

The C-terminal, ER resident domain of LMF1 is necessary for efficient exit of LPL from the ER, but the role of the N-terminal domain is unknown [2,3]. To identify the minimal LMF1 construct that can support LPL maturation, we made two N-terminal truncations of LMF1 (Fig. 1A). To ensure that these truncation variants were properly targeted to and retained in the ER, we added a short, N-terminal motif from the human invariant chain isoform Iip33 previously shown to target a plasma membrane protein to the ER [13]. This Iip33 segment replaced N-terminal portions of LMF1 in two truncation constructs named TM3 and TM5 (Fig. 1A). TM5 is comprised of only the C-terminal ER domain whereas TM3 has this domain plus loop C, which was shown to be important for binding to LPL [10]. These truncations allowed us to test the role of loop C and the C-terminus of LMF1 independently. Full length (FL) LMF1, TM3 and TM5 all have C-terminal His tags for uniform detection, and all were transiently transfected in *cld*/*cld* cells. Membrane fractions from these cells were analyzed by Western blot for LMF1. The FL, TM3, and TM5 LMF1 variants were detected at the expected masses of 60, 43, and 27 kDa (Fig. 1B). TM5 was highly expressed and so 5-fold less protein was loaded to allow detection of the other LMF1 variants. No LMF1 was detected in the soluble fraction, showing that the truncation versions localize to the membrane.

Next, C-terminally V5-tagged LPL was co-expressed with each of the three LMF1 constructs in *cld*/*cld* cells to determine the minimal domain of LMF1 sufficient for LPL maturation. Heparin was added to the media to induce release of LPL from the cell surface into the media. LPL is expressed in cells with all LMF1 constructs, as it is detected in the lysate and pellet fractions (Fig. 1C). However, LPL is only secreted when FL LMF1 is present (Fig. 1C, media fraction). Taking into account that only 1/5th of the TM5 pellet was loaded, there is more LPL in the pellets of TM3 and TM5 than in the FL LMF1 pellet fraction. This suggests that when FL LMF1 is not present, LPL can't fold properly and is not able to exit the ER, as shown for cells lacking LMF1 [18]. Taken together, the release of LPL into the media only with FL LMF1 and the accumulation of LPL in the pellets when co-expressed with the TM3 and TM5 truncations indicate that FL LMF1 is needed for the maturation process of LPL.

3.2. Localization of LMF1 constructs by fluorescence microscopy

To ensure that the N-terminal ER retention signal from Iip33 properly targeted both LMF1 truncation variants to the ER we tested for co-localization with an ER marker (mCh-KDEL) by immunofluorescence. COS-7 cells were co-transfected with LMF1, TM3, or TM5 and mCh-KDEL. Fig. 2 shows that LMF1 co-localizes with mCh-KDEL. The merge of the two bottom panels of Fig. 2 shows that both LMF1 truncation variants co-localize with mCh-KDEL as well as FL LMF1 does. We conclude that although TM3 and TM5 are not sufficient to promote LPL maturation, both truncations are present in the ER.

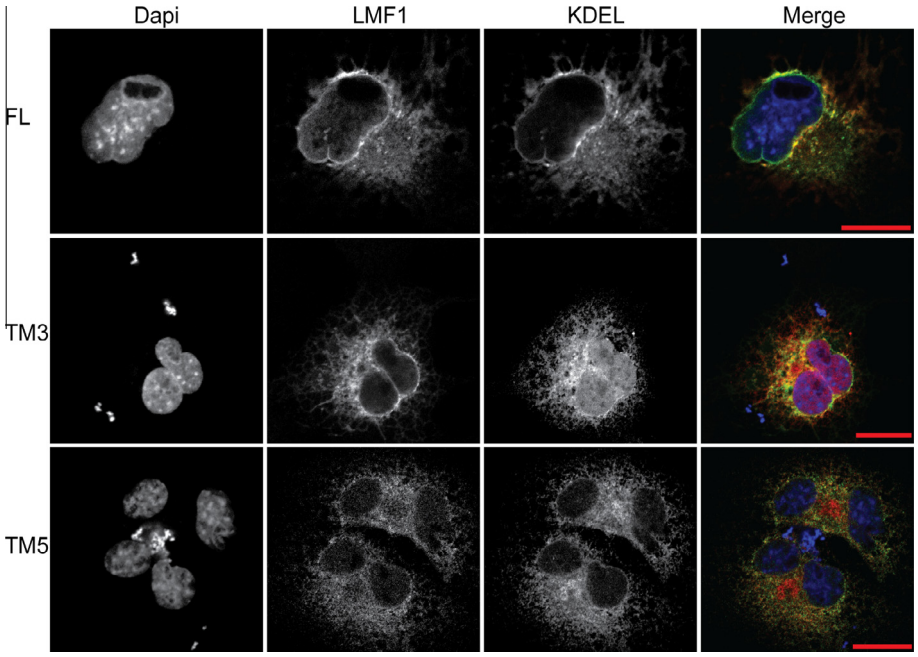

Fig. 2. LMF1 truncation variants localize to the ER. Immunocytochemistry of the LMF1 variants (green) was compared to the ER marker mCh-KDEL (red) in COS-7 cells. All LMF1 constructs show perinuclear staining characteristic of the ER as is confirmed by co-localization with mCh-KDEL. Scale bars represent 20 μm.

3.3. Membrane topology of LMF1 constructs

Truncation of the N-terminus of LMF1 could result in insertion of the protein into the ER with an incorrect orientation. We performed a protease protection assay (PPA) to determine the orientation of the LMF1 truncations in the ER. In this assay, the plasma membrane is selectively permeabilized with digitonin followed by trypsin treatment [15]. Trypsin cleaves membrane domains not protected within the lumen of organelles. COS-7 cells transfected with CD3δ-YFP (CD3δ with a YFP tag exposed to the cytosol) served as a positive control for trypsin cleavage (Fig. 3A, left panel). CD3δ with a CFP tag protected by the ER lumen (CFP-CD3δ) was a negative control to ensure that the organelles were preserved at the digitonin conditions used. Fig. 3A shows complete loss of CD3δ-YFP signal within 2 min of trypsin exposure whereas incubation with digitonin alone does not result in YFP degradation. The right panel of Fig. 3A shows no degradation of the 50 kDa CFP-CD3δ band, confirming that the assay conditions leave the ER membrane intact.

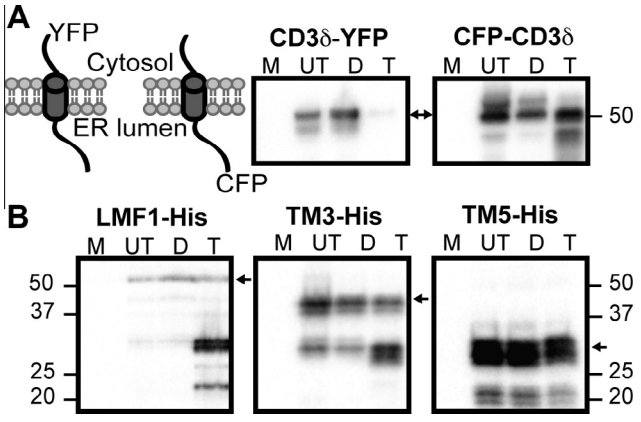

Fig. 3. Topology of LMF1 truncations. (A) CD3δ-YFP and CFP-CD3δ were used as positive and negative controls for trypsin cleavage, respectively. The middle and right panels show the Western blots for expression and the PPA. Lane 1 was mock (M) transfected, lane 2 shows untreated cells (UT), lane 3 has digitonin (D) addition for 3 min, and lane 4 has trypsin (T) for 2 min (after a 1 min incubation with digitonin). Arrows indicate the FL version of each construct. (B) Expression and PPA for WT LMF1 and the two truncation constructs. Lanes are labeled as in A.

We next tested if FL, TM3 and TM5 LMF1 were properly oriented in the ER. If these proteins have the correct topology, their C-terminal His-tags will be trypsin resistant. Unlike CFP-CD3δ, which has ER-protected CFP attached to a single transmembrane domain, LMF1 should show some cleavage products after trypsin treatment. Human LMF1 has 5 transmembrane domains with 16 cytosolic trypsin cleavage sites (PeptideCutter). Trypsin could cut LMF1 multiple times but only the products with a C-terminal His-tag will be detected. Following trypsin addition to cells expressing FL LMF1, we detect bands corresponding to both the full protein (60 kDa) and cleavage products. The cleavage products, ranging from 27 to 20 kDa, are likely due to the 7 trypsin sites in loop D of LMF1 (Fig. 3B, left panel). After trypsin treatment uncleaved TM3 was present at 43 kDa in addition to lower molecular weight cleavage products (Fig. 3B, middle panel). For TM5 (Fig. 3B, right panel); uncleaved TM5 appears at about 27 kDa. The band profile is not altered upon trypsin addition because no exposed cytosolic loops are expected in this construct. Retention of the majority of the signal from the His-tag following trypsin treatment indicates that TM3 and TM5 have their C-termini located in the ER. Thus, the Iip33 ER retention signal targets the LMF1 truncation constructs to the ER with the same membrane orientation as WT LMF1.

3.4. Low level LMF1 expression is sufficient for LPL secretion

The relative expression levels of *LMF1* mRNA have been compared in different tissues, but little is known about LMF1 protein levels [2]. We generated an antibody against LMF1 to measure its cellular levels. LMF1 was expressed in SF9 cells and purified to homogeneity as shown by coomassie staining and gel filtration (Fig. 4A). Purified protein was used to raise antibodies against LMF1 in chickens. This antibody could detect as little as 17.5 fmoles of purified LMF1 (Fig. 4B). However, Western blots using this antibody failed to detect a specific band for LMF1 in the *cld/wt*

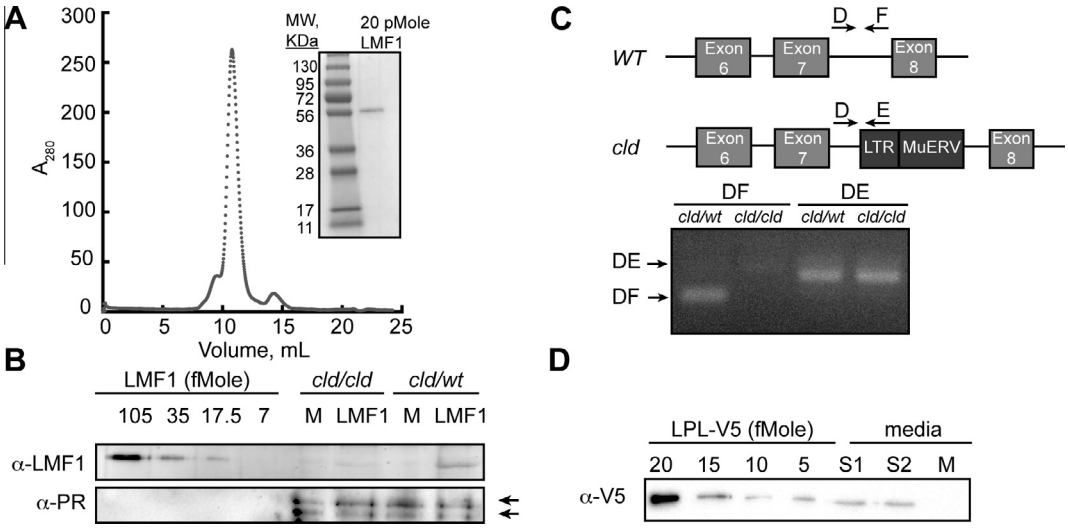

Fig. 4. LMF1 purification and cellular levels. (A) Gel filtration trace of purified LMF1 with a coomassie stained gel. (B) Western blot showing that α-LMF1 antibody can detect as little as 17.5 fmole of purified LMF1. Cells are mock transfected (M) or transfected with a plasmid expressing LMF1. PR is a loading control for the pellet fraction. Arrows indicate both PR isoforms. (C) The diagram at the top indicates the oligos (D–F) used to test for the insertion of the murine endogenous retrovirus into intron 7 of *LMF1*. PCR products, below, show that *cld*/*wt* and *cld*/*cld* cells have the expected genotype. (D) Western blot for quantification of LPL-V5 released from *cld*/*wt* cells. S1 and S2 indicate two replicate media collections.

vs. *cld*/*cld* cells (Fig. 4B). When we transfected a plasmid expressing *LMF1* into the cells, we observed a band at the expected molecular weight of LMF1 (Fig. 4B).

Because we could not detect endogenous LMF1 in *cld*/*wt* cells, we wanted to ensure that both cell lines had the expected genotype. We confirmed the correct genotype of these cells by analysis of the genomic DNA. Genomic DNA was harvested and tested by PCR for insertion of the murine endogenous retrovirus into intron 7 of *LMF1*, which defines the *cld* mutation (Fig. 4C) [2]. PCR products of the expected size were observed (Fig. 4C) indicating that a WT copy of *LMF1* is present in the *cld*/*wt* but not the *cld*/*cld* cells, as expected.

We cannot report the exact number of LMF1 molecules per cell, but can calculate an upper limit. The assays in Fig. 4B used 1×10^4 cells. Additionally, the membrane fraction of a greater number (1×10^6) of *cld*/*cld* and *cld*/*wt* cells was harvested, solubilized, and probed for LMF1 (data not shown). Based on these cell counts and the detection limit of our antibody, we can conclude that there are less than 10,000 molecules of LMF1 per cell, a relatively low expression level. A proteomic analysis of mammalian cells indicates that approximately 75% of proteins are expressed at higher levels [19]. Indeed, LMF2 was identified in this analysis, but LMF1 wasn't [19]. We next quantified the amount of LPL-V5 secreted from transiently transfected *cld*/*wt* cells into fresh media three hours after heparin addition (Fig. 4D). Purified LPL-V5 served as a standard for quantification. These experiments revealed that on average, 500,000 molecules of LPL were secreted/cell. Thus, each molecule of LMF1 can promote the maturation of at least 50 molecules of LPL.

4. Discussion

Previous studies on LMF1 have focused on the C-terminus. In patients with defective LMF1, mutations are located in the C-terminus and *in vivo* co-IP's show that loop C of LMF1 binds to LPL [2,3,10]. Based on these findings, we hypothesized that LMF1's C-terminus is not sufficient for the maturation of dimeric lipases but the C-terminus in combination with loop C would promote lipase maturation. To test this hypothesis, we made truncations of LMF1 starting from the N-terminus. Because LPL must be properly folded to enter the secretory pathway [8], we tested LPL secretion into the media in cells expressing only the truncated variants of LMF1. These data indicate that FL LMF1 is required for the maturation process of LPL. Because the ER retention signal of LMF1 is not known, we used the ER retention signal of lip33 to ensure ER localization of the truncated variants. To demonstrate that the TM3 and TM5 LMF1 truncations did not compromise ER localization and the luminal orientation of the C-terminus, we co-localized LMF1 with an ER marker and performed a protease protection assay. The results of these combined experiments demonstrate that TM3 and TM5 LMF1 have the same localization and topology as FL LMF1. However, they cannot promote the maturation of LPL, indicating that the N-terminus has an important, but unknown function.

We generated a polyclonal antibody to measure LMF1 protein levels. Although this antibody was very sensitive it could not detect endogenous levels of LMF1. We calculated that there are at most 10,000 molecules of LMF1 per cell, and that each molecule of LMF1 assisted in the maturation of at least 50 molecules of LPL. These data hint at LMF1 function. LMF1 protein is present at low levels compared to general chaperones like GRP78, Calnexin, and Calreticulin, which are present at over 1,000,000 molecules per cell [19]. It thus performs a specialized maturation function that will be the focus of future research.

It is widely accepted that LMF1 is required for the maturation of dimeric lipases, but it is not known if LMF1 coordinates the activities of other interacting partners. FL LMF1 is required for lipase maturation, but only loop C and the C-terminus have a known role. This suggests that other domains of LMF1 could interact with binding partners required for lipase maturation. Supporting this idea, we were unable to detect a strong, direct interaction between purified LPL and LMF1 *in vitro* (data not shown). Additionally, real time-PCR analysis shows that LMF1 mRNA is expressed at higher levels in tissues that lack dimeric lipases compared to tissues expressing dimeric lipases [2]. Future studies will be needed to determine if LMF1 requires interacting partners to promote lipase maturation as well as if LMF1 has a role other than lipase maturation in other tissues.

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