A quantitative assay measuring the function of lipase maturation factor 1

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Abstract  Newly synthesized lipoprotein lipase (LPL) and related members of the lipase gene family require an endoplasmic reticulum maturation factor for attainment of enzyme activity. This factor has been identified as lipase maturation factor 1 (Lmf1), and mutations affecting its function and/or expression result in combined lipase deficiency (cld) and hypertriglyceridemia. To assess the functional impact of Lmf1 sequence variations, both naturally occurring and induced, we report the development of a cell-based assay using LPL activity as a quantitative reporter of Lmf1 function. The assay uses a cell line homozygous for the cld mutation, which renders endogenous Lmf1 nonfunctional. LPL transfected into the mutant cld cell line fails to attain activity; however, cotransfection of LPL with wild-type Lmf1 restores its ability to support normal lipase maturation. In this report, we describe optimized conditions that ensure the detection of a complete range of Lmf1 function (full, partial, or complete loss of function) using LPL activity as the quantitative reporter. To illustrate the dynamic range of the assay, we tested several novel mutations in mouse Lmf1. Our results demonstrate the ability of the assay to detect and analyze Lmf1 mutations having a wide range of effects on Lmf1 function and protein expression.—Yin, F., M. H. Doolittle, and M. Péterfy. A quantitative assay measuring the function of lipase maturation factor 1. J. Lipid Res. 2009. 50: 2265–2269.

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Lipase maturation factor 1 (Lmf1) has been identified as the protein affected by the combined lipase deficiency (cld) mutation in the mouse (1). Mice homozygous for cld exhibit severe chylomicronemia due to the deficiency of lipoprotein lipase (LPL) activity (2). LPL is synthesized and secreted by a wide variety of cell types, and it represents the rate-limiting step in the tissue influx of circulating triglyceride-derived fatty acids (3). The severity of the LPL deficiency in cld mice is similar to homozygous LPL knock-out mice (4), and both exhibit neonatal lethality resulting from chylomicronemia-derived ischemia and the abolishment of triglyceride-derived fatty acid influx (2). Besides LPL deficiency, enzyme activities for several lipase family members are also diminished in cld mice, including hepatic lipase (1, 5). Thus, LMF1 has become a candidate gene for hypertriglyceridemia and related phenotypes associated with cld in human populations. In this regard, it is essential to have a quantitative assay to determine the functional consequence of LMF1 sequence polymorphisms that are currently being identified worldwide in patients exhibiting hypertriglyceridemia. Furthermore, such an assay would be an invaluable tool in uncovering Lmf1 structure/function relationships using site-directed mutagenesis and related strategies that require the ability to assess the impact of induced sequence variation on Lmf1 function.

The cld mutation causes a C-terminal truncation of Lmf1 that abolishes its function as a posttranslational maturation factor for LPL (1). Thus, in cld mutant cells, LPL protein is synthesized normally, but it never attains activity and remains as an inactive protein trapped in the endoplasmic reticulum, where it is eventually degraded (6). However, when a human or mouse wild-type Lmf1 cDNA expression construct is cotransfected with LPL in cld mutant cells, LPL matures normally to an active lipase that is eventually secreted to the medium (1).

The ability to complement lipase maturation by transfection of target Lmf1 constructs in cld mutant cells

Abbreviations: cld, combined lipase deficiency mutation; hLmf1wt, full length human Lmf1 (567 amino acid); hLmf1Y430N, human Lmf1 truncated at position 439; Lmf1, lipase maturation factor 1; LPL, lipoprotein lipase; mLmf1cld, mouse Lmf1 truncated at position 361; mLmf1G181E, mouse Lmf1 containing a G/E substitution at position 181; mLmf1N430A, mouse Lmf1 containing a N/A substitution at position 430; mLmf1Y439X, full length mouse Lmf1 (574 amino acid); SEAP, secreted form of alkaline phosphatase; wt, wild type.
MATERIALS AND METHODS

The cld mutant cell line

The cld mutant cell line used in the assay has been described previously (7). It represents a hepatocyte-derived cell line derived from 16- to 18-day-old fetal cld/cld mice immortalized by transfection with the large SV40 T antigen [pSV3neo (8)], and it is available upon request. The cells lines were maintained at split ratios of 1:20 in DMEM-10% FBS and included passage numbers 25–53. For the immortalization of cld mutant cells (1), however, Y439× represents a complete loss-of-function mutation that is qualitative in nature (all or none) and thus relatively easy to detect. Our original assay did not have the quantitative dynamic range to detect mutations that cause only a limited loss of function, or those that only partially affect Lmf1 protein expression. In this report, we describe an optimized version of our original assay that has the ability to detect Lmf1 mutations that affect function or protein levels over a wide dynamic range. We demonstrate the utility of the assay using several induced mutations in mouse Lmf1.

Expression constructs and Lmf1 target sequences

The LPL expression construct represents a full-length human LPL cDNA subcloned into the pcDNA6/V5-His expression vector (Invitrogen). The C terminus of the expressed LPL protein is fused with a V5 epitope tag as described (9).

All human and mouse Lmf1 sequences were subcloned into the pcDNA3.1 expression vector (Invitrogen); the expressed Lmf1 proteins contained an N-terminal c-myc epitope tag (1). The following human LMF1 target sequences were used: wild-type Lmf1 (hLmf1wt) encoding the full-length 567 amino acid protein, and the Y439× nonsense mutation, removing 127 amino acids from the C terminus of Lmf1 (hLmf1Y439×) (1). The following mouse Lmf1 target sequences were used: wild-type Lmf1 (mLmf1wt) encoding the full-length 574 amino acid protein; the naturally occurring cld mutation, removing 214 residues from the C terminus of the Lmf1 protein (mLmf1cld) (1); an induced missense mutation substituting Ala for Asn at amino acid 430 of Lmf1 that is part of the single Asn-Ala-Ser glycosylation site in wild-type Lmf1 (mLmf1N430A); and a missense mutation causing a Glu for Gly substitution at residue 181 of the Lmf1 protein (mLmf1G181E). The G181E mutation was identified in a clonal library of ENU-induced mutations in mouse Lmf1.

Overview of the Lmf1 functional assay. The first step of the assay is cotransfection of the cld mutant cell line with an Lmf1 target sequence and two reporter constructs, LPL and SEAP. The second step quantitates LPL activity, Lmf1 protein levels, and SEAP activity, and the latter used to correct for differences in transfection efficiency. It is essential that the Lmf1 target sequence is expressed at levels that fall within the sensitive range of LPL activity (see text and Figs. 2 and 3).
catalyzes the hydrolysis of \( \text{p-Nitrophenyl phosphate} \) producing a yellow end product read spectrophotometrically at 405 nm. For each assay, OD\(_{405\text{nm}}\) readings were taken over 30–240 min to establish the linear range for the assay, which was then used for normalization of LPL activity levels.

LPL activity was measured using a lecithin-stabilized radiola- beled triolein (glycerol tri[9,10(\(n\))-\(^{3}H\)]oleate) substrate as described (10). Each mU of LPL activity is equivalent to 1 nmol free fatty acid released per minute. Secreted LPL activity is given as mU/ml of medium, normalized to SEAP activity per ml (i.e., mU/SEAP). Because SEAP activity is based on OD\(_{405\text{nm}}\) measurements, normalized LPL activity is a relative value and thus given as an arbitrary number (see Figs. 2 and 3).

For detection of Lmf1 protein, a biotinylated rabbit anti-c- myc polyclonal antibody (Gene Tex) was used at a dilution of 1:2,500; LPL protein was detected using a biotinylated rabbit anti-LPL antibody (a kind gift from André Bensadoun) at 1:2,000. Biotinylated antibodies were detected using horseradish peroxidase-conjugated Streptavidin (Invitrogen) at 1:50,000 and visualized using a chemiluminescent substrate (ECL+®; GE Healthcare). Quantitation of Lmf1 band intensity was performed using the software NIH Image 1.63.

**RESULTS AND DISCUSSION**

Our assay is based on the ability of transfected Lmf1 to restore LPL activity in \( cld \) mutant cells; thus, LPL activity becomes a reporter of Lmf1 function. Because these cells do not endogenously synthesize LPL, they are cotransfected with LPL along with a second reporter construct, SEAP (Fig. 1). LPL restoration by an Lmf1 target sequence is compared with a range of values whose maximum is defined by wild-type Lmf1 expressing full function as an LPL maturation factor and a minimum defined by Lmf1 carrying a loss-of-function mutation. Thus, an Lmf1 target sequence to be tested requires comparison to both wild-type and mutant Lmf1 controls, each representing separate cotransfection experiments. To account for differences in transfection efficiency occurring in separate experiments, LPL activity is normalized to SEAP activity, and this value is used as the readout of Lmf1 function.

**Determining the sensitive range of the assay**

The sensitive range of the assay ensures the detection of a wide range of Lmf1 function (full, partial, or complete loss of function) using LPL activity as a quantitative reporter.
In this range, the quantitative reporter (LPL activity) responds proportionally to changes in Lmf1 protein levels (or function), as shown in Fig. 2A. When increasing amounts (0–20 ng) of wild-type human Lmf1 (hLmf1\textsuperscript{wt}) plasmid are cotransfected with a constant amount (0.8 µg) of LPL plasmid, levels of LPL activity are directly dependent on transfected Lmf1 plasmid levels until they become saturating at ~10 ng (Fig. 2A). Notably, increasing the amount of hLmf1\textsuperscript{wt} plasmid in the transfection increased Lmf1 protein levels proportionally (Fig. 2B). The assay is most sensitive when expressed Lmf1 protein levels fall within the linear portion of the LPL activity curve (highlighted in Fig. 2A). A similar sensitive range was determined for mouse Lmf1 (Fig. 3), showing that both human and mouse Lmf1 target sequences can be tested successfully in the assay. As expected, LPL protein levels are relatively unchanged, because the amount of LPL plasmid used in the transfection is held constant (Fig. 3B).

Figures 2A and 3A indicate that the ratio of target Lmf1 to LPL plasmid should be chosen within the sensitive range of LPL activity, ensuring that the full dynamic range of the assay is utilized. Such a ratio was used to compare hLmf1\textsuperscript{wt} to hLmf1\textsuperscript{Y439\times}, the latter containing a C-terminal deletion causing cld and hypertriglyceridemia in a human patient (1). In this case, 5 ng of both Lmf1 plasmids were used while the amount of LPL plasmid remained constant (0.8 µg), representing an hLmf1\textsuperscript{wt}-LPL ratio falling within the predetermined sensitive range (Fig. 2A). As shown in Fig. 2C, the secretion curves of LPL activity arising from hLmf1\textsuperscript{wt} and hLmf1\textsuperscript{Y439\times} target sequences confirms Y439\times as a mutation causing loss of function. In general, we recommend that the protein level of an Lmf1 target sequence should be less than or equal to a wild-type Lmf1 control whose sensitive range has been predetermined (see below).

Analyzing the function of Lmf1 mutant proteins

To illustrate the dynamic range of the assay, we tested several novel mutations in mouse Lmf1. These included two induced missense mutations, G181E and N430A, and the naturally occurring cld truncation (1). N430A removes the single glycosylation site in mouse Lmf1, while G181E is adjacent to a putative transmembrane-spanning region. Both Asn and Gly at positions 430 and 181 are conserved amino acids; however, glycosylation occurs only in mouse Lmf1 (data not shown), suggesting that loss of N-linked glycosylation by N430A may be unimportant. The effect of G181E on Lmf1 function is unknown, but its juxtaposition near a transmembrane domain could disrupt proper membrane insertion, affecting Lmf1 membrane topology.

In an initial comparison, equivalent plasmid amounts were used for all transfections (5 ng) based on a wild-type Lmf1 control predetermined to be within the sensitive range of the assay (Fig. 3A). The secretion curves of the three Lmf1 mutant plasmids compared with the wild-type control are shown in Fig. 4A, whose slopes are replotted in Fig. 4B as LPL activity secreted per minute. While the
N430A mutation is comparable to the wild-type control. Lmf1 carrying either G181E (mLmf1\textsuperscript{G181E}) or cld (mLmf1\textsuperscript{cld}) exhibit a significant decrease in their ability to restore LPL activity levels (Fig. 4B). Notably, the assay is able to detect a distinct difference between all three mutations by showing that G181E is intermediate in its severity when compared with N430A and cld. Regardless of the Lmf1 sequence tested, LPL protein expression remained relatively unchanged (Fig. 4C), indicating that G181E and cld affect LPL activity in a posttranslational manner.

Decreased Lmf1 protein levels and loss of Lmf1 function could account for the observed decrease in the ability of mLmf1\textsuperscript{G181E} and mLmf1\textsuperscript{cld} to restore LPL activity (Fig. 4B). Indeed, the G181E and cld mutations cause markedly reduced Lmf1 protein levels when compared with N430A and the \textit{wt} control (Fig. 4D). Thus, both mutations likely cause increased Lmf1 protein turnover, possibly due to misfolding and/or improper membrane topology. To offset these low Lmf1 protein levels in the assay, increased amounts of plasmid for mLmf1\textsuperscript{G181E} and mLmf1\textsuperscript{cld} were used in the transfection while maintaining the preestablished plasmid levels for the mLmf1\textsuperscript{wt} control. As shown in Fig. 5A, LPL activity was restored by mLmf1\textsuperscript{G181E} in a dose-dependent manner; in contrast, mLmf1\textsuperscript{cld} remained non-functional even when using highly elevated plasmid amounts in the assay. Based on these results, G181E clearly causes reduced Lmf1 protein levels, but it is difficult to assess whether it also affects Lmf1 function. To eliminate Lmf1 protein level as a variable, the readout of the assay was changed from LPL activity to Lmf1-specific activity, defined as LPL activity restored per unit Lmf1 protein. As shown in Fig. 5B, Lmf1-specific activity clearly shows that G181E does not affect Lmf1 function, which is in stark contrast to Lmf1 truncated by cld. Thus, G181E causes reduced Lmf1 protein expression without affecting function, whereas cld clearly represents a loss-of-function mutation. We hypothesize that G181E may decrease the efficiency of proper Lmf1 membrane insertion, resulting in increased protein turnover. However, some fraction of mLmf1\textsuperscript{G181E} attains a native state, and these molecules remain stable and function normally as a lipase maturation factor. Importantly, the N430A, G181E, and cld mutations illustrate the ability of the assay to detect and analyze mutations having a wide range of effects on Lmf1 function and protein expression.

**REFERENCES**

1. Peterfy, M., O. Ben-Zeev, H. Z. Mao, D. Weissglas-Volkov, B. E. Aouizerat, C. R. Pullinger, P. H. Frost, J. P. Kane, M. J. Mallow, K. Reue, et al. 2007. Mutations in LMF1 cause combined lipase deficiency and severe hypertriglyceridemia. \textit{Nat. Genet.} 39: 1483–1487.

2. Reue, K., and M. H. Doolittle. 1996. Naturally occurring mutations in mice affecting lipid transport and metabolism. \textit{J. Lipid Res.} 37: 1387–1405.

3. Merkel, M., R. H. Eckel, and I. J. Goldberg. 2002. Lipoprotein lipase: genetics, lipid uptake, and regulation. \textit{J. Lipid Res.} 43: 1997–2006.

4. Coleman, T., R. L. Seip, J. M. Gimble, D. Lee, N. Maeda, and C. F. Semenovitch. 1995. COOH-terminal disruption of lipoprotein lipase in mice is lethal in homozygotes, but heterozygotes have elevated triglycerides and impaired enzyme activity. \textit{J. Biol. Chem.} 270: 12518–12525.

5. Paterniti, J. R., Jr., W. V. Brown, H. N. Ginsberg, and K. Arzt. 1983. Combined lipase deficiency (cld): a lethal mutation on chromosome 17 of the mouse. \textit{Science.} 221: 167–169.

6. Briquet-Laugier, V., O. Ben-Zeev, A. White, and M. H. Doolittle. 1999. cld and lec23 are disparate mutations that affect maturation of lipoprotein lipase in the endoplasmic reticulum. \textit{J. Lipid Res.} 40: 2044–2058.

7. Boedeker, J. C., M. H. Doolittle, and A. L. White. 2001. Differential effect of combined lipase deficiency (cld)/cld in human hepatic lipase and lipoprotein lipase secretion. \textit{J. Lipid Res.} 42: 1858–1864.

8. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. \textit{J. Mol. Appl. Genet.} 1: 327–341.

9. Ben-Zeev, O., H. Z. Mao, and M. H. Doolittle. 2002. Maturation of lipoprotein lipase in the endoplasmic reticulum. Concurrent formation of functional dimers and inactive aggregates. \textit{J. Biol. Chem.} 277: 10727–10738.

10. Briquet-Laugier, V., O. Ben-Zeev, and M. H. Doolittle. 1999. Determining lipoprotein lipase and hepatic lipase activity using radiolabeled substrates. \textit{Methods Mol. Biol.} 109: 81–94.