Coexpression of novel furin-resistant LPL variants with lipase maturation factor 1 enhances LPL secretion and activity

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Abstract  LPL is a secreted enzyme that hydrolyzes triglycerides from circulating lipoproteins. Individuals lacking LPL suffer from severe hypertriglyceridemia, a risk factor for acute pancreatitis. One potential treatment is to administer recombinant LPL as a protein therapeutic. However, use of LPL as a protein therapeutic is limited because it is an unstable enzyme that is difficult to produce in large quantities. Furthermore, these considerations also limit structural and biochemical studies that are needed for large-scale drug discovery efforts. We demonstrate that the yield of purified LPL can be dramatically enhanced by coexpressing its maturation factor, LMF1, and by introducing novel mutations into the LPL sequence to render it resistant to proteolytic cleavage by furin. One of these mutations introduces a motif for addition of an N-linked glycan to the furin-recognition site. Furin-resistant LPL has previously been reported, but is not commonly used. We show that our modifications do not adversely alter LPL’s enzymatic activity, stability, or in vivo function. Together, these data show that furin-resistant LPL is a useful reagent for both biochemical and biomedical studies.—Wu, M. J., A. Wolska, B. S. Roberts, E. M. Pearson, A. R. Gutgsell, A. T. Remaley, and S. B. Neher. Coexpression of novel furin-resistant LPL variants with lipase maturation factor 1 enhances LPL secretion and activity. J. Lipid Res. 2018. 59: 2456–2465.

Methods

LPL is a secreted lipase that both hydrolyzes triglycerides packaged within lipoproteins and promotes the uptake of triglyceride-rich remnant lipoproteins in the liver (1). Together, these actions make LPL a vital enzyme for the clearance of triglycerides from circulation (2, 3). LPL deficiency (LPLD) is a rare but serious condition caused by homozygous or combined heterozygous loss-of-function mutations in LPL (4). Individuals with LPLD suffer from pronounced accumulation of triglyceride-rich lipoproteins in the plasma, which puts affected individuals at high risk for recurrent, acute pancreatitis (5). Apart from strictly limiting dietary fat, the only treatment option was a gene therapy product known as Glybera, which consists of a gain-of-function LPL variant (LPLS447X) delivered via adenovirus-associated virus vectors (6). However, at a cost of close to $1 million per patient, Glybera proved too expensive and was withdrawn from the market (7). Volanesorsen, an antisense oligonucleotide drug targeting ApoCIII, decreased triglycerides in individuals with familial chylomicronemia syndrome, but is not currently Food and Drug Administration (FDA)-approved (8). Additional treatment options for LPL-related diseases are therefore needed.

Protein therapeutics are a key class of medicines utilized to treat multiple diseases. FDA-approved therapeutics include monoclonal antibodies, replacement enzymes, coagulation factors, hormones, and other plasma proteins. A classic example of this technology is the use of purified insulin for the treatment of both T1D and T2D, which has been in use for nearly 100 years (9). There are also a number of metabolic disorders that have enzyme-replacement therapy (ERT) treatments such as Gaucher’s disease, Fabry disease, and Wolman disease (10). Although there are

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many successful protein therapeutics on the market, their success relies on both their clinical utility and their ability to be produced in a cost-effective manner.

ERT could be an attractive treatment option for LPLD, but there are many outstanding challenges to its implementation. One challenge is that LPL contains two N-linked glycans and five disulfide bonds and, thus, must be produced in mammalian host cells to ensure proper processing of these posttranslational modifications. The yield of LPL produced in cultured mammalian cells is notoriously poor. This is due to both inefficient folding and susceptibility to cleavage by the furin protease (11). With respect to inefficient folding, LPL folding in the endoplasmic reticulum (ER) is marked by the formation of folding-incompetent, insoluble aggregates (12). LPL, and related dimeric lipases endothelial lipase (EL) and HL, require a specialized factor known as lipase maturation factor 1 (LMF1) for proper folding (13). LMF1 is an ER-resident, transmembrane protein that is needed for the production of secretion-competent LPL (14).

Furin is a serine endoprotease that belongs to the proprotein convertase family of enzymes. Proprotein convertases catalyze the maturation of secreted precursor proteins and hormones by cleaving off an activity-blocking prodomain at a consensus motif (15). The furin recognition motif is RX-(K/R)-R↓, where X is any residue and ↓ represents the cleavage site. LPL contains the furin recognition motif RAKR at residues 294–297 (residues numbered from the signal peptide cleavage site) (11). Furin inactivates, rather than activates, LPL by cleaving apart the N-terminal catalytic domain from the C-terminal partner binding domain (11). EL is also susceptible to furin cleavage (16). We, and others, observe that in the medium of cultured cells expressing LPL, the amount of inactive LPL cleavage fragments exceeds the amount of active, full-length protein. Therefore, a possible solution to improving LPL yield is to inhibit furin protease activity (11, 17).

Previous studies have exploited both the addition of a furin inhibitor or site-directed mutagenesis of the furin motif to increase EL and LPL yield. One type of inhibitor is a variant of the protease inhibitor α1-antitrypsin, α1-antitrypsin Portland (α1-PDX), which covalently and irreversibly inhibits furin (18). Coexpression of α1-PDX with EL was shown to prevent its proteolysis, but this also prevents proteolysis of many other furin substrates necessary for proper cell function (16). An alternative to global inhibition is to block the specific furin cleavage of LPL by mutating its consensus furin cleavage site. A previous study reported that an R297A mutation within the LPL furin motif (LPLR297A) both prevented cleavage and increased the amount of full-length LPL in the medium (11). However, the R297A mutation reduced the total amount of LPL secreted into the medium. Data from a second study using LPLR297A show an enhanced level of full-length protein in the medium relative to LPL, but the amount of LPLR297A in the cell pellet is also increased, indicating possible problems with intracellular trafficking (17).

Currently, few groups use furin-resistant variants of LPL, such as LPLR297A, for routine biochemical studies, despite the increase in yield. This may be due to concerns of whether LPLR297A function is similar to LPL or why this variant is inefficiently secreted. Potentially, LPLR297A could be slightly destabilized, causing it to be retained in the cell by quality-control mechanisms, thus decreasing the total amount of secreted protein. Alternatively, furin cleavage of LPL could serve some important, but uncharacterized, function in LPL trafficking. In order to better characterize mechanisms to enhance the yield of purified LPL protein, we tested the roles of α1-PDX, novel mutations conferring furin resistance, and coexpression with LMF1 on LPL production. Our furin-resistant LPL variants, R297N and R297S/S298C, show enhanced secretion when additional LMF1 is coexpressed in HEK293 cells. We fully characterize these LPL variants for activity in vivo and in vitro, inhibition, stability, and subcellular trafficking. We show that coexpression of LMF1 with furin-resistant LPL results in a dramatic increase in the yield of fully functional LPL useful for biochemical or biomedical applications.

MATERIALS AND METHODS

Molecular cloning

Human LPL with a 6× polyhistidine tag with or without an internal ribosome entry site followed by human LMF1 was cloned into pCDNA5/FRT/TO (Thermo Fisher Scientific). Furin-resistant variants were generated by PCR using QuickChange to introduce mutations R297N and R297S/S298C as per the manufacturer’s instructions. The dual-color (DC) LPL construct comprised the LPL signal peptide followed by citrine fluorescent protein, LPL, and cerulean fluorescent protein. The final construct was expressed in pCDNA 3.1. Final sequences are available upon request. Furin-resistant (R297N) DC LPL was generated using QuikChange. A plasmid for the expression of α1-PDX was a kind gift from Akihiko Tsuji of Tokushima University.

Cell culture and protein expression

For protein expression, LPL-containing plasmids were stably integrated into Flp-In™T-REx™ HEK293 cell lines as per manufacturer’s instructions (Thermo Fisher Scientific). All stable cell lines were maintained in Gibco DMEM, 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine (complete medium). For experiments analyzing LPL glycosylation, cells were grown in Gibco DMEM without glucose, 1% FBS, 1% penicillin/streptomycin, and 1% L-glutamine, and 0, 4,500, or 10,000 mg/l added glucose. Culture medium contains 1% penicillin/streptomycin, 1% L-glutamine, 1% FBS, 10 units/ml heparin, and 2 µg/ml tetracycline. Cells were given expression medium at 80% confluency. Five T175 flasks were used per each LPL variant for protein production. For protein production, 25 ml of expression medium was added to each T175 flask with medium collection every 24 h for 6 days. A total of 750 ml of expression medium was collected per LPL variant for purification.

Protein purification and quantification

LPL was purified over two tandem 1 ml HiTrap Heparin Sepharose High Performance Columns (GE HealthCare Life Science) washed with 75 ml of 850 mM NaCl, 10% glycerol, and 20 mM Bis-Tris (pH 6.5) and eluted over a 5 ml gradient to 1.5 M NaCl, 10% glycerol, and 20 mM Bis-Tris (pH 6.5). If needed, LPL was concentrated using an Amicon 30K centrifugal, aliquoted,
flash-frozen in liquid nitrogen, and stored at −80°C until use. ANGPTL4 was purified as described (19).

**LPL activity and inhibition assays**

Lipase activity was measured using a SpectraMax M5 microplate reader (Molecular Devices) in black-walled, clear-bottom, 96-well plates (Corning). Assays were conducted in a total 100 µl reaction volume containing 2.5 nM LPL in assay buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.2% FA-free BSA, 0.01% Triton X-100, and 1 mM deoxycholate) and a concentration of 10 µM of the fluorescent substrate 1,2-di-O-α-lauryl-rac-glycero-3-(glutaric acid 6-methyloxysulfurin ester (DGGR; Sigma). Substrate hydrolysis was measured as the initial increase in fluorescence over 60 s, using an excitation wavelength of 529 nm, and reading emission at 600 nm with a 590 nm cutoff filter. Assays were measured in triplicate at room temperature. LPL-ANGPTL4 inhibition assays were done in the presence of 0, 0.4, 0.8, 1.5, 3.9, and 5.0 µM ANGPTL4 over multiple concentrations of DGGR. V_{max} and K_{m} values were calculated using the Michaelis-Menten equation, and the K_i value for ANGPTL4 inhibition was calculated from a nonlinear, global fit of the data using the program Mathematica, essentially as described (19).

**Western blot analysis**

Media and purified proteins were separated by 12% SDS-PAGE, transferred to 0.22 µm PVDF membrane (Bio-Rad), and blocked with 5% nonfat milk in TBST (20 mM Tris-Cl, pH 7.6, 150 mM NaCl, and 0.01% Tween 20). LPL variants were probed with a mouse anti-His Ab (Bio-Rad) at a 1:5,000 dilution and HRP-conjugated anti-mouse antibody (Southern Biotech) at 1:5,000 dilutions for detection. Western blots were developed using WesternBright ECL (Advansta) on a Bio-Rad Chemi-doc MP imager. Samples that were deglycosylated were treated with PNGase F (NEB) according to the manufacturer’s instructions prior to SDS-PAGE.

**Microscopy**

A total of 1.2 µg of the WT or LPL^R297N^ DC plasmids were transfected into HEK293 cells with 0.8 µg of mCherry-tagged Sec61, SI, or Lamp1 plasmid for cellular colocalization images. Sec61 was used to label the ER, SI was used to label the trans-Golgi network, and Lamp1 was used for the lysosomes. Cells were fixed in 4% paraformaldehyde and stained with 1:1,000 Alexa Fluor 594 anti-mouse (Invitrogen) secondary antibodies. A Zeiss 710 laser-scanning confocal microscope with 40x/1.4 plan apo lens was used to capture z-stack images of individual cells containing all three fluorescent channels. Images were deconvolved with Bitplane Autoquant 3 and analyzed in Fiji with the JACoP plugin. Mander’s overlap coefficient was used for the colocalization analysis between channels.

**Animal studies**

C57Bl/6 mice were maintained on a regular rodent chow diet (NIH-31 chow diet; Ziegler Brothers, Inc., Gardner, PA). On the day of the study, mice were fasting for 7 h with full access to water. Mice received 20% Intralipid® (Fresenius Kabi, Uppsala, Sweden) by ip injection in a single bolus of 1 ml (20, 21). After 1 h, either saline buffer or LPL in a single dose of 2 µg/g body weight (in a sterile saline buffer at a total volume of ≤250 µl) was injected iv. Blood samples were obtained from the retroorbital plexus with heparinized capillary tubes at time points −1, 0, 1, 3, 6, and 24 h and placed into tubes with EDTA (final concentration 4 mM). Plasma separation for triglyceride assays was obtained after blood centrifugation at 1,000 g for 20 min at 4°C. Plasma levels of triglycerides were measured enzymatically (Wako Chemicals). Mice were returned to metabolic cages with food after 7 h from the fat challenge test. The study was approved by the National Heart, Lung and Blood Institute Animal Care and Use Committee (protocol no. H-0050).

**RESULTS**

Combining furin resistance and LMF1 optimizes LPL yield

We tested several different strategies aimed at increasing the yield of full-length LPL. These techniques included coexpression of LPL with LMF1, coexpression of LPL with the furin inhibitor, α1-PDX, and mutations within LPL’s furin cleavage site. Because previous reports indicated that LPL^R297A^ does not dramatically increase the overall yield of LPL, we opted to replace the positive-charged R297 residue with a polar, rather than hydrophobic, amino acid. We first made the mutation R297N (LPL^R297N^), which should not only block furin cleavage but also introduce a consensus site (NX-S/T) for addition of an N-linked glycan (22). We hypothesized that addition of an N-linked glycan in the furin recognition site would block furin interaction. Moreover, glycoengineering of therapeutic proteins is a common strategy to decrease their susceptibility to proteolysis, increase their activity, and enhance their in vivo half-life (23). In the event that an additional N-linked glycan on site R297 interferes with LPL function, we also made a combined R297S/S298C mutation (LPL^R297S^S298C^) that should prevent furin cleavage without introducing novel post-translational modifications. These mutations are shown in Fig. 1A, B (11).

We detected LPL by Western blot against an added C-terminal His epitope, so that only the full-length protein and C-terminal cleavage product were visible. As expected, cell culture medium containing LPL alone had a significantly greater amount of C-terminal cleavage product than full-length protein (Fig. 1C, lane 1). When LPL was coexpressed with α1-PDX, there was a slight increase in full-length protein accompanied with a reduced amount of cleavage product (Fig. 1C, lane 2). LPL coexpression with LMF1 gave a similar increase in full-length protein as coexpression with α1-PDX, but had no change in the amount of cleavage product in comparison to LPL alone (Fig. 1B, lane 3). Lastly, we coexpressed LPL^R297N^ and LPL^R297S^S298C^ with LMF1, which resulted in a dramatic increase in full-length protein with no detectable cleavage product (Fig. 1B, lanes 4 and 5). We also tested LPL, LPL^R297N^, and LPL^R297S^S298C^ without LMF1 (supplemental Fig. S1). LPL^R297N^ appeared to have a slightly higher molecular mass than LPL, likely due to the addition of an N-linked glycan. LPL has two native sites for N-linked glycans, and the LPL^R297N^ mutation introduces a third (24). In order to determine whether this third site is glycosylated in LPL^R297N^, we grew the cells in conditions of limited glucose (25). This decreases the intracellular pool of the substrate for glycosyltransferases, UDP-N-acetylglucosamine (26). As a result,
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not all sites for N-linked glycosylation are occupied with oligosaccharide, and distinct bands for different occupancy can be readily observed by Western blot. When cells expressing LPL were grown under limiting glucose conditions, two distinct bands were observed (Fig. 1D). For LPL<sup>R297N</sup> cells grown under the same glucose-limiting conditions, a third, upper band was also observed (Fig. 1D). When samples were treated with PNGase, an enzyme that removes N-linked glycans, both LPL variants displayed the same apparent molecular mass (Fig. 1E). Thus, the LPL<sup>R297N</sup> mutation resulted in the addition of an N-linked glycan.

To quantify the increase in LPL yield afforded by coexpression of LMF1 with the LPL<sup>R297N</sup> and LPL<sup>R297N</sup> variants, we compared purification of LPL from HEK293 cells stably expressing LPL alone and cells coexpressing LMF1 and LPL, LPL<sup>R297N</sup>, or LPL<sup>R297N</sup>. An equal volume of conditioned medium was collected from the four different cell lines and purified using heparin-sepharose chromatography. As shown in the UV trace in Fig. 2, coexpression of LMF1 with LPL or either of the furin-resistant LPL variants resulted in a larger LPL peak than expression of LPL alone. The area under the UV peak was calculated across several independent purifications for each LPL variant, and these values are listed in Table 1. LPL activity in peak fractions was also tested (Fig. 2, gray bars). The total yield for each purification was calculated using activity measurements against a bovine LPL standard curve. The yield for each LPL variant averaged from multiple independent purifications is listed in Table 1. The yield of all LPL variants coexpressed with LMF1 was compared with LPL expressed alone. The fold increase was higher when calculated by activity (13-fold to 20-fold increase) than when calculated based on UV peak area (2-fold to 3-fold increase; Table 1). This is because a larger fraction of the total protein in the peak is LPL for variants coexpressed
with LMF1. The relative purity of all LPL variants can be seen in the gels shown in supplemental Fig. S2. After the heparin column purification, LPL alone was not sufficiently concentrated to visualize on a gel or to take accurate activity measurements. It was thus concentrated using a spin concentrator, which resulted in considerable sample loss. Upon coexpression with LMF1, LPL variants were concentrated enough to be used directly without further manipulation.

Furin resistance does not alter LPL activity

Loss-of-function mutations at dozens of positions throughout LPL have been identified in hypertriglyceridemic patients, indicating that mutations in LPL are not generally tolerated. Although LPL R297N and LPL R297S S298C can be purified with an enhanced yield relative to LPL, these variants are only useful if they have equivalent enzymatic activity to LPL. We therefore compared the enzymatic activity of LPL, LPL R297N, and LPL R297S S298C coexpressed with LMF1.

We quantified active LPL protein concentration using an activity-based probe (Fig. 3A), such that equal amounts of active lipase were used in each assay (27). Lipase activity was analyzed using a synthetic, long-chain lipase substrate, DGGR, which becomes fluorescent upon hydrolysis by LPL (28). As shown in Fig. 3B, the initial rate of DGGR hydrolysis by each LPL variant was measured at increasing substrate concentrations and fit to the Michaelis–Menten equation. This analysis yielded indistinguishable kinetic parameters ($V_{\text{max}}$ and $K_m$) for the LPL variants. Thus, LPL, LPL R297N, and LPL R297S S298C are equally able to engage and hydrolyze substrate.

LPL and furin-resistant LPL have equal stability

LPL is notoriously unstable due to thermal unfolding (19). Therefore, we compared the thermal stability of LPL, LPL R297N, and LPL R297S S298C. We incubated LPL and LPL

| LPL Variant | UV Peak Area, mAU/ml Medium | Average Yield, μg |
|-------------|-----------------------------|-------------------|
| LPL         | 0.26 ± 0.18                 | 5.7 ± 8.8         |
| LPL + LMF1  | 0.51 ± 0.15                 | 78.7 ± 47.2       |
| LPL R297N + LMF1 | 0.60 ± 0.06 | 115.4 ± 69.0     |
| LPL R297S, S298C + LMF1 | 0.81 ± 0.36 | 82.0 ± 54.0     |

Each purification started with 750 ml of conditioned medium.

![Graphs A-F](https://via.placeholder.com/150)
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variants at 25°C and monitored loss of LPL activity over time. All LPL variants had a similar, rapid loss of activity, indicating that enhanced LPLR297N and LPL R297S S298C production cannot be attributed to enhanced thermal stability (Fig. 3C). LPL has several physiologically important macromolecular inhibitors, including angiopoietin-like protein 4 (ANGPTL4). To determine whether the LPL variants were differentially affected by ANGPTL4, we performed in vitro LPL inhibition assays. All LPL variants were equivalently inhibited by ANGPTL4 in a noncompetitive manner (Fig. 3D–F).

Intracellular trafficking of furin-resistant LPL

The physiological significance of LPL cleavage by the furin protease is unknown. Nonetheless, the furin recognition site in LPL is well conserved evolutionarily (Fig. 1A), indicating a possible physiological purpose. Proprotein convertases usually activate their substrates and can alter their trafficking. However, the cleaved N-terminal domain of LPL is inactive against long-chain substrates, indicating that furin cleavage of LPL has the opposite effect to what is typically observed (29). Therefore, we asked whether furin cleavage affects intracellular trafficking of LPL. Furin cycles between the trans-Golgi network, endosomes, and the cell surface, where it may encounter substrates in any of these compartments (30). To determine whether furin-sensitive and furin-resistant LPL are differentially trafficked, we made DC LPL and LPLR297N (DC-LPL or DC-LPL R297N), using two fluorescent labels (Fig. 4A). These constructs comprise a signal peptide that targets LPL to the ER, followed by citrine fluorescent protein on the N terminus of LPL and a cerulean fluorescent protein on the C terminus. The DC arrangement should reveal where furin cleavage occurs and whether this cleavage affects trafficking. We tested for lipase activity in the medium of cells expressing DC-LPL and DC-LPL R297N and found that these LPL variants were secreted and active (supplemental Fig. S3).

To determine where furin cleavage occurs, we initially analyzed DC-LPL and DC-LPL R297N by Western blot both in cell lysate and in conditioned medium. As shown in Fig. 4B, for DC-LPL, only faint bands for the two cleavage products are seen in the cell lysate. However, there are strong bands for both the N- and C-cleavage products (middle and bottom bands, respectively) in the medium. In the case of DC-LPL R297N, cleavage product is not seen in either the lysate or the medium. Together, these data indicate that furin most likely cleaves LPL at the cell surface, rather than inside the cell. We next analyzed colocalization of both the citrine and cerulean fluorophores in the DC-LPL variants using fixed cells. We found that both fluorophores were well colocalized in LPL- and LPLR297N-expressing cells (Fig. 4B, C). These data indicate that the N- and C-terminal domains of LPL are not separated within the cell and sorted to different subcellular compartments.

Next, we investigated whether the LPL R297N mutation affects its intracellular trafficking. To do so, we used confocal

| TABLE 2. Average kinetic data for each variant using DGGR substrate |
|--------------------------|-----------------|-----------------|
| LPL Variant             | \( V_{\text{max}} \) (RFU/sec) | \( K_{\text{m}} \) (µM) | \( K_{\text{i}} \) (µM) |
| LPL + LMF1              | 1.06 ± 0.18     | 0.67 ± 0.58     | 2.6 ± 1.00    |
| LPL R297N + LMF1        | 1.22 ± 0.09     | 0.79 ± 0.52     | 1.69 ± 0.90   |
| LPL R297S, S298C + LMF1 | 1.2 ± 0.18      | 0.65 ± 0.51     | 1.59 ± 0.93   |

\( V_{\text{max}} \) and \( K_{\text{m}} \) values were calculated using the Michaelis–Menten equation, and the \( K_{\text{i}} \) value for ANGPTL4 inhibition was calculated using a nonlinear, global fit of the data. The average is calculated from four replicates for all samples. RFU, relative fluorescence units.
microscopy to measure colocalization of the citrine and cerulean LPL fluorophores with fluorescent markers for the ER, Golgi, and lysosome. In DC-LPL R297N-expressing cells, both the N-terminal citrine and the C-terminal cerulean fluorophores were significantly more colocalized with the ER marker than LPL (Fig. 5A, B). These data indicate that DC-LPL R297N is more abundant in the early secretory system than LPL. Introduction of site for N-linked glycan addition to LPL R297N may explain the enhanced colocalization of this variant with the ER marker. This increased ER colocalization does not seem to have a deleterious effect on overall secretion (Fig. 1C). Next, we analyzed colocalization of the N-terminal citrine and the C-terminal cerulean fluorophores of both DC-LPL and DC-LPL R297N with Golgi and lysosomal markers. For DC-LPL and DC-LPL R297N, both fluorophores were equally colocalized with Golgi and lysosomal markers (Fig. 5C–F). These data indicate that LPL trafficking through the late secretory system is unaffected by furin resistance.

In vivo activity of furin-resistant LPL

To ensure that furin-resistant LPL is active in vivo, we compared purified bovine LPL, human LPL, and human LPL R297N, using a mouse model of acute hypertriglyceridemia (20) (Fig. 6A). Mice were injected i.p. with a bolus of intralipid, causing acute, severe hypertriglyceridemia, reaching levels between 4,000 and 5,000 mg/dl (Fig. 6B). Fluids in the peritoneal cavity dynamically exchange between the cavity and the lymphatic system and the general circulation, such that the intralipid entered the circulation in a manner reminiscent of chylomicrons (31). Following intralipid injection, the mice then received an iv injection of either PBS, bovine LPL, human LPL, or human LPL R297N. Plasma triglyceride levels were monitored for 24 h postinjection. Treatment with all three forms of LPL resulted in a marked reduction of plasma triglyceride levels relative to control PBS injection. Overall, the average triglyceride levels between each LPL test condition were somewhat variable, but were only statistically significant at 6 h. At this time point, the plasma triglyceride values of mice treated with human LPL and LPL R297N were 51 ± 55 and 231 ± 118 mg/dl, respectively (P < 0.01; see enlarged graph in Fig. 6C).

DISCUSSION

LPL is the key enzyme in regulating plasma triglyceride levels. Genetic loss of LPL results in severe hypertriglyceridemia, making LPL an attractive candidate for ERT. However, both thermal instability and poor production yield limit the potential use of LPL as a protein therapeutic. In addition, poor production yield of LPL limits structural and biochemical studies that are crucial for large-scale drug-development efforts. In this study, we demonstrate that we can enhance the yield of recombinant LPL produced in mammalian cells by coexpressing LPL and LMF1 and mutating the furin protease recognition site. Mutation of the furin recognition site in LPL does not alter its enzymatic properties, inhibition by ANGPTL4, or thermal stability. Furthermore, furin-resistant LPL lowers plasma triglycerides in a mouse model of severe hypertriglyceridemia. Likewise, furin-resistant LPL R297N does not show dramatically different subcellular localization, with equivalent localization to the Golgi and lysosomes; however, slightly more LPL R297N was observed in the ER relative to LPL.

The reasons that LPL R297N shows increased ER localization relative to LPL are not entirely clear. Furin cycles between the trans-Golgi network, the endosomes, and the cell surface, and so it is unlikely that alterations in LPL R297N interactions with furin cause this change in ER localization (30). One possibility is that the addition of an N-linked glycan to LPL R297N results in a longer residence in the ER, which perhaps aids in its complete folding. Most importantly, we see equivalent stability and a dramatic overall increase in LPL R297N secretion; therefore, the higher colocalization of LPL R297N in the ER relative to LPL does not seem to have any negative consequences.

Another mystery is why there were more triglycerides in the blood of the mice treated with LPL R297N relative to control LPL at the 6 h time point. Previous studies showed that when rats are injected with LPL, the enzyme is taken up from the blood by the liver fairly rapidly (32). For example, when I125-labeled LPL was injected into rats, only about 40% of the LPL could be recovered from the blood after 30 min (33). These studies did not extend to 6 h, and so we do not know the amount and activity of exogenous LPL remaining at this time point. One possibility is that the LPL R297N had the highest quantity of active LPL/total LPL (Fig. 2). For the experiments shown in Fig. 6, mice were treated with equivalent amounts of active LPL, but more inactive LPL was likely present in the LPL preparation relative to the LPL R297N preparation. LPL also acts as a bridge to promote the uptake of triglyceride-rich remnant lipoproteins in the liver, and lipase activity is not required for this function (34). At 6 h, the triglyceride clearance facilitated by remnant lipoprotein uptake could have been enhanced by the greater amount of total LPL in the LPL preparation.

We found that the majority of the LPL cleavage fragments were in the conditioned medium, rather than in the cell lysate (Fig. 4B). Additionally, our microscopy data showed that in DC-LPL, the N-terminal citrine and C-terminal cerulean fluorophores were strongly colocalized in the cell (Fig. 4C). In combination, these data suggest that in the HEK293 cells used in these studies, furin cleavage of LPL occurs on the cell surface. This phenomenon has been observed before. The zinc metalloendopeptidase, Pro-ADAMTS9, was shown to be cleaved by furin at the cell surface in HEK293 cells (35). A recent study suggests that LPL may be processed by furin intracellularly in adipocytes (36). These differences in the location of LPL cleavage by furin are likely due to the different cell types used in the two studies.

Previous reports have shown that furin-resistance moderately increases the amount of full-length LPL secreted from cells in culture (11, 17). However, furin-resistant mutants are rarely used in biochemical LPL studies. Here, we have
Fig. 5. Trafficking of LPL and LPL\textsuperscript{R297N} diverges in the ER. A, B: Colocalization of the citrine and cerulean fluorophores with mCherry Sec61-\textbeta, an ER marker, was calculated. For LPL\textsuperscript{R297N}, both N- and C-terminal fluorophores colocalized with the ER marker more frequently than LPL. C, D: Colocalization of the N- and C-terminal fluorophores with mCherry SIT, a marker of the Golgi, was indistinguishable between LPL and LPL\textsuperscript{R297N}. E, F: Colocalization of the N- and C-terminal markers for both LPL and LPL\textsuperscript{R297N} with the lysosome was not significantly different. For all samples, Mander’s overlap coefficient for colocalization was used. Significance was calculated using a two-tailed Student's \textit{t}-test. For analysis, 14 cells per condition were used for ER, 23 cells per condition were used for Golgi, and 11 cells per condition were used for lysosome. All scale bars are 10 \textmu M.
generated and characterized two new furin-resistant LPL variants, LPL R297N and LPL R297S S298C. The LPL R297N mutation was significant at 6 h as determined by a two-tailed Student’s t-test. For B and C, points are the average value observed for n = 4–7 mice, and error bars represent the standard deviation. Lines are a smoothed fit and were added to guide the eye between points.

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