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Mechanisms of lipase maturation

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Abstract

Lipases are acyl hydrolases that represent a diverse group of enzymes present in organisms ranging from prokaryotes to humans. This article focuses on an evolutionarily related family of extracellular lipases that include lipoprotein lipase, hepatic lipase and endothelial lipase. As newly synthesized proteins, these lipases undergo a series of co- and post-translational maturation steps occurring in the endoplasmic reticulum, including glycosylation and glycan processing, and protein folding and subunit assembly. This article identifies and discusses mechanisms that direct early and late events in lipase folding and assembly. Lipase maturation employs the two general chaperone systems operating in the endoplasmic reticulum, as well as a recently identified lipase-specific chaperone termed lipase maturation factor 1. We propose that the two general chaperone systems act in a coordinated manner early in lipase maturation in order to help create partially folded monomers; lipase maturation factor 1 then facilitates final monomer folding and subunit assembly into fully functional homodimers. Once maturation is complete, the lipases exit the endoplasmic reticulum and are secreted to extracellular sites, where they carry out a number of functions related to lipoprotein and lipid metabolism.

Keywords

chaperone; endoplasmic reticulum; endothelial lipase; glycosylation; hepatic lipase; lipase maturation factor 1; lipoprotein lipase; protein folding

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Lipase function & molecular structure

This article focuses on the maturation (i.e., folding and assembly) of a family of extracellular lipases that play essential roles in circulating lipid metabolism. The family is comprised of

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evolutionarily conserved members that include pancreatic lipase (PL; gene name *PNLIP*), lipoprotein lipase (LPL; gene name *LPL*), hepatic lipase (HL; gene name *LIPC*) and endothelial lipase (EL; gene name *LIPG*). All are secreted *N*-linked glycoproteins that hydrolyze two principal lipid substrates, triglycerides (TGs) and phospholipids, but with widely varying efficiencies [1]. PL and LPL are largely TG lipases, while HL can hydrolyze both substrates and EL is principally a phospholipase. Unlike many other lipases, these lipases cleave their substrates at the *sn*-1 position, releasing fatty acids and monoglycerides or lysophospholipids [1,2]. While this article mainly focuses on mechanisms of lipase maturation, lipase folding and assembly require an appreciation of lipase function and molecular structure, which will be briefly summarized, along with the role of these lipases in disease. For a more comprehensive discussion of these topics, a number of excellent reviews are available [2–18].

Pancreatic lipase functions in the absorption of dietary fat; it is secreted by pancreatic acinar cells, acting in the intestinal lumen to hydrolyze bile-emulsified TGs. The released fatty acids and monoglycerides are taken up by intestinal enterocytes, and then re-esterified into TGs and packaged, along with vitamin and cholesteryl esters, into the hydrophobic core of chylomicrons. During fasting, the liver synthesizes a similar TG-rich lipoprotein termed VLDL [19]. In the circulation, TGs sequestered in the core of chylomicrons and VLDL are inaccessible to tissues except through the action of LPL. This lipase is synthesized by a variety of cell types including adipocytes and myocytes from skeletal muscle and heart, respectively. Once secreted, LPL is transported to the lumenal face of capillaries, where it is bound to the surface of the endothelium by heparin sulfate proteoglycans and the recently identified GPIHBP1 [20] – a glycosylphosphatidylinositol-anchored protein that acts as a platform for LPL lipolysis of TG-rich lipoproteins. At this site, TGs are hydrolyzed by LPL into its more polar products (i.e., free fatty acids and monoglycerides), which are then accessible to subjacent tissues through receptors such as CD36 [6]. Once lipolyzed by LPL, chylomicrons and VLDL are released from the capillary beds of peripheral tissues and circulate to liver sinusoids where they become further hydrolyzed by HL [11]. The result is the creation of lipoprotein remnants with much of their TGs removed – a form that is then taken up by the liver via receptor-mediated endocytosis. This process is also assisted by LPL and HL; in a noncatalytic fashion, these lipases act as ligands that facilitate uptake of remnant particles by the LDL-receptor related protein [3,9,11].

Along with their roles in the metabolism of TG-rich lipoproteins, the lipases also have important functions in the formation and metabolism of HDL. For example, the hydrolysis of TG-rich lipoproteins by LPL causes the core to shrink, leaving redundant surface lipids which, along with ApoA1, break off and form nascent HDL [3,4]. In addition, HL and EL have important roles in HDL remodeling and uptake that are essential to the process of reverse cholesterol transport [11,14]. Indeed, all the lipases emerge as important genetic determinants of circulating lipid levels in human populations and are thought to contribute to a number of pathogenic states including atherosclerosis and inflammation, obesity and insulin secretion and sensitivity (discussed later) [11,21–27]. However, regardless of the role, lipases can function only when secreted, a process that first requires their maturation in the endoplasmic reticulum (ER).

Lipase maturation involves the folding of newly synthesized polypeptides into fully functional enzymes, a process requiring attainment of a full 3D conformation. While only PL has a solved crystal structure [28], domain-exchange experiments indicate that all family members share folding domains with structures very similar to PL [17]. Indeed, the crystal structure of PL has been used to model LPL [8], EL [29] and HL (Figure 1A). All members of the lipase family share a basic tertiary structure comprised of two folding domains, dividing the lipase protein into a large N-terminal domain and smaller C-terminal domain separated by a short ‘hinge’ region (Figure 1A). The primary function of the N-terminal domain is catalysis, while the C-

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terminal domain provides ancillary functions aiding catalysis by facilitating the binding of lipid substrates and enzyme cofactors, while also providing binding sites for GPIHBP1 and heparan sulfate proteoglycans [2].

While the C-terminal domain is comprised of a series of β-sheets that form a sandwich-like fold [8], the N-terminal domain features the active site cleft covered by a lid domain. Upon association with the lipid substrate, the lid opens to provide access to the Ser/Asp/His catalytic triad (Figure 1B, colored red, white and blue). The active site cleft is comprised of secondary structures termed the α/β hydrolase fold, a key feature of esterases, lipases and thioesterases [30,31]. It is comprised of a fan of internal β-sheets surrounded by α-helices, and comprised of amino acids that are conserved between all members of the lipase family (Figure 1A, white). Notably, the residues unique to HL are found to be located on the surface (Figure 1A, green ribbons), including hydrophobic residues at both N- and C-terminal domains that are seemingly in contact with the aqueous phase (Figure 1A, arrows). While such an arrangement would be thermodynamically unstable, these surface hydrophobic residues are shielded when two monomers assemble into a homodimer, and appear to form intermolecular contact points that may hold the monomers together in a head-to-tail arrangement (Figure 1B).

Notably, among the lipase family members, only PL functions as a monomer [2,32]; LPL, HL and EL are active only as noncovalent homodimers with monomers arranged in a head-to-tail orientation [29,33,34]. As discussed later, we believe that this difference in subunit structure marks a fundamental divergence in the maturation pathway between PL and the rest of the lipase family. Specifically, LPL, HL and EL probably share a common pathway that is tailored to the unique characteristics of monomer structure (e.g., surface hydrophobic residues) required for successful homodimer assembly. In fact, unlike PL, the folding and assembly of all three lipase homodimers require a specialized maturation factor: lipase maturation factor 1 (LMF1; see later). Moreover, the evolutionary relationships between the lipase family members suggest that PL diverged from an ancestral lipase prior to HL, LPL and EL, which share a more recent root [35]; like PL, the ancient bacterial and fungal lipases also function as monomers [2]. Indeed, it is tempting to speculate that a specialized maturation pathway co-evolved with this more recent branch of the lipase gene family to facilitate attainment of its unique structural requirements.

Lipases in disease

Lipase maturation, along with other transcriptional, post-transcriptional and post-translational mechanisms [3,4,9], regulates the expression and secretion of lipase activity, and thus contributes to pathophysiological conditions that have been attributed to lipase deficiency, overexpression and altered function. For example, naturally occurring mutations in mice and humans that affect LPL [36,37], LMF1 [38] or the extracellular lipolytic platform GPIHBP1 [39], result in LPL deficiency, which in turn causes massive hypertriglyceridemia (chylomicronemia) and low HDL levels. Although such mutations are rare, it is now clear from genome-wide association studies that variation in LPL is among the strongest genetic determinants of plasma TG and HDL-C levels in normal human populations [40–43]. Thus, genetic variation of both *cis*- and *trans*-acting factors affecting LPL activity levels, including lipase maturation, can elicit profound changes in plasma lipid levels that are known to contribute to atherosclerosis [44], pancreatitis [45] and metabolic syndrome [46,47].

In addition to LPL deficiency, genetically engineered mouse models have also shed light on the pathophysiological consequences of LPL overexpression in various tissues [48]. Transgenic mice expressing elevated LPL activity in skeletal muscle [49] and liver [50] demonstrate that TG oversupply to these tissues – with concomitant decreased delivery of circulating TGs to adipose tissue – causes insulin resistance [27,51,52] and resistance to diet-

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induced obesity [53]. Consistent with these mouse models, genetic variation in the human *LPL* gene has been associated with insulin resistance [54,55], as well as obesity [56,57], in some human populations. LPL overexpression in the heart causes cardiomyopathy [58] owing, at least in part, to the accumulation of toxic lipid intermediates [59], which raises the possibility of a potential role for LPL in cardiac lipid accumulation and dilated cardiomyopathy associated with obesity and diabetes [60–62]. In mouse models, LPL deficiency and overexpression, specifically in macrophages, is associated with respective protection against, or acceleration of, atherosclerosis [63,64], raising the possibility of an involvement of LPL in cardiovascular disease beyond its effects on plasma lipid levels. Indeed, a recent genetic analysis demonstrated association between a polymorphism of the *LPL* gene and myocardial infarction [65].

Similar to LPL, rare forms of HL deficiency [66,67] as well as commonly occurring variation in HL [40–43], have been linked to altered plasma TG and cholesterol levels. As HDL appears to be the primary lipoprotein affected by HL, the role of this enzyme in atherosclerosis and coronary artery disease has been intensively investigated (for a review, see [11]). These studies suggest both pro- and antiatherogenic properties of HL, and indicate complex, context-dependent involvement of HL in cardiovascular disease [13]. EL is also emerging as an important factor in the control of lipoprotein metabolism and inflammation in humans [23, 24,68–70]. EL has recently been identified as an important determinant of plasma HDL-C levels [40–43], reflecting its primary role in HDL catabolism. Taken together, it is clear that LPL, HL and EL contribute significantly to circulating lipid levels and related metabolic traits; moreover, factors such as lipase maturation are now emerging as important determinants in their expression.

Lipase glycosylation & processing in the endoplasmic reticulum

The majority of newly synthesized proteins in the ER transverse the secretory pathway with at least one glycan chain covalently attached to an asparagine residue in the consensus sequence Asn-X-Ser/Thr (NXS/T), where X is any amino acid except proline [71–73]. The lipases are no exception; members of the lipase gene family contain at least one *N*-linked glycan chain in each folding domain. Glycosylation has multiple roles: site-specific glycosylation decreases regional hydrophobicity, increasing solubility and influencing local folding behavior; glycan chains also act as ligands, guiding proteins to appropriate chaperones in the ER. Such ligands are uncovered when glycan chains are processed through the specific removal of glucose and mannose residues by ER-specific glycosidases. For example, glucose cleavage controls binding and release from lectin chaperones (see later), while mannose trimming limits the time allotted for protein folding before initiating ER-associated degradation (ERAD) [71,72,74].

The importance of site-specific glycosylation in lipase maturation can be appreciated by the conservation of NXS/T sites residing in both N- and C-terminal folding domains of LPL, HL and EL (Figure 2A; dark circles). By contrast, PL is the only lipase family member lacking such conserved sites (Figure 2A), suggesting that its maturation may diverge in some aspects from other members of the lipase family. In fact, PL folding is much more efficient than HL and LPL [75], and its maturation proceeds without the apparent need for robust interaction with ER-folding factors [76]. Unlike PL, the maturation of LPL, HL and EL requires folding and assembly into homodimers, which increases their structural complexity. Maturation of these lipases proceed efficiently only if nascent chains engage with specific folding factors whose interactions are controlled in part by site-specific glycosylation of the N-terminal domain. In particular, *N*-linked glycosylation at positions 43, 56 and 62 of LPL, HL and EL (Figure 2A, arrows) is critical for efficient maturation. Studies have demonstrated that removal of these conserved sites by site-directed mutagenesis results in severe maturation defects, as evidenced by ER retention of lipase proteins and, at least for LPL and HL, the abolishment of enzyme activity [77–79]. These critical glycosylation sites are adjacent to a pair of cysteine

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residues forming the first disulfide bridge, and near the lid domain that shields hydrophobic residues within the active site cleft [8,18]. Moreover, *in vitro* LPL refolding experiments indicate that establishing native tertiary structure to the N-terminal domain is the rate-limiting step in the formation of the homodimer [80]. Thus, folding of the N-terminal domain requires the intramolecular assembly of key peptide surfaces whose efficiency can be greatly increased if chaperones are present in order to limit nonproductive interactions (see later).

Along with the addition of glycan chains to conserved sites in the N-terminal domain, their initial processing by ER glucosidases is critical for efficient lipase maturation. Glycosylation occurs by transferring a preformed 'high-mannose' glycan chain from dolichol phosphate to the NXS/T sequence of a growing polypeptide chain by the oligosaccharyl transferase complex in the ER [72]. As shown in Figure 2B, the carbohydrate composition of this preformed glycan chain is predominated by nine mannose residues (circles) topped by three glucose residues (triangles). After transfer to the growing polypeptide chain, glucosidases I (GI) and II (GII) sequentially remove the glucose residues (Figure 2B). Removal of the two outermost glucose residues results in a monoglucosylated high-mannose chain that binds with high affinity to the ER chaperones calnexin or calreticulin (CNX/CRT). Cleavage of the last glucose residue by GII releases the glycan chain from CNX/CRT; however, it can rebind after addition of a glucose by the ER lumenal protein UDP-glucose: glycoprotein glucosyltransferase 1 (UGGT; Figure 2B). Thus, the innermost glucose residue acts as a ligand on *N*-linked glycoproteins that promotes its engagement with CNX/CRT; its release and reattachment is controlled by GII and UGGT, respectively. Using such a mechanism, nascent *N*-linked glycoproteins can undergo repeated rounds of chaperone binding (also known as cycling) in order to increase folding efficiency. As expected, inhibition of CNX/CRT binding by inhibiting ER glucosidases results in deficient lipase maturation, as evidenced by loss of lipolytic activity and lipase aggregation [75,81–83]. These results underscore the importance of lectin chaperones in lipase folding (see later), and explain why specific glycan attachment sites have remained conserved during the evolution of the lipase gene family.

Lipase maturation factors

Chaperone and folding factors aid in the maturation of most proteins, from the time they first emerge from the Sec61 translocon (i.e., cotranslocationally) to the end of their folding and assembly cycle in the ER lumen (i.e., post-translationally). These factors also provide surveillance of maturation, known as ER quality control [72,84–87], ensuring that immature proteins remain in the ER until their folding is complete. Moreover, they direct terminally misfolded proteins to degradative pathways, and provide safeguards against massive protein misfolding by regulating the ER stress response [88]. As most proteins utilize the functions provided by these ER factors during the course of their maturation, they are considered 'general' maturation factors. However, some proteins also engage with specialized proteins when folding or assembly requires the attainment of unique structural characteristics; these are often known as 'client-specific' factors. Indeed, lipase maturation requires both.

A recent proteomics study found that HL maturation uses general maturation factors originating from both major chaperone systems operating in the ER: the CNX/CRT and binding protein (BiP)/Grp94 systems [76]. Both systems are comprised of component factors that form large, multiprotein complexes in the ER [89]. Table 1 lists some of the component factors that were found to associate with HL during its sojourn in the ER. In particular, HL associates with the lectin chaperone CNX, as would be expected from the maturation defects encountered when glucosidase inhibitors prevent binding of HL and LPL to CNX (discussed earlier); other studies using co-immunoprecipitation have also detected HL–CNX association [75,90]. Moreover, the maturation efficiency of transfected human LPL in insect cells is greatly increased by cotransfection with mammalian CNX/CRT [91]. Along with CNX, components of the CNX/

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CRT system were also identified as being associated with HL during its maturation [76], including UGGT, GII and ERp57 (Table 1). UGGT and GII provide repeated access of HL to CNX (discussed earlier), while ERp57 functions in the formation (oxidation), elimination (reduction) and transfer (isomerization) of disulfide bonds among cysteine residues of the CNX-bound nascent polypeptide.

Along with the CNX/CRT system, HL also associates with BiP/Grp94 (Table 1). Unlike CNX, BiP (also termed the 78-kDa glucose-regulated protein; Grp78) binds directly to the polypeptide backbone of nascent and misfolded proteins, a process independent of *N*-linked glycosylation [92–94]. BiP has an affinity for extended hydrophobic domains encountered in unfolded or misfolded proteins, with a preference for alternating aromatic and hydrophobic amino acids [95], estimated to occur on average every 36 residues [72]. Thus, it is not surprising that most proteins bind transiently with BiP during their sojourn in the ER. Indeed, BiP was found to co-isolate with every member of the lipase gene family (including PL) during a proteomics screen [Doolittle M, Unpublished Data, 76]. Along with BiP, components of the BiP/Grp94 system were also found to associate with HL (Table 1), including the 94 kDa glucose-regulated protein Grp94, protein disulfide isomerase and peptidyl-prolyl *cis-trans* isomerase (PPIase) [76]. PPIase converts *cis* peptidyl–prolyl bonds into the favored *trans* configuration comprising the majority of peptide linkages in native proteins; notably, such isomerization is a rate-limiting step in the *in vitro* refolding of chemically denatured LPL [80]. UGGT is also found to be associated with BiP [89], as with HL [76]; thus, while serving a function within the CNX/CRT chaperone cycle (Figure 2B), UGGT is also a component of the BiP/Grp94 chaperone system. Besides its role in protein maturation, BiP has additional functions, including ER quality control, regulation of the ER-stress response, and targeting terminally misfolded proteins to ERAD [86,92–94].

Besides general factors, lipase maturation also requires a client-specific chaperone, which has recently been identified as the protein affected by the combined lipase deficiency (*cld*) mutation. Mice homozygous for *cld* exhibit massive hypertriglyceridemia immediately after birth [38,96], and die shortly after owing to complications arising from the absence of LPL activity [97]; HL activity is also diminished [38,96,98]. The lack of lipase activity is not due to decreased levels of lipase mRNA or protein; rather, newly synthesized LPL remains inactive and is retained in the ER [83,99–101]. In fact, the inactive LPL protein expressed in *cld*/*cld* cells is highly aggregated, resembling misfolded LPL resulting from *lec23* [83] – a mutation affecting glucosidase I activity and thus preventing proteins from entering into the CNX cycle (Figure 2B). Nevertheless, the *cld* mutation is not genetically localized to lipase structural genes, lectin chaperones or any other general factors discussed above [102]. Moreover, the effects of *cld* appear to be limited to lipase maturation, as no other affected nonlipase proteins have been identified. We have recently shown that, along with LPL and HL, EL maturation is also defective in *cld*/*cld* cells [Peterfy M, Unpublished Data], increasing the ‘combined lipase deficiency’ phenotype to include all members of the lipase gene family that are known to form homodimers. Conspicuously, the maturation of PL, which is functional as a monomer, is unaffected by the mutation [38,99]. By screening genes within the critical chromosomal region of *cld*, only one candidate gene, a hypothetical transmembrane protein (Tmem112), was found to rescue the lipase maturation defect occurring in *cld*/*cld* cells. The gene was renamed ‘lipase maturation factor’ to reflect its function in mice [38]. Loss-of-function mutations (Y439X and W464X) in the human *LMF1* ortholog also causes combined lipase deficiency [38,103], verifying its function in humans as well.

As demonstrated by immunofluorescence microscopy [38], LMF1 colocalizes with CNX in the ER membrane. Figure 3 illustrates the topology of this client-specific LMF, which is a polytopic, multipass membrane protein localized exclusively to the ER [104]. LMF1 contains a large evolutionarily conserved domain of unknown function, referred to as DUF1222 in the

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Pfam database (Figure 3, thick line) [105]. This domain is found in a number of hypothetical proteins from bacteria to humans, with LMF1 being the first member of the DUF family with a known function. LMF1 has five α-helical transmembrane domains dividing the protein into three domains facing the cytoplasm, and three others oriented toward the ER lumen (Figure 3). The *cld* mutation causes truncation of the large C-terminal domain (Figure 3, arrow), as do Y439X and W464X nonsense mutations (Figure 3, ovals), suggesting that this domain is essential in carrying out the function of lipase maturation. Moreover, we have recently identified loop C as the site where both LPL and HL physically bind to LMF1 (Figure 3, arrow); notably, PL associates very poorly to this site. Thus, two domains important in carrying out the function of lipase maturation, loop C and the C-terminal domain, reside within DUF1222; both face the ER lumen where they are in proximity to nascent lipase polypeptides.

Mechanisms of lipase maturation

While the key factors in lipase maturation are now fairly well established (see earlier), the mechanisms involved in the process have yet to be fully elucidated. Nevertheless, as shown in Figure 4, a hypothetical model can be envisioned based on evidence gleaned from *in vivo* studies detailing the maturation of well-studied protein substrates, such as viral and some host proteins [72,106,107], and from *in vitro* studies monitoring the structure of folding intermediates occurring during the refolding of chemically denatured LPL [80,108,109]. In particular, LPL refolding experiments have demonstrated that folding of the smaller C-terminal domain happens quickly and completely, whereas folding of the N-terminal domain is much less efficient and occurs through folding intermediates exhibiting a degree of disordered tertiary structure [80]. Thus, the N-terminal domain would be expected to be subject to much more misfolding *in vivo*, with its proper folding constituting the rate-limiting step in lipase maturation. It is not surprising, therefore, that glycosylation at the conserved site in the N-terminal domain, as opposed to the C-terminal domain, is required for lipase maturation (Figure 2; see arrows).

The mechanism of lipase maturation can be considered to occur in two fundamental stages: the first stage would involve folding of the monomer (Figures 4A–E), which begins soon after the N-terminus of the lipase emerges through the Sec61 translocon (i.e., cotranslocationally); the second stage would entail assembly of fully folded monomers into homodimers (Figure 4F). *In vitro* evidence indicates that the first stage is rate limiting [80]; however, lipase maturation takes hours *in vitro*, as opposed to minutes *in vivo* [82]. Such disparate kinetics strongly indicates the involvement of chaperones and folding factors that facilitate folding of the monomer *in vivo*. While the second stage occurs rapidly *in vitro* (and likely *in vivo* as well), the product of the second step, lipase homodimers, are considered to be in a high-free-energy state that is inherently unstable and subject to dissociation [80,108]. Thus, both homodimers and folding intermediates are envisioned as being associated with chaperones and folding factors in the ER lumen, acting to stabilize and catalyze these two main steps in lipase maturation (Figure 4). Indeed, disruption of Ca^{2+} levels in the ER, an ion required for proper chaperone function [72], causes cessation of LPL maturation until appropriate levels are restored [110].

Folding of the monomer begins with chaperone stabilization of the lipase N-terminal domain as it emerges into the ER lumen through the Sec61 translocon (Figure 4A). Such stabilization would eliminate intermolecular associations leading to nonproductive aggregation [111], and would delay folding until lipase translation is finished. While either BiP or CNX/CRT could carry out this function, we believe CNX is the likely chaperone, based on the proximal position of the first conserved glycosylation site within the lipase N-terminal domain (Figure 2). Proteins with glycan attachment sites falling within approximately the first 50 amino acids (such as LPL, HL and EL) have been found to engage with CNX prior to BiP; proteins without

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such *N*-glycans at their N-terminus tend to associate with BiP first [72,107]. While both CNX and CRT carry out very similar functions, CNX is the lectin chaperone of choice during HL maturation [76]. CNX is better situated for cotranslational association with target proteins, since CNX (unlike CRT) is a membrane-bound protein, as is the Sec61 translocon, and only CNX is found to be associated with the ribosome during protein synthesis [112]. As the protein disulfide isomerase ERp57 physically associates with CNX, it seems likely that formation of intramolecular disulfide linkages also begins at this time (Figure 4A).

Glucosidase II cleavage would release the full-length lipase polypeptide into the ER lumen, which would likely be captured quickly as an unfolded intermediate by the BiP/Grp94 multi-protein complex (Figure 4B). Among its component proteins, the PPIase would catalyze the rate-limiting isomerization of *cis* peptidyl–prolyl bonds to the *trans* configuration. Upon release from the BiP–Grp94 complex, folding of the lipase polypeptide would happen quickly, with establishment of the β-sandwich fold comprising the C-terminal domain occurring efficiently and completely [80]. By contrast, the N-terminal domain would remain in a 'molten globule' state (Figure 4C). In LPL-refolding experiments, such a state was found to exhibit a native-like secondary structure, as determined by far-UV circular dichroism, but a less-ordered tertiary structure, as assessed by increased intrinsic tryptophan fluorescence and elevated binding of the hydrophobic molecule 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid [80]. In Figure 4C, we consider such an intermediate as a 'partially folded' monomer with the potential to form either a fully folded monomer (Figure 4E) or to undergo misfolding (Figure 4H), which would prevent further maturation. We propose that LMF1 would capture a partially folded monomer exhibiting native structure (Figure 4D) but not misfolded forms; rather, misfolding would cause the monomer to recycle back to CNX through UGGT (Figure 4G) or to enter into the ERAD pathway (Figure 4H). The slow kinetics of HL maturation suggests that CNX cycling indeed occurs [75], and this is supported by the finding that HL associates with UGGT [76]. UGGT can sense localized regions of polypeptide disorder in misfolded proteins [113,114], adding a glucose residue to the high-mannose chain in order to re-establish CNX binding (Figure 2B). Unlike HL, however, repeated CNX cycling of LPL seems unlikely, owing to the much faster kinetics of LPL maturation [81]. In fact, LPL aggregates appear in pulse experiments nearly simultaneously with the formation of homodimers; these aggregates exhibit properties of terminally misfolded forms (Figure 4I), including intermolecular disulfide bonding, ER retention and eventual ERAD [81].

Lipoprotein lipase refolding *in vitro* is capable of forming homodimers with an efficiency of approximately 40%, a process taking hours at 25°C and requiring the presence of Ca^{2+} ions and other stabilizing factors [80]; notably, the fully folded monomer was not detected *in vitro*, indicating that it is a very short-lived intermediate. In the case of *in vivo* lipase maturation, there are several reasons to suspect that chaperone association would stabilize the partially folded intermediate (Figure 4D), the short-lived fully folded monomer (Figure 4E) and the fully functional homodimer (Figure 4F). First, *in vivo* lipase maturation occurs with an efficiency of approximately 70% at 37°C [75,81] – a temperature that greatly reduces the efficiency of LPL refolding *in vitro* by increasing LPL aggregation [80]. Aggregation occurs by inappropriate intermolecular interactions that are prevented by chaperone association [111]. Second, the short-lived nature of the fully folded monomer may result from surface hydrophobic regions required for noncovalent homodimer association (Figure 1A, arrows); such surface hydrophobicity would be thermodynamically unstable unless shielded from the aqueous environment by chaperone association (Figure 4E). Finally, the high-free-energy state of the homodimer favors its rapid dissociation into misfolded monomers unless stabilized *in vitro* by binding to factors such as heparin [80]. However, studies have indicated that the LPL dimer is extraordinarily stable in the ER lumen [81,82], suggesting that an ER-specific stabilizing factor prevents such dissociation *in vivo* (Figure 4F).

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What is the stabilizing factor that facilitates these later steps in lipase maturation, as shown in Figure 4D–F? Considering that such steps involve the attainment of structural features unique to lipase proteins, we propose that they are chaperoned by the client-specific factor LMF1. LMF1 has the ability to bind lipases in the ER, and in its absence, LPL homodimers decline severely while LPL aggregates increase dramatically [83]. Thus, without functional LMF1, maturation steps Figure 4D–F occur very inefficiently indeed. By contrast, early lipase maturation steps occurring through CNX and BiP seem to be independent of LMF1. For example, LPL in *cld*/*cld* cells is properly glycosylated and processed [83], indicating that these early maturation steps remain unaffected (Figure 2). Thus, LMF1 most likely functions in later stages of lipase maturation, when lipase monomers and homodimers may need to be shielded from an ER environment conducive to their misfolding, dissociation and aggregation.

Conclusion

Lipase maturation is a process occurring in the ER that involves the folding and assembly of newly synthesized (nascent) lipase polypeptides into fully functional enzymes. The structural similarities of LPL, HL and EL, particularly in their requirement for a homodimer configuration, suggest that these lipase family members share a similar maturation pathway. This pathway utilizes the two major chaperone systems operating in the ER, the CNX and BiP/Grp94 multiprotein complexes, which probably stabilize and catalyze folding of nascent lipase polypeptides into partially folded monomers. Final maturation of the partially folded monomers to fully assembled homodimers may require the client-specific chaperone, LMF1. This lipase-specific chaperone may also stabilize the homodimer from its dissociation into a lower free energy state of misfolded monomers. The absence or functional loss of any of these ER chaperones, such as LMF1, results in combined lipase deficiency that has profound impacts on pathophysiological phenotypes such as hypertriglyceridemia.

Future perspective

The most challenging aspect of lipase maturation is the elucidation of LMF1 function, both as a maturation factor and as a candidate in lipase regulation. As a maturation factor, a number of questions remain unanswered. What is the precise role of LMF1 in the mechanism of lipase folding; does it stabilize the partially folded monomer and homodimer as proposed in Figure 4? What are the functions of the various LMF1 domains in this and related processes, such as ER localization? The polytopic nature of LMF1 suggests a complexity of functions involving associations with both cytoplasmic and ER lumenal proteins; what is the identity of these binding partners and how do they assist in lipase maturation? Is LMF1 also involved in the effective exit of homodimers from the ER, possibly by associating with the cytosolic coat protein II vesicles budding from the ER?

As a result of its essential role in lipase maturation, LMF1 may regulate lipase activity levels *in vivo*. It is the only known factor affecting the activity and secretion of every member of the lipase gene family except PL. The consequence of LMF1 deficiency on systemic and cell-associated phenotypes is a challenge for the future. The *cld*/*cld* mouse has limited utility in this regard, owing to the lethal nature of LPL deficiency occurring shortly after birth. Thus, inducible and tissue-specific models of LMF1 deficiency are needed to overcome the lethality and to ascertain cell-associated phenotypes in isolation from overwhelming systemic effects, such as massive hypertriglyceridemia. Furthermore, LMF1 may have roles other than lipase maturation; unlike lipase proteins, it is expressed in nearly all tissues and early during development [Peterfy M, Unpublished Data]. Moreover, naturally occurring *LMF1* splice forms exist that lack the DUF1222 domain, and thus cannot function in lipase maturation. What are the functions of these splice variants, and what are the roles of LMF1 in tissues or during developmental times when lipases are not expressed? Could LMF1 be involved in some aspect

of ER homeostasis, such as contributing to the ER-stress response? While questions abound concerning LMF1 function, expression and regulation, it is abundantly clear that it plays a pivotal role in lipase maturation.

Executive summary

Lipase function & molecular structure

- Lipase maturation is defined as the folding and assembly of a family of proteins consisting of pancreatic lipase (PL), lipoprotein lipase (LPL), hepatic lipase (HL) and endothelial lipase (EL).
- While all lipases hydrolyze triglycerides and/or phospholipids, each has specific functions *in vivo*, including absorption of dietary fats (PL), remodeling and uptake of lipoproteins (LPL, HL and EL) and regulation of fatty acid influx in adipose tissue, muscle and the heart. The lipase monomer is divided into an N- and C-terminal folding domain. Except for PL, monomers must assemble into homodimers in order to form fully functional enzymes.

Lipases in disease

- Lipoprotein lipase, HL and EL have emerged as some of the strongest genetic determinants of circulating triglyceride and HDL-C levels in human populations.
- Mutations affecting *trans*-acting factors that affect lipase expression, such as lipase maturation, can also have profound affects on triglyceride and HDL-C levels.
- Mouse models of LPL overexpression indicate that lipase dysregulation can contribute to metabolic traits related to obesity, cardiomyopathy and insulin resistance.

Lipase glycosylation & processing in the endoplasmic reticulum

- Lipoprotein lipase, HL and EL exhibit conserved glycosylation sites in both folding domains. Glycosylation of conserved N-terminal sites is necessary for efficient lipase maturation.
- Glycan processing by glucose trimming is an early but essential step in lipase maturation, facilitating cycles of lipase binding and release of chaperone calnexin (CNX) from the endoplasmic reticulum (ER).

Lipase maturation factors

- The folding and assembly of lipases in the ER requires general and specific maturation factors.
- General factors include the CNX/calreticulin and BiP/Grp94 chaperone systems, each comprised of multiple folding factors.
- The ER membrane protein lipase maturation factor (LMF) 1 is a lipase-specific factor required in LPL, HL and EL folding and assembly.

Mechanisms of lipase maturation

- Maturation begins cotranslationally by association of growing lipase chains with CNX; after its translation and release, the unfolded lipase is likely captured by the BiP/Grp94 chaperone system.

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- Folding of the large N-terminal domain is a rate-limiting step, and proceeds through partially folded intermediates that can misfold prior to final folding and assembly.
- Final lipase folding and assembly produce fully functional homodimers, a process likely aided by LMF1. LMF1 may also stabilize lipase homodimers until their exit from the ER.

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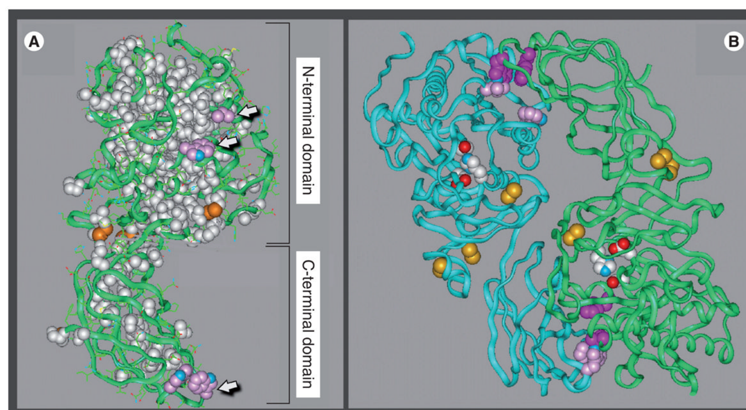

Figure 1. Structural model of hepatic lipase

(A) The hepatic lipase (HL) monomer is modeled after the crystal structure of pancreatic lipase (PL). Model building and short molecular dynamics runs were performed on a Silicon Graphics Indigo 2 computer with Insight II and Discover 2005 software (Accelrys Inc., CA, USA). The side chains of HL were mapped on to the backbone according to a multiple sequence alignment for HL and PL generated by using the MULTALIN multiple alignment algorithm. The location of the two major folding domains of the HL model is indicated by brackets. Amino acids that are conserved between HL and PL and are depicted in a space-filling form as white; the green ribbons are comprised of residues unique to HL. Also shown are conserved disulfides (orange); arrows point to side chains of surface-located hydrophobic residues (pink). **(B)** The noncovalent HL homodimer, with the two individual monomers is depicted as ribbons colored blue and green. The side chains of active-site residues are colored red, white and blue; conserved disulfides are highlighted orange; and the side chains of hydrophobic residues comprising putative dimer interaction sites are shown in pink and purple.

Models are courtesy of Keith Munson and Howard Wong.

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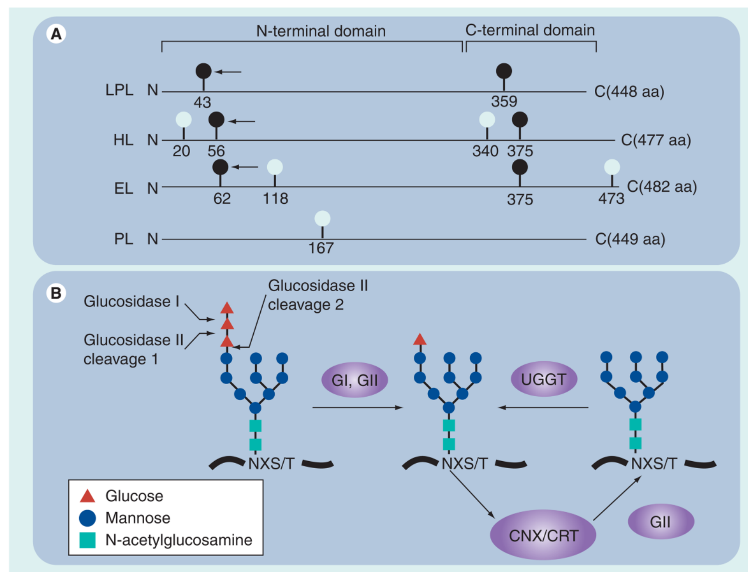

Figure 2. Glycosylation sites and processing events that are important in lipase maturation

(A) Glycan attachment sites (consensus sequence NXS/T) among members of the human lipase gene family are depicted by light and dark balls. Dark balls represent evolutionarily conserved sites. The arrow points to sites of glycosylation essential in lipase maturation. Also shown are the relative positions of both N- and C-terminal folding domains. **(B)** Cleavage sites for GI and GII on the unprocessed high mannose chain; this chain is added to NXS/T sites shortly after they emerge from the ribosome during translation elongation. GI and GII cleavages occur rapidly and result in a processed monoglucosylated chain that can bind to CNX or CRT cotranslationally; release occurs after the second cleavage by GII. If the nascent protein becomes misfolded, a single glucose can be added to the unglucosylated chain by the ER lumenal protein, UGGT 1; thus, the lipase can reattach to CNX/CRT in a process termed chaperone cycling.

aa: Amino acid; CNX: Calnexin; CRT: Calreticulin; EL: Endothelial lipase; ER: Endoplasmic reticulum; G: Glucosidase; HL: Hepatic lipase; LPL: Lipoprotein lipase; PL: Pancreatic lipase; UGGT: UDP-glucose: glycoprotein glucosyltransferase.

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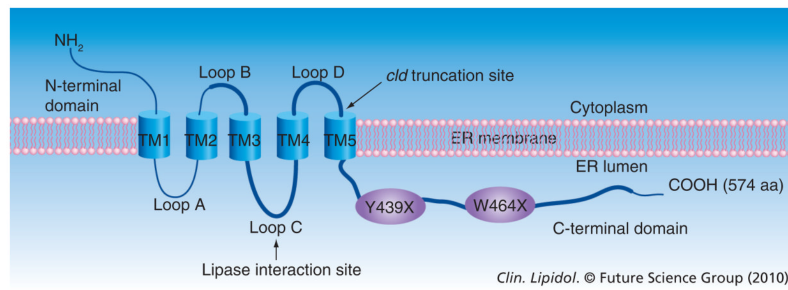

Figure 3. Structure of the recently identified lipase maturation factor 1

The five α-helical transmembrane segments divide the protein into six separate domains. The approximate size of each domain is 49 aa, N-terminal domain; 56 aa, loops A, B; 71 aa, loop C; 46 aa, loop D; and 188 aa, C-terminal domain. The evolutionarily conserved domain of unknown function (DUF1222) is depicted as a thick line. Arrows point to the *cld*-induced truncation site and the site of lipase interaction. Y439X and W464X are nonsense mutations causing combined lipase deficiency in human patients. aa: Amino acid; ER: Endoplasmic reticulum; TM: Transmembrane.

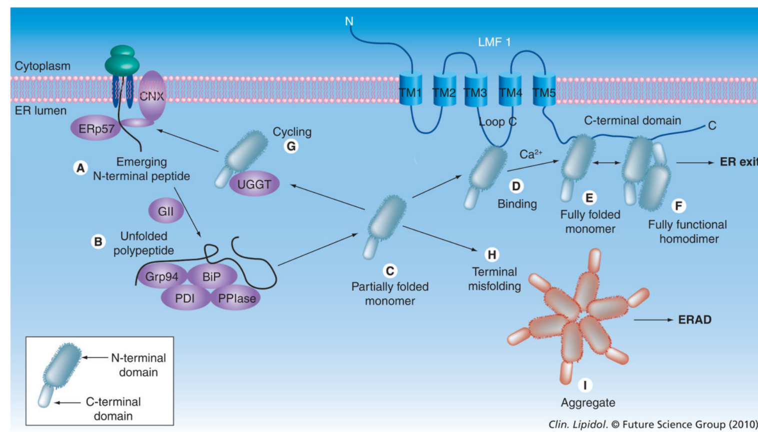

Figure 4. Model of lipase maturation

The N- and C-terminal folding domains comprising the lipase monomer; wavy lines indicate an N-terminal domain that is partially folded **(C, D)** or misfolded **(G, I)**. Only the homodimer exhibits lipolytic activity and exits the ER; all other lipase forms are inactive and are retained in the ER. Terminally misfolded forms, such as the aggregate, are destined for ERAD. BiP: 79 kDa binding protein; CNX: Calnexin; ER: Endoplasmic reticulum; ERAD: Endoplasmic reticulum-associated degradation; ERp56: 57 kDa endoplasmic reticulum protein; GrP94; Grp94: 94 kDa glucose-regulated protein; HL: Hepatic lipase; PDI: Protein disulfide isomerase; PPIase: Peptidyl-prolyl cis–trans isomerase; TM: Transmembrane; UGGT: UDP-glucose: glycoprotein glucosyltransferase 1.

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Table 1

General factors in lipase maturation.

Chaperone system	Component	Location	Functions in lipase maturation
CNX/CRT	CNX	ER membrane	Stabilize unfolded and misfolded forms; provide platform for folding factors.
	UGGT, GII	ER lumen	Chaperone cycling, providing repeated access of HL to CNX.
	ERp57	ER lumen	Oxidation, reduction and isomerization of disulfide bonds.
BiP/Grp94	BiP, Grp94	ER lumen	Stabilize unfolded and misfolded forms; entry into the ERAD pathway.
	PDI	ER lumen	Oxidation, reduction and isomerization of disulfide bonds.
	PPIase	ER lumen	Isomerization of peptidyl–prolyl bonds.

BiP: 78-kDa binding protein; CNX: Calnexin; CRT: Calreticulin; ER: Endoplasmic reticulum; ERAD: Endoplasmic reticulum-associated degradation; ERp57: 57-kDa endoplasmic reticulum protein; GII: Glucosidase II; Grp94: 94-kDa glucose-regulated protein; HL: Hepatic lipase; PDI: Protein disulfide isomerase; PPIase: Peptidyl-prolyl cis–trans isomerase; UGGT: UDP-glucose: glycoprotein glucosyltransferase 1.