Maturation of Lipoprotein Lipase in the Endoplasmic Reticulum

CONCURRENT FORMATION OF FUNCTIONAL DIMERS AND INACTIVE AGGREGATES*

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The maturation of lipoprotein lipase (LPL) into a catalytically active enzyme was believed to occur only after its transport from the endoplasmic reticulum (ER) to the Golgi apparatus. To test this hypothesis, LPL located in these two subcellular compartments was separated and compared. Heparin affinity chromatography resolved low affinity, inactive LPL displaying ER characteristics from a high affinity, active fraction exhibiting both ER and Golgi forms. The latter forms were further separated by β-rinic chromatography and were found to have comparable activities per unit of LPL mass. Thus, LPL acquired its fully functional form in the Golgi apparatus.

Active LPL, regardless of its cellular location, exhibited comparable activities per unit of LPL mass. Thus, LPL must reach a functional conformation in the ER. LPL must therefore be hereby marked "WITHDRAWN July 22, 2019." This article has been withdrawn by Osnat Ben-Zeev and Mark H. Doolittle. Hui Z. Mao could not be reached. Fig. 3A contained several duplicated regions. Figs. 3, B and C; 5B; 6, B and C; 7, A and B; and 8, A and B, were inappropriately manipulated.

Most circulating dietary- and liver-derived fatty acids arrive at the heart, adipose tissue, and skeletal muscle esterified as triglycerides located within the hydrophobic core of chylomicrons and very low density lipoproteins. At the luminal surface of tissue capillaries, the esterified fatty acids are then released following the hydrolysis of core triglycerides by lipoprotein lipase (LPL). LPL is synthesized by the tissue parenchymal cells and is secreted to the surface of the capillary endothelium, where it binds to heparan sulfate proteoglycans (1, 2). The free fatty acids released by the action of LPL are subsequently taken up by the subjacent tissue for storage or oxidation.

The acquisition of enzymatic activity by newly synthesized LPL, a process known as maturation, has remained an unresolved question. Mature, fully functional LPL is a homodimer that binds heparin with high affinity (2, 3). As a secreted protein, nascent LPL is vectorially transported from its site of synthesis at the cytoplasmic face of the ER into the lumen, where asparagine-linked glycosylation takes place. It has been well documented that initial glucose trimming from these nas-
it was shown that LPL was retained in the ER because the mutations rendered it misfolded and transport-incompetent rather than a defect in transport impeding LPL maturation (13).

In the present study, a prime objective was to study whether LPL maturation occurs in the ER by attempting to isolate a fully functional LPL fraction that exhibited only ER processing. Our characterization of such a form corroborates the fact that LPL becomes fully active in the ER, and only this transport-competent form enters the Golgi en route to secretion. Our findings also establish the existence of an inactive LPL pool in the ER. However, this pool is not a precursor to the active form and does not acquire a native conformation. Rather, it contains "off-pathway," misfolded LPL molecules that form large aggregates. These aggregates are degraded in the ER, conforming to the stringent quality control mechanism of the cell. However, when the cells were stressed by reducing disulfide bonds, a large, inactive LPL complex exhibiting different properties from the aggregate was formed. Under these conditions, the functional dimer itself coalesced, losing enzyme activity in the process. Unlike the normal aggregates occurring in control cells, the stress-generated complex was found to be LPL arrayed with several folding chaperones; upon return of the cells to normal conditions, the complex dispersed to reform fully functional, transport-competent enzyme.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Media—**All cell cultures were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (for CHO and EcR-CHO cells) or 10% (for 3T3-L1 adipocytes), antibiotics (50 units/ml penicillin, 100 units/ml streptomycin), 0.1 mM nonessential amino acids, 4 mM pyruvate. CHO cells were the proline auxotroph derivatives (Pro5) obtained from the ATCC (Manassas, VA). EcR cell line (Invitrogen) was maintained as described above. The entire coding region of human LPL (including the termination codon of human LPL cDNA) was replaced with a modified version of the cis-acting SP1 elements, the latter elements incorporated by regulated expression of the epitope-tagged LPL construct was achieved using the pIND(SP1) edysyme-inducible expression vector (Invitrogen). The entire coding region of human LPL (including the epitope tag) was isolated as a HindIII/BamHI fragment. This fragment was cloned into the pcDNA6/V5-His B/LPL vector to achieve enhanced expression levels. The pcDNA6/V5-His B, which was subcloned in pcDNA6/V5-His (Invitrogen). The entire coding region of human LPL in frame with the V5-epitope tag of pcDNA6/V5-His B/LPL, and this fragment cloned into the pIND(SP1) construct was sequenced in its entirety and used to transiently transfected CHO cells.

**Transfection, Selection, and Harvesting of Cells—**CHO and EcR-CHO cells were stably transfected with pcDNA6/V5-His LPL and pIND-LPL, respectively, using the calcium phosphate method (14). The selection medium contained 10 μg/ml blasticidin S HCl (CHO) or 50 μg/ml hygromycin (EcR-CHO). LPL-expressing colonies were identified by assaying medium for LPL activity following incubation for 16 h with 10 units/ml heparin (CHO), or 10 units/ml heparin and 10 μg/ml pison-teron A (EcR-CHO). LPL-expressing EcR-CHO colonies were further screened for their potential to undergo inducible expression in response to increasing concentrations of pison-teron A (1–10 μM; 16-h incubation). Only colonies that responded with at least a 4-fold increase in LPL activity were selected. pcDNA6/V5-His H2b was transiently transfected into CHO cells using the reagent PuGENE 6 (Roche Molecular Biochemicals), according to information supplied by the manufacturer. Based on a β-galactosidase control expression vector, about 20–30% of CHO cells were transfected using this reagent. The CHO cells were analyzed 24 h post-transfection.

Cells were subcultured onto 100-mm plates and propagated in 8 ml of medium; however, experiments were conducted using 3 ml of medium to increase the concentration of secreted LPL. Unless otherwise indicated, fresh medium containing heparin (10 units/ml; Sigma) was added to the culture medium 4 h prior to experiments and was also included at the same concentration during experimental procedures. Incubations of LPL-transfected cells with various inhibitors were carried out for up to 3 h, as the secretion of the dimer and turnover of the aggregate occur with a half-life of about 30 min. Experiments with the H2b subunit of the asialglycoprotein receptor were carried out for 6 h, due to the slower turnover rate of this protein.

**Chromatography and Centrifugation—**Heparin Sepharose chromatography was carried out as previously described (15). For measuring high affinity LPL fraction (LPL), the cell monolayer was treated with neuraminidase. The LPL-containing 0.1% Triton X-100 buffer of the preceding chromatography was collected as the column fraction eluted with 0.2 M galactose (Sigma) in modified Eagle's medium containing 10% fetal bovine serum. The collection of LPL was carried out using a 12-ml linear 20% linear sucrose gradient as described previously (15). Generally, each fraction was treated to each gradient consisted of a cell lysate derived from CHO cells; centrifugation was carried out at 200,000 × g for 22 h at 4 °C. Generally, centrifugation, fractions of 0.48 ml were manually collected from the top. To ensure maximal recovery of the large LPL aggregate that sedimented to the bottom of the tube during centrifugation, the last fraction was vigorously shaken prior to removal from the gradient tube.

**Cell Labeling—**LPL-expressing CHO cells were grown in 175-cm² flasks to confluence. After washing cells in PBS, prewarmed Dulbecco's modified Eagle's medium containing 0.1 mM glutamate and 1 mM sodium pyruvate, but no methionine, cysteine, or serum, was added, and cells were allowed to equilibrate for 20 min. Tran35S-label (American Pharmacia Biotech) was added to a final concentration of 130 Ci/ml. The labeling reaction was stopped after 5 or 20 min of pulse time by quickly removing the labeling medium and replacing it with 25 ml of ice-cold stopping solution (PBS containing 15 μg/ml cycloheximide). The stopping solution was gently swirled over the cell monolayer for 30 s and removed, and an additional wash with stopping solution was applied. The cells were harvested by scraping into 10 ml of fresh stopping solution. The cells were centrifuged as described above, and the result cell pellets were stored at –80 °C.

**Glycosidase Digestions—**Samples selected for endoglycosidase H (endo H) digestion were adjusted to pH 5.9 by the addition of 0.1 M sodium phosphate buffer, pH 5.5. If samples contained NaCl concentrations of >0.75 M, they were dialyzed at least 2-fold with 50 mM sodium phosphate buffer, pH 5.9. To facilitate endo H digestion, proteins were denatured by adding SDS to 0.5% and heating the sample in a boiling water bath for 2 min. After chilling on ice, 10 milliliters of endo H (Roche Molecular Biochemicals) were added, and the samples were incubated at 37 °C for 18–20 h.

Samples selected for endo-β-N-acetylglucosaminidase D (endo D) digestion were adjusted to pH 6.5 and digested at 37 °C for 18–20 h. To facilitate endo D digestion, proteins were denatured by adding SDS to 0.5% and heating the sample in a boiling water bath for 2 min. After chilling on ice, 10 milliliters of endo D (Calbiochem) and incubation at 37 °C for 18–20 h. A positive control (LPL containing only endo D-sensitive, Man₇ oligosaccharides) was always included to ensure that endo D digestion occurred.

Neuraminidase digestion was used for samples subjected to β-rinic chromatography (see above). Since these samples contained 1.5 μg NaCl,
Intracellular LPL Is Located Primarily in the ER—In the absence of heparin in the culture medium, although LPL is secreted from the cells, a significant amount remains attached to the cell surface through interaction with heparan sulfate proteoglycans. Only a fraction of LPL is present in the medium, where it accumulates with time. Thus, after 4 h of incubation in the absence of heparin, about 60% of the total LPL activity expressed in those cells to enzyme residing in the ER (see Fig. 1). However, since published evidence suggested that LPL in the ER was inactive (6, 10, 11), it was possible that the expressed activity was solely derived from the small Golgi pool present in those cells.

As a first step in resolving this uncertainty, LPL from CHO cells incubated for 4 h in heparin was subjected to heparin-Sepharose chromatography. We have shown previously that this procedure can separate inactive from active LPL by elution at increasing ionic strengths (13). Indeed, as shown in Fig. 2 (top), an inactive LPL fraction eluted at a lower salt concentration (0.75 M NaCl) than active LPL, which bound with higher affinity and eluted at 1.5 M NaCl (see activity profile and the subjacent Western blot of eluted LPL protein). Endo H digestion of the peak fractions revealed that low affinity, inactive LPL was exclusively located in the ER, whereas high affinity, active enzyme was localized in both ER and Golgi compartments.

To determine whether LPL in both ER and Golgi contributed to the catalytic activity of the high affinity fraction, the two LPL forms were separated based on their organelle-specific glycan structures. This was accomplished by β-rin chromatography. As the β-rin toxin specifically binds galactose, it interacts only with Golgi-processed (complex) glycoproteins, provided that their penultimate galactose residues have been exposed. Accordingly, the terminal sialic acid of LPL from the active sample was removed by neuraminidase digestion prior to its application onto a β-rin-agarose column. It was expected that the unbound fraction would contain LPL exhibiting high mannose glycans, while the bound sample, eluting at 0.2 M galactose, would contain LPL bearing complex sugars.
As shown in Fig. 2 (bottom), β-rin chromatography separated the high affinity fraction into two peaks of activity: one that emerged in the flow-through and a second peak that bound to the column and eluted at 0.2 M galactose. The equivalent of 2 milliunits of LPL activity from each peak was subjected to Western blotting following incubation with or without endo H; the flow-through peak was also subjected to endo D digestion (see below). Endo H digestion confirmed that the flow-through contained only LPL with high mannose glycans, whereas the galactose eluate contained only LPL with complex glycan chains. Scanning densitometry of the undigested bands was carried out in order to compare relative specific activities (LPL activity per unit of enzyme mass), and these were found to be similar: 1.0 for the flow-through and 0.85 for the galactose eluate. Thus, high mannose LPL was at least as active as LPL bearing complex glycan chains. Finally, to ascertain that the high mannose form indeed represented LPL residing in the ER, this fraction was subjected to endo D (endo-β-N-acetylglucosaminidase D) digestion. This enzyme cleaves only the Man₅ form of glycan chains, which, although it is endo H-sensitive, is characteristic of glycoproteins that have reached the cis-Golgi compartment and have been trimmed by the Golgi mannosidase I (18). As evident from the resistance of high mannose LPL to endo D digestion, this pool of lipase had not yet reached the cis-Golgi and must represent LPL located in the ER. Consequently, the ER must also be the machinery necessary for maturation of LPL to its functional form. Strikingly, however, the ER also contained a substantial pool of inactive LPL that eluted from the column with 0.75 M NaCl (Fig. 2, top). Thus, we decided to characterize the inactive form of LPL.

Active LPL Is Highly Dimeric, whereas Inactive LPL Is Highly Monomeric. Therefore, it was anticipated that LPL with low affinity to heparin is a monomer (19–21). Indeed, the resistance of high mannose LPL to endo D digestion suggested that LPL with low affinity to heparin is a monomer. Moreover, since all secreted LPL is active and eluted from heparin-Sepharose at 0.75 M NaCl (see Fig. 2, bottom), it was anticipated that LPL residing in the cis-Golgi compartment would be exclusively in the dimeric form as suggested by others (9). Thus, our next objective was to characterize the inactive form of LPL.

Fig. 3. Separation of cell-associated forms of LPL. A, separation of high-affinity LPL was sequentially eluted from heparin-Sepharose columns with 0.2 and 1.5 M NaCl (see Fig. 2). Aliquots of the peak fractions were subjected to endo H analysis for assessment of subcellular location. Low affinity LPL eluted from the column as a mixture of high mannose forms and complex forms, consistent with an exclusive ER location. While a high mannose form was also present in the 1.5 M NaCl fraction, the additional presence of a complex form in this fraction indicates that high affinity LPL is located in both ER and Golgi compartments. B, separation of high affinity LPL into ER and Golgi forms. β-Ricin chromatography was used to isolate LPL into ER and Golgi forms based on organelle-specific glycan structures. The LPL complex form, containing a penultimate galactose residue due to Golgi processing, binds β-rin with high affinity after removal of terminal sialic acid residues by neuraminidase (see “Experimental Procedures”). It remains bound to the column until elution with 0.2 M galactose (arrow). In contrast, the LPL high mannose form characteristic of ER glycoproteins emerges as unbound protein in the column flow-through. Note that both the high mannose and complex high affinity forms of LPL exhibit activity, suggesting that both possess a native conformation. The high mannose LPL fraction was also subjected to endo D digestion, to verify that the glycan chains had not yet been trimmed to Man₅ by cis-Golgi mannosidase. Indeed, the resistance of the high mannose form to endo D digestion confirmed its exclusive location in the ER.

2 O. Ben-Zeev, H. Z. Mao, and M. H. Doolittle, unpublished data.
The possibility was also considered that aggregation resulted from LPL overexpression since the cell line employed in this study was clonally selected to express high LPL activity. Accordingly, LPL was stably transfected into an ecdysone-inducible CHO cell line (EcR-CHO) and expressed at increasing levels by induction with ponasterone A (24). When LPL in these cells was subjected to sucrose gradient sedimentation, an aggregate was present at all levels of expression, and its amount increased proportionally with increasing levels of the LPL dimer (Fig. 3C). Moreover, a significant pool of inactive aggregate was also detected in differentiated 3T3-L1 adipocytes, cells that are untransfected but endogenously express LPL. Taken together, these experiments rule out the possibility that the aggregate was an artifact resulting from transfection and/or overexpression.

**Aggregated LPL Is Not a Precursor of the Native Enzyme**—The co-localization in the ER of inactive, aggregated LPL together with its active, native form suggested that they might share a precursor-product relationship. Although aggregation is often the result of irreversible misfolding, it has also been shown to occur as an intermediary step between the nascent polypeptide chain and the properly assembled, native protein (25, 26).

To test for a precursor-product relationship, cells were pulse-labeled for 5 or 20 min with [35S]methionine. At each time point, the cell lysates were subjected to sucrose gradient cen-
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Fig. 4. Pulse labeling of LPL indicates concurrent formation of LPL dimer and aggregate. LPL-expressing CHO cells were pulse-labeled with [35S]methionine/cysteine for 5 or 20 min. Lysates of the labeled cells were subjected to sucrose gradient centrifugation, and the fractions containing dimer and aggregated LPL were identified by Western blotting (data not shown). Aliquots of dimer and aggregated LPL were immunoprecipitated followed by endo H digestion. The resulting samples were subjected to SDS-PAGE followed by immunoblotting with horseradish peroxidase-conjugated anti-V5 antibody. The resulting samples were subjected to autoradiography to assess incorporated label. The dimer fraction in contrast to the exclusive high mannose composition of the aggregate (see Western blot panel), auto radiograph panel in Fig. 4). Both dimer and aggregate fractions were detected by Western blot analysis as described under "Experimental Procedures." Comparison of LPL in cells and medium following inhibition of de novo protein synthesis indicates secretion of the dimer but intracellular degradation of the aggregate.

As shown in Fig. 4 (Western blot panel), steady state levels of the LPL dimer consisted of the expected mixture of Golgi and high mannose forms, whereas the aggregate existed only as its anticipated high mannose form. Analysis of radioactivity indicated that even after a short pulse time of 5 min, the extent of [35S]methionine incorporation was equivalent in the dimer and aggregated forms (see autoradiograph panel in Fig. 4). Both newly synthesized LPL forms were still in the ER, evident from their complete endo H susceptibility. At the longer pulse time (20 min), the dimer contained both endo H-sensitive and -resistant forms, indicating the normal progression of functional LPL to the Golgi; in contrast, the aggregate remained at all times in the ER.

The incorporation of label per unit of LPL mass (i.e., radio- specific activity) at the two pulse times was calculated for LPL in the ER, represented by the high mannose forms. If the aggregate was a precursor of the dimer, it should incorporate label prior to the appearance of label in the dimer. As shown in the graph of Fig. 4, no such delay was found, although, within the time frame of the experiment, an expected lag between a potential precursor and its product could have been detected. This finding kinetically ruled out the possibility that the active dimer in the ER was formed from the inactive aggregate.

Degradation of the Aggregate and Secretion of the Dimer—Since the inactive LPL aggregate was not converted into a dimer and, based on endo H susceptibility, was restricted from advancing into the secretory pathway, it followed that it must be degraded within the ER. To test this hypothesis, the fate of the dimer and aggregate was followed after arrest of de novo protein synthesis. CHO cells pretreated with heparin for 4 h were incubated for increasing time periods with heparin and cycloheximide (Cx). The expected secretion of active LPL was shown by the progressive appearance of increasing amounts of LPL activity in the medium balanced by a proportional decrease of activity in the cells (Fig. 5A). After 1 h, most of the activity could be accounted for in the medium, whereas slightly

3 As radiospecific activity increased through 20 min of pulse time in both aggregate and dimer, [35S]methionine incorporation into their respective pools had not yet reached steady state. Only at steady state would a precursor-product relationship be impossible to detect.
more than 10% remained in the cells. As the combined activity (cells plus medium) remained constant, activity was not lost within the time period analyzed. Indeed, commensurate with activity, the LPL dimer was secreted to the medium without any apparent loss of protein during 60 min (Fig. 5B). However, unlike the dimer, the LPL aggregate disappeared from the cells and did not appear in the medium (Fig. 5B), indicating its degradation in the ER.

We raised the question of whether the selective degradation of the LPL aggregate was a consequence of its retention in the ER, where it was exposed to proteolytic factors, while the dimer “escaped” degradation by being secreted. To test this possibility, the cycloheximide experiment was repeated in the presence of cycloheximide (data not shown), this finding ruled out the possibility that the degradation of the aggregate required synthesis of its replenishment by that portion of precursor normally undergoing proteasomal degradation. Similar results were obtained with an array of other proteasomal inhibitors: lactacystin (50 μM), clasto-lactacystin β-lactone (10 μM), N-acetyl-leucyl-leucyl-methioninal (100 μM), and N-acetyl-leucyl-leucyl-norleucinal (100 or 200 μM). Thus, the LPL aggregate must be degraded in the ER via a nonproteasomal pathway. Lysosomal degradation of the aggregate was also considered; however, no effect was seen when the cells were incubated with leupeptin (LP; 10 μM) or chloroquine (100 μM) (Fig. 7B, see LP as representative). Since no accumulation of LPL aggregate was observed when either proteasomal or lysosomal inhibitors were used in the absence of cycloheximide (data not shown), this finding ruled out the possibility that the degradation of the aggregate required synthesis of a short lived protein. However, when cells were depleted of ATP by incubation in 2-deoxy-D-glucose, the rate of degradation was drastically reduced (Fig. 7B, 2DG). Thus, the LPL aggregate is turned over by an energy-dependent pathway that is other than proteasomal or lysosomal.

Since interchain disulfide bonds often occur within aggregates of misfolded proteins, their presence in the LPL aggregate was evaluated by comparing its migration in SDS-PAGE under reducing and nonreducing conditions (with and without β-mercaptoethanol). The LPL dimer, whose migration was unaffected by the presence or absence of β-mercaptoethanol was included as a control. Indeed, even in the absence of reducing, the dimer fraction migrated during SDS-PAGE to the expected...
57-kDa region of the gel. However, under these nonreducing conditions, the bulk of the aggregate fraction was not detected in this region of the blot (Fig. 7C) and in fact was even unable to penetrate the stacking gel. Thus, it appeared that misfolded LPL in the ER associates into large aggregates that feature interchain disulfide bonds.

Disruption of Disulfide Bond Formation and Its Effect on the LPL Dimer and Aggregate—The presence of interchain disulfide bonds within the aggregate suggested that reduced conditions in the environment of the ER might cause dissociation of the aggregate into LPL monomers. To generate reducing conditions, we employed DTT, a membrane-permeable reductant that abolishes formation of disulfide bonds without affecting most other cellular functions, including secretion (31).

When CHO cells were incubated with Cx and 4 mM DTT, LPL activity declined drastically. Thus, after 1 h, 10% of the original activity was recovered in the cells, and 4% was recovered in the medium; after 2 h, only 3% activity remained in the cells, and no activity could be detected in the medium. Concurrent with the activity, the LPL dimer disappeared, but levels of the
aggregate increased (Fig. 8A, left panel). The simultaneous increase in the amount of aggregate with decreased dimer levels suggested that the aggregate was generated from the dimer, implying that the origins of the aggregate after 2 h in DTT were different than in untreated cells (i.e. at time 0). To ascertain that the source of the DTT-generated aggregate was indeed the dimer, cells were preincubated with Cx and BFA for 3 h, conditions that allow the original aggregate to degrade while retaining the LPL dimer in the ER (see Fig. 6B). At this point (t = 0), DTT was added to the culture, and the incubation continued for an additional 2 h. As shown in Fig. 8A (right panel), in the absence of de novo protein synthesis, DTT induced the gradual disappearance of the dimer with the simultaneous appearance of an LPL aggregate. This finding confirmed that the aggregate formed under perturbed redox conditions was generated from the dimer, in striking contrast to the de novo origins of the aggregate in untreated cells.

The aggregating effect of DTT on native LPL was reversed when DTT was removed from the culture. Thus, when cells incubated for 2 h with Cx and DTT were washed and the medium was replaced with Cx alone (t = 0), there was a gradual decrease in the levels of aggregate, with a concomitant appearance of active dimer that was rapidly secreted (Fig. 8B, histogram). This finding indicated that DTT treatment did not cause irreversible misfolding of the dimer to create the aggregate; rather, DTT arrested it in a condition that permitted proper reassembly upon withdrawal of the reducer.

Since the LPL dimer was present as both ER and Golgi forms, the question arose as to the source of the dimer in the DTT-induced aggregate in the cells. Susceptibility to endo H clearly demonstrated that the aggregate was ultimately created from both forms. Instead, the aggregate was generated solely from the dimer residing in the ER (left panel, Cells). A dimer that secreted unhindered even in the presence of DTT (Medium). Thus, creation of the DTT-generated aggregate most likely explains the origin of the aggregate upon removal of the reducer. This property distinguishes it from the aggregate originating from untreated cells, which appears to be trapped in a conformation destined for degradation.

Association with Chaperones — The nature of the DTT-generated LPL aggregate is that it was associated in the ER with resident folding chaperones. Since DTT treatment induced a prolonged association of a number of misfolded proteins with calnexin and BiP (32–34), we tested the association of the LPL aggregate with these chaperones. We also included in the analysis ERp57 and calreticulin, because ERp57 is directly involved in disulfide bond oxidation and isomerization and works in concert with both calnexin and calreticulin (35). Last, PDI was included, since it may promote “anti-chaperone” activity under reducing conditions by forming large, insoluble aggregates from misfolded proteins (36).

The aggregate fraction from untreated and DTT-treated cells was immunoprecipitated with antibodies to the various chaperones, and the presence of LPL in these precipitates was determined by immunoblotting (Fig. 9). Included on the blots was an aliquot of the aggregate fraction before immunoprecipitation (Fig. 9, control lane) to confirm that equivalent amounts of aggregate were sampled from DTT-treated versus untreated cells. As shown in the upper panel, only a minimal association of the LPL aggregate was detected among the chaperones tested. In stark contrast, a substantial amount of LPL aggregate from DTT-treated cells was associated with calnexin and BiP, and, to a lesser extent, with PDI. Clearly, the nature of the two aggregates is different, as evidenced both by their different origins and by dissimilar associations with ER chaperones. The association of chaperones with the DTT-induced aggregate most likely explains the origin of the aggregate upon removal of the reductant. This property distinguishes it from the aggregate originating from untreated cells, which appears to be trapped in a conformation destined for degradation.

Fig. 9. Differential association of the original LPL aggregate and DTT-generated aggregate with ER chaperones. Similar amounts of LPL aggregate from untreated cells and from cells treated with DTT for 2 h were immunoprecipitated with antibodies against an array of ER chaperones. Normal rabbit IgG (rab. IgG) was used as a negative control. Co-immunoprecipitated LPL was detected by Western blot analysis using an epitope-specific antibody (see “Experimental Procedures”). Proportional aliquots of samples used for these experiments were included on the blot (“control”) to ensure that similar amounts of aggregate from untreated and DTT-treated cells were compared.
that LPL acquired full catalytic activity in the ER. The approach of drug- or stress-induced conditions was avoided, and we attempted instead to isolate an active form from the ER of normal, untreated cells. This was accomplished by a combination of heparin-Sepharose and β-ricin chromatography (Fig. 2). LPL bearing both ER- and Golgi-type glycans was enzymatically active, and, most important, both forms had similar specific activity (milliunits/LPL mass). Thus, we were able to demonstrate for the first time in a direct manner that LPL becomes fully functional in the ER.

While we found that LPL maturation occurs in the ER, we also found that this compartment contained a substantial amount of inactive LPL with low affinity to heparin (Fig. 2). We questioned whether this form could be the proposed monomeric precursor to active LPL (41, 46), since it has become common belief that LPL with low affinity to heparin is monomeric (19–23). However, we found that low affinity LPL, rather than being a monomer, migrated on sucrose gradients as a high molecular weight aggregate. We further showed that the LPL aggregate was confined to the ER, that it was neither an artifact of centrifugation nor overexpression in CHO cells, and that it was also present in 3T3-L1 cells that endogenously express LPL (Fig. 3).

Aggregation is not an unusual feature of proteins in the ER. Rather, there is increasing evidence that aggregation is a common by-product of protein folding in the ER, resulting from poor solubility of folding intermediates (28, 47). But a tendency of newly synthesized proteins to aggregate has also been observed during the normal folding pathway, where aggregation proceeds to dissociation and protein translocation into a native configuration (26, 48–50). Because of these precedents and because it has been histologically demonstrated that LPL maturation occurs through ER, we entertained the possibility that LPL aggregation might be a precursor of the maturation events in the present study. However, no kinetic evidence was obtained that the formation of dimeric enzyme was detected upon disappearance of LPL (Fig. 4). Rather, active LPL was secreted from the cell in association with the aggregate (Fig. 5). This observation was corroborated when the experiment was conducted in the presence of BFA, where secretion was abolished. In this case, as the aggregate disappeared, neither the amount of enzymatic activity nor dimeric LPL mass changed significantly over a period of 3 h (Fig. 6).

Third, DTT treatment clearly displayed the nature of an inactive LPL complex possessing the ability to generate active LPL dimer (Fig. 5B), contrasting sharply with the characteristics of the original aggregate that was eventually degraded. Thus, the LPL aggregate is not a precursor form but presumably originates from nascent molecules that have reached a “dead end” conformation, off the normal folding pathway (28). As opposed to the correctly folded and properly assembled active LPL, which was stable regardless of whether it was secreted or retained in the cell, the aggregate was retained in the ER and eventually degraded. The fact that the aggregate did not accumulate, despite its continued production, must be due to quality control mechanisms that recognize and target improperly folded polypeptides to degradation (28, 45, 51). The disposal of aberrant proteins, known as ER-associated protein degradation (ERAD) (52), efficiently removes these forms from the secretory pathway.

The precise ERAD pathway of the LPL aggregate remains to be determined. Unlike a majority of misfolded and/or unassembled proteins, such as H2b, that are degraded via ubiquitin-proteasome proteolysis (53, 54), the disappearance of the LPL aggregate was not affected by the presence of proteasome inhibitors (Fig. 7A). Thus, the LPL aggregate belongs to a class of misfolded proteins that undergo ER-associated degradation by mechanisms distinct from the proteasomal pathway. Other examples of proteins in this class include the human thiperoxidase (55) and misfolded forms of α1-antitrypsin (56, 57).

Lyosomal degradation of the LPL aggregate was also examined, although misfolded aggregates in the ER are usually retained by quality control mechanisms and thus become transport-incompetent (45). Nevertheless, the lysosomal pathway was considered, since a mutant form of human LPL lacking activity (G142E) was reported to be degraded in this manner (58). However, in contrast to this mutant form of LPL, lyosomal degradation of the aggregate was ruled out (Fig. 7B).

The depletion of ATP greatly reduced the rate of degradation of the LPL aggregate (Fig. 7B), implying that the process was energy-dependent. Several possibilities linking energy depletion with inefficient ERAD of LPL may be considered. For instance, some ER chaperones possessing ATPase activity, notably BiP and PDI (59, 60), have been shown to associate with abnormal or excess proteins and target their degradation in a pre-Golgi compartment (61). However, we were unable to detect a robust association of BiP and PDI with the LPL aggregate (Fig. 7A). A second possibility is that enzyme-specific ERAD of the LPL aggregate (Fig. 7B) is also an ATP-dependent process, since this is an ATP-dependent degradation site of the LPL aggregate. Thus, the cytosol contains proteases incompletely characterized as part of the evolutionarily conserved tricorn protease at the core (64, 65) and tripeptidyl peptidase II (66).

While we have not detected in our studies aglycosylated or deglycosylated LPL4 (features indicating retrotranslocation to the cytosolic degradation), such intermediates are often difficult to detect even when examining proteins that are known to undergo proteasomal degradation (30). Presumably, this is because such intermediates are rapidly turned over and are present in the cytoplasm in very small amounts. Thus, it is still a possibility that the LPL aggregate might be targeted to the cytoplasm for degradation.

The presence of interchain disulfide bonds in the LPL aggregate (Fig. 7C) suggested that the LPL aggregate resulted from poorly soluble nascent polypeptides or partially folded intermediates coming into close contact (67). However, the LPL complex induced by DTT treatment was very different from the original aggregate, both in its origin and its fate (Fig. 8). The DTT-induced complex was generated from dimeric LPL and was readily dissociated back into functional dimers when DTT was removed. In contrast, the original aggregate, which was not a precursor of the dimer, was recognized by the ER as aberrant and consequently targeted for degradation. We assume that this degradation occurred even in the presence of DTT, since the latter blocks disulfide bond formation without adverse effects on most cellular functions, including ATP synthesis (68). Thus, the “aggregate” present after 2 h in Cx plus DTT (or in Cx plus DTT and BFA) was presumably all generated from the preexisting dimer.

We investigated the association of LPL in the DTT-generated complex with chaperones, since DTT often evokes interactions with ER folding factors (69). Moreover, it was clear that formation of the complex occurred exclusively in the ER, since LPL...
located in the Golgi was readily secreted even in the presence of DTT (Fig. 8C). We found that this inactive complex was largely associated with BiP and calnexin and, to a lesser degree, with PDI. This was in contrast to the original LPL aggregate, where only weak associations were found (Fig. 9). The robust association in DTT-treated cells most likely resulted from the unfolded protein response that became activated by the accumulation of reduced proteins (including LPL) in response to the altered redox potential in the ER. The unfolded protein response elicits transcriptional up-regulation of many genes encoding folding factors in the ER and the cytoplasm (52, 70–72). The combination BiP/calnexin has been implicated in the folding of a number of proteins, both in the normal folding pathway (73) and in the unfolded protein response, where interactions are often enhanced (32, 33). Interestingly, DTT promotes association of proteins with calnexin regardless of their glycosylated state (32, 33), although normally glucose trimming to form monoglycosylated proteins is essential before an interaction with calnexin can occur. This could explain the DTT-induced calnexin association with LPL, although the latter had already acquired a functional conformation and was presumably no longer glucosylated. Moreover, a DTT-induced complex between LPL and BiP/calnexin presumably occurred even in BFA-treated cells (Fig. 8A), when LPL had already acquired a complex glycan structure (see Fig. 6A).

PDI was another chaperone detected at low levels in the original LPL aggregate but showing increased associations with LPL in the DTT-generated complex (Fig. 9). Like BiP and calnexin, PDI is an important component of the quality control machinery in the ER that assists proteins both in proper folding and degradation (75). However, at low concentrations, PDI may express anti-chaperone activity, promote misfolding and degradation (76). Indeed, interactions of PDI with BiP are specifically incorporated into the unfolded lysozyme, with PDI functioning as a chaperone molecule with multiple molecules of unfolded proteins (77). A similar situation may effect the low levels of PDI and BiP in the original LPL aggregate. In contrast, the presence of an aggregate that is a product of the complex formation of an LPL aggregate with higher levels of PDI (Fig. 9). It has been shown that PDI can bind BiP to unfolded proteins in an inactive form (35); calnexin may even impede PDI-induced refolding of proteins as has been shown for RNase B (78). Thus, it may be that the complex in DTT-treated cells is a mixture of PDI-associated or calnexin/BiP-associated LPL molecules. Future experiments will be required to address this issue.

In conclusion, our findings suggest LPL aggregation as a normal yet dead end pathway that can occur when the nascent enzyme attempts to undergo folding in the ER. Perhaps aggregation provides a simple, efficient means to segregate misfolded LPL from the folded, transport-competent dimer solely on the basis of different solubility. Whether this process plays a regulatory function in the availability of LPL for lipid metabolism remains to be determined.

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