Calreticulin Promotes Folding/Dimerization of Human Lipoprotein Lipase Expressed in Insect Cells (SF21)*

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Lipoprotein lipase (LPL) is a non-covalent, homodimeric, N-glycosylated enzyme important for metabolism of blood lipids. LPL is regulated by yet unknown post-translational events affecting the levels of active dimers. On co-expression of LPL with human molecular chaperones, we found that calreticulin had the most pronounced effects on LPL activity, but calnexin was also effective. Calreticulin caused a 9-fold increase in active LPL, amounting to about 50% of the expressed LPL protein. The total expression of LPL protein was increased less than 20%, and the secretion rates for active and inactive LPL were not significantly changed by the chaperone. Thus, the main effect was an increased specific activity of LPL both in cells and media. Chromatography on heparin-Sepharose and sucrose density gradient centrifugation demonstrated that most of the inactive LPL was monomeric and that calreticulin promoted formation of active dimers. Higher oligomers of inactive LPL were present in cell extracts, but only monomers and dimers were secreted to the medium. Interaction between LPL and calreticulin was demonstrated, and the effect of the chaperone was prevented by castanospermine, an inhibitor of N-glycan glucose trimming. Our data indicate an important role of endoplasmic reticulum-based chaperones for the folding/dimerization of LPL.

Lipoprotein lipase (LPL) catalyzes triglycerides in the circulating plasma lipoproteins, such as chylomicrons and very low density lipoproteins (1–3). The enzyme is produced and secreted mainly by adipocytes and muscle cells, but it is also produced in other cells such as macrophages and certain neurons. LPL is transported by yet unclear mechanisms to the vascular endothelium, where it is attached to cell surfaces through interaction with heparan sulfate proteoglycans (HSPG). The products of lipolysis are taken up for storage or oxidation in adjacent tissues. In addition, LPL can mediate internalization of intact lipoproteins by bridging them to cell surface receptors like the LDL receptor-like protein or HSPG.

LPL, along with pancreatic lipase, hepatic lipase (HL), and endothelial lipase, belongs to the gene family of mammalian triacylglycerol lipases (2, 4). The three-dimensional structure of LPL has not yet been determined. Based on homology with pancreatic lipase, it has been proposed that LPL has two structurally distinct regions, a larger N-terminal domain containing the active site and a smaller C-terminal domain with other important functions, such as binding to lipids, LDL receptor-like protein, and HSPG (5, 6).

Catalytically active LPL is a non-covalent, homodimeric glycoprotein (7, 8). In the rough endoplasmic reticulum (ER), human LPL is co-translationally glycosylated at amino acid residues Asn43 and Asn359 (9). Studies using site-directed mutagenesis and inhibitors of transport between ER and Golgi have shown that core glycosylation at residue Asn43 is necessary for activation and release of LPL from the ER, but glycosylation at Asn359 is not necessary (9–11). A number of studies have suggested that proper folding, dimerization, and activation of LPL occurs already in the ER (12–18), but no specific factors involved in the acquisition of the active conformation have yet been identified.

Molecular chaperones are a group of proteins that interact transiently with unfolded and misfolded proteins, thus preventing improper protein–protein interactions until the new protein has found its native fold. When the correct three-dimensional structure has been reached, the molecular chaperones are released (19, 20). It has been pointed out that addition of N-linked carbohydrate is often a necessary step to maintain protein solubility in the ER (19). Trimming of glucose residues is required for interaction of the nascent protein with the lectin-like ER chaperones calnexin (CNX) and calreticulin (CRT) (21–23). In cells treated with tunicamycin, an inhibitor of N-linked glycosylation, or with castanospermine (CST), an inhibitor of ER glucosidases, mostly inactive LPL is formed, and the inactive LPL protein is retained within the cells (13, 14, 24). This suggests that N-linked carbohydrate chains play important roles in the initial folding of LPL. Doolittle and co-workers (25) demonstrated that the chaperone CNX is down-regulated in mice with functional LPL and HL deficiency due to the cld/cld mutation and more recently (18) that ER chaperones may be involved in prevention of aggregation of newly synthesized LPL. In accordance, Boedeker et al. (26) found that CNX increased the efficiency of export of active human HL from the ER of transfected Chinese hamster ovary cells and also demonstrated interaction of HL with the chaperone. Thus a role for ER chaperones in LPL folding and activation is implicated but has as yet not been directly studied.
The original purpose for our work was to produce sufficient amounts of pure human LPL for studies of the enzyme with biophysical methods. The baculovirus system had previously been used successfully for production of pancreatic lipase and hormone-sensitive lipase (27, 28). In our hands the insect cells produced and secreted mostly monomeric, inactive LPL. Based on the possible need of LPL for folding assistance, we speculated on the possibility of using the high level of active LPL that insect cells might lack the molecular chaperones or folds that promote folding/dimerization of LPL in mammalian cells. To test this we co-infected the cells with LPL and a selection of folding assistant factors, including CNX, CRT, protein-disulfide isomerase (PDI), immunoglobulin heavy chain binding protein (BiP), and the foldase ERp57. Our results demonstrate that CRT, especially CRT, enhance the specific activity of human LPL, whereas synthesis of LPL protein and its secretion to the culture medium were not much affected. By co-immunoprecipitation we demonstrated direct interaction between LPL and CRT, and this interaction was prevented by treatment of the cells with CST. Based on these findings we conclude that CRT is a potential candidate to assist folding of LPL in tissues.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin was from Leo Pharma AB, Malmö, Sweden. Endo-β-N-acetylglucosaminidase H (Endo H), CST, and the alkaline phosphatase-conjugated rabbit anti-chicken antibody were from Sigma. The baculovirus transfer vector PFAsBacI and cell culture medium SF900 was from Invitrogen. SF21 insect cells were from Invitrogen. Penicillin-streptomycin and phosphate-buffered saline were from Invitrogen. Bovine calf serum was from PAA Laboratories, Australia. Bovine LPL and human LPL (hLPL), used in enzyme-linked immunosorbent assay and Western blotting were purified from bovine milk or human plasma in our laboratory (29, 30). Heparin-Sepharose was prepared as described previously (37). Protease inhibitor tablets (Complete Mini) were from Roche Applied Science, and the polyvinylidene difluoride membranes were manufactured by Gelman Science. Alkaline phosphatase-conjugated goat anti-rabbit antibody was from Bio-Rad. Recombinant baculoviruses expressing ER<sub>57</sub>, CRT, CNX, PDI, or BiP were prepared as described previously (32–38). Antibodies against each of the chaperones were from StressGen (Victoria, Canada). Lyophilized cell powder of *Staphylococcus aureus* was from Sigma.

**Construction of Recombinant Baculoviruses**—The baculovirus transfer vector PFAsBacI was used to subclone the 1447-bp hLPL cDNA fragment into the HindIII and XhoI sites. This was achieved by using the PCR technique with cDNA-hLPL as a template (kindly provided by Dr Taco Bruin, Amsterdam, The Netherlands). The upstream primer contained 18 nucleotides complementary to the 5′-untranslated region upstream of the ATG start codon, as well as an additional restriction site for XhoI. The downstream primer encoded 18 nucleotides of the C terminus of LPL cDNA containing a stop codon, as well as a restriction site for HindIII. The resulting 1.45-kb fragment was digested with XhoI and HindIII and was subsequently ligated into the plasmid PFAsBacI, and a recombinant baculovirus was constructed exactly as described by the manufacturer. Recombinant viruses were isolated after several rounds of plaque purification and were amplified using standard techniques.

**Expression of Human LPL and the Molecular Chaperones in Insect Cells**—Culture and infection of the insect cells (SF21) were performed exactly as described by the manufacturer. The medium used in all experiments was SF 9001, supplemented with 5% bovine calf serum. To study the effect of the molecular chaperones on the expression of LPL, 1 × 10<sup>6</sup> cells/well in 5-mm wells, 1 ml of medium/well) were infected with baculoviruses at a density of infection of 1. Triplicate wells were used for each infection, and cultures were grown at 27°C. Heparin, 10 IU/ml culture medium, was used throughout the expression to release LPL from the cells and to stabilize the enzyme in the medium. The virus titer was 1 × 10<sup>8</sup> plaque-forming units/ml in all experiments. In some experiments 1.8 × 10<sup>6</sup> cells/flask (150 cm<sup>2</sup>) was used, as indicated in the figure legends. Harvesting of Cells—The culture medium was removed, and the cells were usually harvested at 96 h after infection. The plates were washed twice with cold phosphate-buffered saline, and cells were scraped in 1 ml of lysis buffer, pH 8.2, containing 10 IU heparin/ml, 0.025 M amino-

**Endo H Digestion**—Culture and infection of the insect cells (Sf21) were performed—rounds of plaque purification and were amplified using standard methods.

**Immunoprecipitation and Western Blotting**—LPL in medium or cell extracts (200 μl) were applied to 3.6 μl of 5–20% linear sucrose density gradients made up in 20 mM Tris-Cl, pH 7.4, containing 1 nmol of fatty acid/min. Samples were assayed in triplicate. For measurement of LPL mass, affinity-purified immunoglobulins (IgY) from chicken, raised against bovine LPL, was used for capture of the antigen during an overnight incubation. Bound LPL was detected with the SDz monoclonal antibody raised against bovine LPL (a kind gift from Dr. John Brunzell, Seattle, WA), followed by peroxidase-labeled anti-mouse IgG. LPL purified from bovine milk was used as standard for the immunoassay because pure human LPL was not available. *Heparin-Sepharose Chromatography*—Chromatography on heparin-Sepharose was carried out as described previously (37). Briefly, 20-ml sample was loaded to the column (1 ml of gel) which had been equilibrated with buffer containing 0.1% SDS, 100 mM Tris-HCl, pH 7.4, and 0.1% 3H<sub>2</sub>oleoylglycerol had been incorporated at the manufactory. Heat-inactivated rat serum was used as the source of apolipoprotein CI and bovine serum albumin as fatty acid acceptor, and heparin was used to stabilize the lipase. One milliunit of lipase activity corresponded to the release of 1 nmol of fatty acid/min. Samples were assayed in triplicate. For measurement of LPL mass, affinity-purified immunoglobulins (IgY) from chicken, raised against bovine LPL, was used for capture of the antigen during an overnight incubation. Bound LPL was detected with the SDz monoclonal antibody raised against bovine LPL (a kind gift from Dr. John Brunzell, Seattle, WA), followed by peroxidase-labeled anti-mouse IgG. LPL purified from bovine milk was used as standard for the immunoassay because pure human LPL was not available.

**Sucrose Gradient Centrifugation**—Duplicate samples of culture medium or cell extracts (200 μl) were applied to 3.6 μl of 5–20% linear sucrose density gradients made up in 20 mM Tris-Cl, pH 7.4, containing 1 nmol of fatty acid/min. Samples were assayed in triplicate. For measurement of LPL mass, affinity-purified immunoglobulins (IgY) from chicken, raised against bovine LPL, was used for capture of the antigen during an overnight incubation. Bound LPL was detected with the SDz monoclonal antibody raised against bovine LPL (a kind gift from Dr. John Brunzell, Seattle, WA), followed by peroxidase-labeled anti-mouse IgG. LPL purified from bovine milk was used as standard for the immunoassay because pure human LPL was not available.

**Materials**—Heparin was from Leo Pharma AB, Malmö, Sweden. Endo-β-N-acetylglucosaminidase H (Endo H), CST, and the alkaline phosphatase-conjugated rabbit anti-chicken antibody were from Sigma. The baculovirus transfer vector PFAsBacI and cell culture medium SF900 was from Invitrogen.
Recombinant Human Lipoprotein Lipase and Molecular Chaperones

The effect of heparin on release of human LPL expressed by insect cells

Cells in 6-well cell culture plates (1.0 × 10^6 cells/well) were infected with viruses containing cDNA for human LPL. Triplicate wells were used for each group. Heparin (10 IU/ml medium) was present throughout the whole expression time or was added 1 h prior to collection of medium at the time indicated in the table. Culture media were collected 72 and 96 h after infection, followed by the assay of LPL activity as described under "Experimental Procedures." The data represent the mean values ± S.D. of three independent experiments performed in triplicate.

| Condition          | Time after infection | LPL activity in medium (milliunits/1 × 10^6 cell) |
|--------------------|----------------------|-----------------------------------------------|
|                    |                      |                                               |
| With heparin       | 72                   | 10 ± 3.5                                      |
|                    | 96                   | 14 ± 6.7                                      |
| Without heparin    | 72                   | 0                                             |
|                    | 96                   | 0                                             |
| Heparin for 1 h prior to collection of medium | 72 | 1.9 ± 0.6 |
|                    | 96                   | 2.6 ± 0.7                                     |

Fig. 1. Analyses of recombinant human LPL by Western blotting and Endo H digestion. A, culture media and cells were collected at 48, 72, and 96 h after infection and were immunoprecipitated using Fab fragments of chicken anti-bovine LPL immunoglobulins, as described under "Experimental Procedures." The precipitates were subjected to SDS-PAGE and Western blotting using intact chicken anti-bovine LPL immunoglobulins and alkaline phosphatase-conjugated rabbit anti-chicken IgG for detection. B, the experiment with Endo H digestion. 1.8 × 10^5 cells (in culture flasks) were infected with 1 × 10^6 plaque-forming units/ml of recombinant viruses containing the human LPL cDNA. Medium and cells were collected 96 h after infection. Following immunoprecipitation, the precipitates were subjected to Endo H digestion. SDS-PAGE and Western blotting analysis were carried out as described above. The last lane shows hLPL isolated from plasma.

Statistics—Data are presented as means ± S.D. Statistical significance between groups was calculated by Student’s t test.

RESULTS

Glycosylated Human LPL Was Expressed in the Baculovirus System—Culture media and cells were collected at different times after infection (48, 72, and 96 h) and were analyzed for LPL by immunoprecipitation and Western blotting (Fig. 1A). A protein with similar mobility as purified human LPL was detected at all time points, both in media and in the cell homogenates, but was not present in non-infected cells (data not shown). The levels of the expressed LPL protein increased with time. The highest level was obtained at 96 h, while after 120 h it was decreased (data not shown). The decrease was probably due to the visible cell lysis that started to occur after 96 h of culture.

To investigate whether the recombinant LPL protein was glycosylated, an experiment with Endo H digestion was performed (Fig. 1B). It was shown previously (40) that LPL from human adipose tissue contains two types of subunits, one fully Endo H-sensitive and one partially Endo H-sensitive. The migration of the expressed LPL protein, both from media and cells, corresponded well to that of human LPL purified from plasma (Fig. 1B). After digestion with Endo H, the mobility was faster than for the undigested proteins. This demonstrated that LPL expressed by the insect cells, whether recovered from cells or from media, was glycosylated and Endo H-sensitive. Only one LPL band was seen after digestion, indicating that the expressed LPL contained only glycan chains of the high mannose type (fully Endo H-sensitive).

The Expressed LPL Was Mostly Inactive, but This Was Not Due to Rapid Loss of Activity—Despite the presence of LPL protein both in cells and in media, only low levels of LPL activity were detected even after 72 and 96 h of culture (Table I). This activity was not present in non-infected cells (data not shown), and it was dependent on apolipoprotein CII in the assay, indicating that it corresponded to the recombinant LPL. No detectable LPL activity was present in culture media without heparin, but some activity appeared when the cells were treated with heparin for 1 h before harvesting (Table I). This indicated that some active LPL was associated with the surface of the insect cells in a heparin-releasable manner and/or that heparin was necessary to stabilize LPL after release to the medium.

To investigate whether the low level of LPL activity was due to rapid inactivation in the media during the long culture time (96 h), conditioned heparin-containing media were incubated without cells but under the same conditions as used with cells. The decline of LPL activity was followed by measurements at various time points. From these data, the half-life of LPL activity was estimated to be around 12 h (data not shown). The specific LPL activity was not higher in cells or media at shorter times after infection. LPL activity increased in parallel with the LPL protein up to 96 h (data not shown) indicating a constant relation between production and inactivation rates.

The Expressed Inactive LPL Was Mostly Monomeric—It has been shown that inactive monomeric LPL has lower affinity for heparin than the active dimeric form. Therefore, chromatography on heparin-Sepharose is widely used to separate inactive forms of LPL from active dimers (18, 37). The extended molecular shape of the LPL subunits precludes separation of monomers and dimers by gel permeation chromatography (7). When culture media from infected insect cells were subjected to chromatography on heparin-Sepharose, a small amount of the LPL protein eluted late in the salt gradient (at 1.1 M NaCl), in the position expected for active dimers (Fig. 2). This peak was associated with lipase activity. The specific enzyme activity of the peak fractions amounted to 270–400 milliunits/μg, which is in the range expected for fully active LPL assayed under our conditions. Most of the LPL protein, about 90%, eluted earlier in the salt gradient (at around 0.5 M NaCl) and was catalytically inactive. A similar elution profile was obtained for LPL in cell extracts as for LPL secreted to medium (data not shown). With cell extracts even more (95%) of the LPL protein eluted in the early inactive peak.

To verify that the inactive LPL that was produced by the insect cells was in fact monomeric, we performed sucrose gradient centrifugations and compared the sedimentation of LPL activity and mass to the behavior of purified bovine LPL that was partially dissociated to inactive monomers by treatment with 1 M guanidinium chloride (Fig. 3). On centrifugation of
culture media, the low amount of LPL activity (Fig. 3B) sedimented as the active, dimeric bovine LPL (Fig. 3A). The main part of the LPL protein in the culture medium was inactive and sedimented more slowly (Fig. 3B), corresponding to the peak of inactive bovine LPL monomers (Fig. 3A). No LPL protein was recovered from the bottom of the centrifuge tubes, demonstrating that the inactive LPL in the medium was not aggregated to higher oligomers (Fig. 3B). In contrast, on centrifugation of cell extracts, about 30% of the LPL protein was recovered in the bottom of the tube (Fig. 3C). Most of the remaining LPL protein sedimented as monomers (Fig. 3C), whereas the small peak of LPL activity sedimented as the active bovine dimeric standard (compare Fig. 3, C and A). These results demonstrated that the cells secreted inactive LPL monomers to the culture medium, whereas aggregated LPL was only found in the cell extracts. Aggregates were retained in the cells and were not formed in the heparin-containing media during the extended culture time. The secretion of inactive, monomeric LPL to the medium indicated a lack of recognition of this form by the ER quality control system and furthermore a dysfunction of ER molecular chaperones to assist folding of LPL.

Molecular Chaperones Increase Specific Activity of LPL—To
study if mammalian molecular chaperones could promote formation of active dimers of LPL under the conditions used above, cDNA for CNX, CRT, BiP, PDI, and the foldase ERp57, all incorporated into separate baculovirus vectors under the control of the polyhedrin promoter, were used in co-expression experiments, as described previously (36) for expression of other proteins in insect cells. LPL was initially expressed individually with CNX, CRT, ERp57, BiP, or the control virus, bv-PVL. The control virus did not express any protein from the polyhedrin promoter but was used to maintain a constant and optimal multiplicity of infection per experiment. Western blotting of cell extracts using specific antibodies against the different molecular chaperones confirmed that they were all expressed (Fig. 4, inset). CRT increased the specific activity of LPL by about 7-fold, and CNX increased the specific activity about 3-fold (Fig. 4). A slight increase in the specific activity of LPL was observed with PDI, whereas co-expression with ERP57 or BiP did not significantly change the specific activity of the expressed LPL.

### Table II

| Sample source          | CRT | LPL protein applied (ng) | LPL protein recovered from centrifuge tube (ng) |
|------------------------|-----|-------------------------|-----------------------------------------------|
|                        |     | Monomer                | Dimer                  | Aggregate                           |
| Cell extract           |     | 320                     | 145                    | 9.0                                 |
| Cell extract +         |     | 340                     | 139                    | 68                                  |
| Culture medium         |     | 130                     | 84                     | 6.0                                 |
| Culture medium +       |     | 145                     | 75                     | 38                                  |

Calreticulin Facilitated Dimerization of LPL but Did Not Influence Secretion or Degradation—Because CRT had the most pronounced effect on LPL activity, we continued studies only with this chaperone. Measurements of LPL mass at 96 h after co-expression with CRT showed that the amount of LPL protein was similar compared with cells infected with only LPL (Fig. 5A). In the same experiment the specific activity of LPL had increased 7-fold in the medium and 8-fold in the cells on co-expression with CRT (Fig. 5B).
Calreticulin is directly associated with LPL. Cells were co-infected by viruses expressing LPL with either control virus (PVL) or viruses expressing calreticulin (1.8 × 10^6 cells/flask). The cells were harvested at 96 h. Samples were immunoprecipitated, and the precipitates were subjected to SDS-PAGE and Western blotting as described in Fig. 1 with an affinity-purified chicken anti-bovine LPL antibody (A) or with rabbit anti-human calreticulin antibody (B). MW is molecular weight standard; hLPL is human LPL purified from plasma; r-hLPL is recombinant human LPL from insect cells; r-hLPL + CRT is recombinant human LPL from insect cells co-expressed with virus containing cDNA for calreticulin, and CRT control is extract from cells infected only with cDNA for CRT directly run on the gel without immunoprecipitation.

To illustrate the effects of CRT on the formation of active LPL, the ratio of inactive to active LPL in media was investigated by heparin-Sepharose chromatography. When CRT was co-expressed with LPL, the peak of LPL activity increased compared with medium from cells without CRT (Fig. 5C). The profile of LPL protein mass (Fig. 5D) changed from having almost all LPL protein in the first inactive peak in the absence of CRT to almost equal amounts of LPL protein in the inactive peak and in the second active peak. In the corresponding cell extracts the profiles of LPL activity and mass in the presence and absence of CRT were similar as shown for the media (data not shown). These results suggested that co-expression with CRT increased the amount of active dimeric LPL at the expense of the levels of inactive monomeric LPL. To take into account effects of CRT also on the formation of aggregated LPL inside the cells, one experiment was analyzed by sucrose density gradient centrifugation (Table II). Although the recovery of LPL protein was only about 70% after the 18 h of centrifugation (Table II). The results indicated that co-expression with CRT reduced the amounts of aggregated LPL in the cells by about 50% (from 66 to 34 ng). This reduction was not sufficient to explain the increase in the dimeric active form of LPL (from 9 to 68 ng in the cells and from 6 to 38 ng in the media). Thus, we conclude that the presence of CRT in the cells, in addition to preventing aggregation, promoted conversion of more of the monomeric form to the active dimeric form. This was reflected in a higher proportion of active dimers secreted to the medium but did not much affect the total recovery of LPL protein in the system.

**Calreticulin Interacts with LPL—**To investigate the possible direct association of CRT with LPL, immunoprecipitation of cell extracts by anti-LPL antibody was carried out, and the precipitates were then analyzed by SDS-PAGE and Western blotting with antibodies against LPL and CRT, respectively. The results showed that CRT was co-immunoprecipitated by immunoglobulins to LPL when LPL and CRT were co-expressed (Fig. 6). Hence, an interaction between LPL and CRT, later broken by the conditions for the SDS-PAGE, was clearly demonstrated.

Another strategy to investigate the possible association of CRT with LPL was to use CST, the inhibitor of glucosidases I or II. The prediction was that if glucose trimming of the N-glycan chains by glucosidases I or II was inhibited by CST, binding of the N-glycans of LPL to CRT should not be possible. Both with and without co-expression with CRT, CST decreased the specific activity of LPL (by 70 and 35%, respectively, Fig. 7). Western blot analyses demonstrated that the amount of expressed CRT was unaffected by CST (data not shown). Thus, these data indicate that the interaction between CRT and LPL depended on the presence of mono-glucosylated N-glycans. Furthermore, the data indicate that the insect cells themselves might produce molecular chaperones, the function of which was also affected by the presence of CST.

**DISCUSSION**

LPL is an important enzyme in blood lipid metabolism (1–3). It directs the flow of lipids into tissues according to the nutritional status of the body. It is known that LPL activity is regulated in a tissue-specific manner. In most studies it has been found that the short time regulation of LPL, at least in adipose tissue, occurs by post-translational mechanisms involving the monomer-dimer conversion (37, 41–43). Preliminary studies using the baculovirus system for expression of human LPL had demonstrated to us that most of the recombinant protein was inactive. In the present study we used insect cells as a model system to investigate the role of mammalian molecular chaperones for formation of active, dimeric LPL. This was done by co-expression of LPL with the different chaperones, using a similar strategy as described previously (36) for the expression of the serotonin transporter protein. We found that the recovery of active, dimeric LPL was enhanced, especially by co-expression with CRT, indicating that folding/dimer-
ization was the limiting step for production of active, recombinant LPL by the insect cells. This may be the case also in systems with expression of LPL in mammalian cells, where inactive monomers of LPL are invariably seen together with active dimers (6, 18, 44–46).

The advantages with the baculovirus system are that insect cells can be grown in large scale in suspension cultures and without serum (47). We found, however, that the expression level of LPL was low compared with other lipases that have been expressed by this method (27, 48). At the most 2–3 μg of LPL/10⁶ cells was obtained, and more than 80% of the expressed LPL was inactive. There were at least two possible explanations for these results. One was that the low synthesis was only apparent and due to rapid degradation of misfolded/aggregated LPL protein (18). Another was that the activation and/or the secretion processes were impaired. We found that the stability of LPL activity in the culture medium containing heparin was acceptable, as evidenced by incubation of conditioned media. The time-half for LPL activity was about 12 h, which did not indicate rapid unfolding/degradation, and no LPL aggregates were found in the media by sucrose density centrifugation. The LPL protein seemed to be properly coreglycosylated, which in other systems has been shown to be a prerequisite for activity (12–18). Furthermore, secretion of the LPL protein was probably not limiting, because the ratio between active and inactive LPL was similar inside and outside the cells.

Co-expression with human CRT had remarkable effects on the activity of LPL, which increased 8–10-fold. Because the total amount of LPL protein increased by only around 20% in the presence of CRT, the main effect of the chaperone was to increase the specific activity of LPL. There was no marked change in the distribution of LPL-protein between cells and media in the presence of CRT. Together these data demonstrated that CRT did not primarily change the rate for degradation of LPL within the cells or the rate for secretion of LPL protein to the medium. Although some aggregates of LPL were found in the cell extracts, monomer was the predominant form of inactive LPL in the cells. In medium we did not find any aggregates at all. It has been proposed that LPL has to be folded into its native, dimeric conformation to be accepted by quality control systems for further transport to Golgi (18). In our system the inactive LPL protein was apparently folded into a rather stable, monomeric form that was fit for secretion to the heparin-containing medium at a rate that was comparable with that of the active dimers of LPL. In the medium the inactive LPL protein existed as separate monomers for many hours at 27 °C. Even after storage of the media at ~80 °C, LPL could be isolated as a symmetrical peak by chromatography on heparin-Sepharose or by sucrose density gradient centrifugation, demonstrating that the monomer was stable and not prone to aggregate. Because the subunit of LPL contains many intra-chain disulfide bonds, primarily misfolded LPL could form improper disulfide bonds within itself or with other proteins, as suggested by Ben Zeev et al. (18). In our hands the correct folding of LPL to the active conformation was not markedly assisted by chaperones like PDI or ERp57, which were expected to be supportive if formation of improper disulfide bonds was the major problem. Thus, we conclude that the main effects of CRT and CNX was to promote correct folding of the LPL subunit during its maturation and/or that the chaperones might directly facilitate dimerization.

We were able to demonstrate, by pull-down assays, that CRT interacted with LPL. This supports the idea that CRT might directly assist LPL folding in vivo. CRT is dependent on monoglucosylated N-glycan chains for interaction with proteins. We used CST, an inhibitor of glucosidase I, to prevent the generation of monoglucosylated LPL. Previous studies (13, 24) have shown that this inhibitor decreases LPL activity. Addition of CST to the insect cells resulted in a marked decrease in the recovery of active LPL, from a specific activity in the medium of 177 ± 5.1 milliunits/μg LPL to 53 ± 4.3 milliunits/μg LPL. Also in the absence of co-expression with CRT the inhibitor decreased the specific activity of LPL by 35%, indicating that the cells might contain CRT-like chaperones, but probably in insufficient amounts for assisting activation of the recombinant LPL.

Kinetic data for inactivation of LPL were used previously to demonstrate that the dimeric form of native LPL is in rapid equilibrium with active monomers, which in turn are prone to irreversible moderate changes in secondary structure leading to loss of activity (8). The dimeric form probably stabilizes the active conformation of the subunits. Isolated monomers have in most studies been found to be completely inactive, prone to aggregate, and to have considerably lower affinity for heparin than the active dimeric form (49). Thus, proper folding of the monomer is most likely the major bottleneck for synthesis of the active form of LPL, and stabilization of the dimer is crucial to preserve activity. Under physiological conditions (37 °C), the stability of LPL is much increased by heparin or heparin-like molecules (49). It has been proposed that interaction with heparin might keep the subunits together in the active dimeric form (5, 50). The propensity of LPL to dissociate, with formation of inactive monomers, was proposed to be a built-in property to restrict the lifetime of the active enzyme when bound to endothelial sites outside the reach of other regulatory mechanisms (8). Nutritional and/or hormonal regulation of ER chaperone expression has been reported (51, 52), supporting the idea that regulation of chaperone activity under certain circumstances could influence LPL activation. Interestingly, evidence for decreased levels of CNX was found in the LPL- and HL-deficient cld/cld mouse strain (25). Furthermore, LPL was found to be misfolded and retained in ER in cells from the cld/cld mice (25, 53), as well as in Lee23 cells that lack α-glucosidase I which is necessary for the function of CRT and CNX (25).

In conclusion we have shown that folding of LPL can be assisted by molecular chaperones like CRT and CNX. This is the first time that a role of chaperones for LPL folding is directly demonstrated. Our findings provide clues to how the post-translational regulation of LPL activity may occur in vivo and suggest useful strategies for efficient production of recombinant LPL.

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