Maturation of Hepatic Lipase

FORMATION OF FUNCTIONAL ENZYME IN THE ENDOPLASMIC RETICULUM IS THE RATE-LIMITING STEP IN ITS SECRETION*

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Among three lipases in the lipase gene family, hepatic lipase (HL), lipoprotein lipase, and pancreatic lipase, HL exhibits the lowest intracellular specific activity (i.e. minimal amounts of catalytic activity accompanied by massive amounts of inactive lipase mass in the endoplasmic reticulum (ER)). In addition, HL has a distinctive sedimentation profile, where the inactive mass overlaps the region containing active dimeric HL and tracts into progressively larger molecular forms. Eventually, at least half of the HL inactive mass in the ER reaches an active, dimeric conformation (t1/2 = 2 h) and is rapidly secreted. The remaining inactive mass is degraded. HL maturation occurs in the ER and is strongly dependent on binding to calnexin in the early co-/post-translational stages. Later stages of HL maturation occur without calnexin assistance, although inactive HL at all stages appears to be associated in distinct complexes with other ER proteins. Thus, unlike other lipases in the gene family, HL maturation is the rate-limiting step in its secretion as a functional enzyme.

Hepatic lipase (HL) is a member of the mammalian lipase gene family that includes lipoprotein lipase (LPL), pancreatic lipase (PL), endothelial lipase (EL), phosphatidylserine phospholipase A1 (PS-PLA1), and the most recently discovered lipase H (1–5). These enzymes are localized in specific organs, suggesting that they have evolved to play distinct roles in lipid metabolism. Thus, HL is synthesized in the hepatocytes and is secreted to the space of Disse, where it is anchored to the cell surface by heparan sulfate proteoglycans. At this site, HL mediates the conversion of high density lipoprotein (HDL3) to HDL2 particles and transforms intermediate density lipoproteins to low density lipoproteins. HL also participates in the conversion of very low density lipoprotein remnants to low density lipoproteins and enhances the uptake of remnant lipoproteins to the liver. Besides the liver, HL is also found on the endothelium of the adrenal and the gonads (6) and in macrophages (9), implying that HL may play a direct role in the pathogenesis of atherosclerosis. LPL, on the other hand, is found in heart, skeletal muscle, and adipose tissue, where it hydrolyzes the triglyceride core of chylomicrons and very low density lipoproteins (10). PL hydrolyzes dietary lipids in the intestine (11). EL localizes primarily to the placenta, lung, and macrophages, and PS-PLA1 localizes to platelets, whereas lipase H is found in the intestine (3, 5, 12). The physiological role of the last three lipases is still under investigation.

Despite the disparate anatomical location and distinct physiological functions of the lipases in this gene family, sequence homologies and gene structure strongly suggest a common folding pattern. HL, LPL, and EL are particularly closely related, whereas PL, PS-PLA1, and lipase H appear to have branched out earlier from the primordial progenitor (1–3, 5, 13). In particular, HL, LPL, and EL share homologous heparin-binding domains and also exhibit two conserved N-linked glycosylation sites, located in the N-terminal and in the C-terminal regions, respectively. At least for LPL and HL, it has been shown that the presence of the conserved N-terminal glycan was obligatory for the formation of active lipase (14, 15). Human HL is distinct from HL in other species, since it contains two additional N-linked glycan chains, whereas EL displays three other potential, but nonhomologous, glycosylation sites.

The requirement for glycosylation at the conserved N-terminal site for optimal HL and LPL enzymatic activity implies that these lipases associate with the lectin chaperones calnexin and/or calreticulin. Nascent proteins bind to these chaperones via the innermost glucose residue of the oligosaccharide chain, following removal of the outer two glucose residues by ER glucosidases I and II (16). Indeed, both HL and LPL activity were inhibited by drugs that prevent trimming of the outer glucose molecules (17–20), and direct evidence of binding of HL to calnexin was also demonstrated (21).

Besides association with calnexin, we have recently investigated additional aspects of LPL maturation (i.e. the process of acquisition of enzymatic activity by newly synthesized lipase) (22). We have demonstrated the concurrent formation of two different LPL pools. One pool became fully active; its maturation occurred in the ER and was rapid, to the extent that no inactive precursor to the active form could be detected at steady state or in pulse-chase experiments. The second LPL pool consisted of inactive, misfolded lipase molecules that associated into large aggregates via interchain disulfide bonds. In accordance with strict quality control, these aggregates were degraded in the ER, whereas the active LPL molecules were readily secreted.

The close homology between LPL and HL suggests that the latter might exhibit similar maturation properties, a hypothesis that was investigated in the present study. Our results...
demonstrate that, like LPL, part of nascent HL remains misfolded and is degraded in the ER, whereas the pool of HL that reaches a functional conformation is secreted. However, in stark contrast to the rapid maturation of LPL, HL achieves an apparent array of other ER factors in complexes of varied size.

EXPERIMENTAL PROCEDURES

Cell Lines and Media—The proline auxotroph derivatives (Pro5) of Chinese hamster ovary (CHO) cells, Lec1 cells, and HepG2 cells were obtained from the American Type Culture Collection. Cell cultures were maintained in the manufacturer's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum, antibiotics (50 units/ml penicillin and 50 µg/ml streptomycin), 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate.

Expression Constructs—The cDNA of human HL and PL was inserted into the pcDNA6/V5-His expression vector (Invitrogen) as previously described for the human LPL construct (22). The termination codons (nucleotides 1,498–1,500 of HL and 1,396–1,398 of PL) were replaced with a BamHI site and cloned into the expression vector as a HindIII/BamHI fragment. This placed the 3'end of human HL and PL in frame with the V5 epitope tag, which was subsequently used for antibody detection of the expressed lipase proteins. For HL, the frame cloning replaced the natural termination codon with glycine followed by a 50-amino acid C-terminal adduct: STSPVWWNSADI-

501

The constructs were driven by a cytomegalovirus promoter and contained a bovine growth hormone polyadenylation site.

Transfection, Selection, and Harvesting of Cells—CHO cells were stably transfected with the pcDNA6/V5-His vector containing the cDNA of HL, LPL, and PL, using the calcium phosphate kit and instructions provided by Invitrogen. The selection medium contained 10 µg/ml blasticidin. The transfectants were selected in medium for HL activity (23), after incubating the cells for 16 h in the presence of 10 units/ml heparin.

Cells were subcultured onto 100-mm plates and kept under blasticidin selection. Prior to experiments, cells were incubated for 6–12 h in medium without blasticidin, containing 10 units/ml heparin.

HepG2 cells were transiently transfected with the pcDNA6/V5-His expression vector using the FuGene 6 transfection agent (Roche Applied Science) with the protocol provided by the manufacturer. Six hours prior to harvesting, fresh medium containing 10 units/ml heparin was added. The cells were harvested 24 h after transfection.

For harvesting, cell monolayers were washed twice with phosphate-buffered saline (PBS) and scraped off. 0.4 ml of PBS cell slurry was centrifuged at 1000 × g for 5 min at 4 °C, and the resulting cell pellets were stored at −80 °C. For analysis, pellets from individual plates were added to the cells (2 mM final concentration), and the slurry was incubated at 37 °C for 2 min) in the presence of 0.4 M taurodeoxycholate (Sigma) and the flow-through was collected. After washing the column with 10 bed volumes of column buffer, HL was eluted in two stages: the addition of 0.5 M NaCl, followed by 1.0 M NaCl in column buffer.

Heparin was added to all eluted fractions to a final concentration of 10 µM. For analysis, each of the fractions was collected in the column buffer. For neuraminidase digestion, 50 milliunits of neuraminidase (Calbiochem) were added to the eluted fractions containing lipase samples.

Glycosidase Digestion—For endoglycosidase H (endo H) digestion, samples were denatured by adding SDS to 0.5% and heated at 95 °C for 2 min. After chilling, 10 milliunits of endo H (Roche Applied Science) were added, and the samples were incubated at 37 °C for 16–18 h.

For neuraminidase digestion, 50 milliunits of neuraminidase (Calbiochem) were added to 1 ml of HL eluate obtaining 7% triheparin-Sepharose chromatography. Incubation was carried out for 90 min on ice prior to β-ricin chromatography.

Lipase Assays and Determination of Specific Activity—The activity of HL and LPL was measured using the respective triolein substrates, prepared by sonication (24, 25). PL was assayed with the substrate used for HL, with the following modifications: A mixture containing 0.8 units of neuraminidase (Sigma), 0.8 µmol of taurodeoxycholate (Sigma), and 0.2 µmol of CaCl₂ was prepared in a final volume of 50 µl in water. Enzyme source and 10 mM Tris-HCl, pH 7.5, were then added to complete the volume to 100 µl, and the reaction was initiated by the addition of 100 µl of substrate.

One milliunit of lipase activity is defined as 1 nmol of free fatty acid (FFA) hydrolyzed per min.

Lipase specific activity was calculated by dividing the activity loaded onto electrophoretic gels by the densitometric value of the band obtained by Western blotting. Since densitometry does not provide an absolute concentration, we report the specific activity relative to that of a sample chosen from the blot that was arbitrarily set to a value of 1.0. Cells in 100-mm plates were washed twice with PBS, rinsed with 1 ml of column buffer, and once with deionized water. Bound lipase samples were incubated overnight with 40 µg of anti-calnexin antibody (Invitrogen; 1:2,500). Quantitation of the lipase bands was performed using the NIH Image analysis program for the Macintosh.
intracellular HL activity accompanied by the broad distribution of mass across the gradient suggested that correct HL folding and assembly in these cells was either impaired or considerably prolonged. To determine whether this abnormal or delayed maturation was HL-specific, we compared the intracellular activity of HL with that of two related lipases, PL and LPL. Thus, cell lysates containing PL, LPL, and HL with known enzymatic activity were subjected to Western blotting (Fig. 2A). The progressive decrease in the migration of LPL and HL relative to PL was due to the difference in carbohydrate content, as human PL contains one glycan chain, LPL has two chains and human HL has four chains. When the carbohydrate was removed by endo H digestion, the migration of the three lipases was similar (data not shown). The amount of protein in each lane was measured by scanning densitometry, and specific activities were calculated. Considering the relative specific activity of PL as 100% (see “Experimental Procedures” for details), intracellular LPL was 55% active, and intracellular HL was only 2% active (Fig. 2A).

In contrast to HL, the relatively high specific activity of PL and LPL suggested that the intracellular lipase protein contained in large part mature, fully functional molecules. This distinction was evident in the sedimentation pattern of the three lipases. As shown in Fig. 2B, a perfect correlation existed in the sedimentation profile of PL enzymatic activity and protein, corresponding to that of an expected monomer (11). The molecular size of the peak fraction was ~59 kDa, in close agreement with the predicted PL molecular mass of 53 kDa. LPL intracellular protein, as was previously described (22), was separated into the fully mature dimer form that overlapped the enzymatic activity and an inactive aggregate at the bottom of the gradient. In contrast, the sedimentation pattern of HL was not so clearly defined. Although the majority of HL protein migrated to a region that could loosely be interpreted as a homodimer, it was associated with very little enzymatic activity. Therefore, protein accumulation at this sedimentation range could at best represent a mixture of some homodimeric, active HL, and inactive HL that is either dimeric or monomeric in association with proteins of similar molecular weight. In addition, as seen here and in Fig. 1, HL protein trailed into larger molecular forms. Clearly, these cells contained very little HL with a fully mature conformation.

Although PL, LPL, and HL are closely related lipases and their tertiary structure (especially that of LPL and HL) is presumably very similar (31, 32), we nevertheless considered the possibility that CHO cells specifically lacked the ability to fold HL. Therefore, we examined the expression of HL in HepG2, a human hepatoma cell line that endogenously expresses HL (18). In order to analyze HL mass, HepG2 cells were transiently transfected with the V5-tagged HL construct. The expression levels of the transfected protein were low, and little activity above that of endogenous HL was detected. Nevertheless, sucrose gradient centrifugation revealed even in these cells the familiar widespread distribution of HL protein (Fig. 2B, bottom panel). Similar sedimentation patterns of HL protein were also observed in mouse cell lines derived from fetal liver or from skin explants, under different levels of expression (data not shown). These results clearly indicate that, regardless of cell type, maturation of HL per se is distinctively different from that of other members of the lipase gene family.

**Inactive HL Is a Precursor of the Native Enzyme**—The presence of vast amounts of inactive HL alongside minimal amounts of active, mature HL suggested that the inactive component represents, at least in part, intermediate(s) in the maturation of the nascent polypeptide. To test this hypothesis, protein synthesis was arrested by incubating cells in the pres-
ence of cycloheximide (Cx), and the fate of the preexisting HL activity and mass was followed.

Even after a prolonged incubation in Cx (8 h), little change occurred in the small amounts of intracellular HL activity (Fig. 3A). Surprisingly, for the first 1 h or so, the secretion of active HL from these cells was comparable with that of control cells. The effect of Cx became apparent only during the second hour, as secretion of activity gradually tapered off. After about 4 h, no more protein was secreted, and the amount of HL activity in the medium remained constant for the rest of the incubation. By that time, however, total HL activity in the cells and medium generated in the presence of Cx had increased almost 6-fold over the initial activity and was only 40% lower than the total activity in control cells.

The HL activity generated de novo under conditions of arrested protein synthesis must have originated from preexisting inactive HL. This was confirmed by following the fate of the HL protein in Cx-treated cells and medium (Fig. 3B). The gradual disappearance of HL protein from the cells was detectable even after the first 15 min of incubation in Cx and continued throughout the experiment (upper panel). Concomitantly, HL protein appeared in the medium (lower panel), indicating a precursor-product relationship.

An accurate view of the dynamics of HL secretion and turnover was obtained by plotting, alongside the activity, the amounts of HL mass shown in Fig. 3B. As shown in Fig. 3C, it became apparent that 50% of the inactive mass present in the cells at time 0 was secreted (see closed and open squares), the remainder of the inactive mass undergoing degradation. Further, the secretion rates of HL activity and mass were closely correlated (open squares and circles), indicating that HL mass was released from the cells only after the inactive protein matured into an active, secretable form. Finally, the rates of HL disappearance from the cell and appearance in the medium were similar, with a $t_{1/2}$ of 2 h (117 versus 123 min, respectively). Since the intracellular activity (i.e., mature HL) remained at very low steady state levels (see dark circles), we conclude that HL maturation is a slow, rate-limiting process relative to secretion. When the protein reaches an active conformation, it is rapidly secreted, whereas HL protein that does not acquire a secretable conformation is rapidly degraded within the cell.

Maturation of HL Occurs in the ER—Considering that the
ER is the main compartment for quality control, we hypothesized that the immature HL precursor undergoes folding and assembly in the ER. Thus, at least part of the small HL enzymatic activity detected in cell lysates was derived from HL located in the ER.

To test this hypothesis, it was necessary to isolate the active HL from the massive amounts of inactive protein residing in the cell and to establish its intracellular location. We utilized the fact that native HL has a high affinity to heparin and is eluted at ionic strength concentrations exceeding 0.75M (33). Thus, the first step in the isolation of active HL was heparin-Sepharose chromatography. As shown in Fig. 4A, the vast majority of HL protein (black circles) did not attach to the heparin-Sepharose matrix and remained in the flow-through. Increasing the ionic strength to 0.5M NaCl resulted in further release of HL protein. In the final step, when the ionic strength was increased to 1.0M NaCl, a very small amount of HL protein emerged. However, HL activity (gray triangles) relative to its mass (i.e. enzyme-specific activity) was low in the flow-through, somewhat higher in the 0.5M NaCl wash, and highest in the 1.0M NaCl eluate (0.02, 0.05, and 1.00, respectively). Based on its high affinity to heparin and the high specific activity, the 1.0M NaCl eluate was assumed to represent the mature, functional form of HL.

The intracellular localization of the fractions eluted from the heparin column was determined by endo H sensitivity. Glycans sensitive to endo H digestion contain a high mannose structure, characteristic of glycoproteins residing in the ER. As expected, all fractions contained high mannose glycans chains, indicative of their localization within the ER (Fig. 4A, see panels below peak fractions). However, prolonged exposure of the film revealed that only the 1.0M NaCl eluate contained, in addition to the predominate endo H-sensitive pool, a small amount of protein containing endo H-resistant glycans (see separate strips below respective fractions). Thus, as evidenced by the very low levels of HL glycans exhibiting endo H-resistant forms, only very small amounts of HL protein reside within Golgi or post-Golgi compartments.

To rule out the possibility that the enzymatic activity measured in the 1.0M NaCl eluate was attributable only to this small amount of HL protein in the Golgi, this fraction was separated from HL located in the ER by β-ricin chromatography. As β-ricin toxin specifically binds galactose (the penultimate sugar residue in Golgi-processed glycoproteins), the terminal sialic acid of HL glycans was removed by neuraminidase digestion. Following application of this sample onto a β-ricinagarose column, the flow-through was recovered, and the Golgi-derived HL was eluted with 0.2M galactose. As shown in Fig. 4B, the majority of the protein remained in the flow-through of the β-ricin column and was entirely endo H-sensitive. Again, no protein could be visualized in the 0.2M galactose through of the β-ricin column and was entirely endo H-sensitive. (34). As shown in Fig. 4C, equal amounts of HL activity from the β-ricin flow-through, CHO medium, and Lec1 medium displayed similar amounts of mass, corresponding to comparable specific activities. Thus, HL maturation occurs in the ER, and, since very little HL was detectable in post-ER compartments, we conclude that upon reaching maturity the functional HL is rapidly secreted.

Castanospermine Inhibits Maturation of Newly Synthesized HL—The maturation of HL in the ER, like that of other glycoproteins, depends on the partial glucose trimming of its carbohydrate side chains. Thus, in the presence of castanospermine or N-methyldeoxynojirimycin, drugs that inhibit the activity of glucosidases I and II, HL activity and secretion were shown to be impaired (17, 18, 21). However, considering that inactive HL protein acquired activity over a period of hours (Fig. 3), it was not clear whether the glucosidase inhibitors affected the maturation of HL in the ER.
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Fig. 4. Isolation of active HL in the ER. A, isolation of intracellular active HL by heparin-Sepharose chromatography. Cell lysates combined from six 100-mm plates were applied onto a heparin-Sepharose column. After collecting the flow-through, HL was sequentially eluted with 0.5 and 1.0 M NaCl. Fractions were analyzed for enzymatic activity (gray triangles), and HL mass was calculated by densitometric scanning of bands obtained by Western blotting (black circles). The mass is reported relative to milliunit activity in the peak fraction eluted at 1.0 M NaCl, since that fraction was fully mature (Fig. 4C), and 1 milliunit represents approximately 1 ng of HL protein (see “Experimental Procedures”). Since the intracellular activity is dramatically low relative to HL mass, the inset shows HL activity alone drawn to scale. For assessment of subcellular location, aliquots from the peak fractions were subjected to endo H analysis (see panels below respective fractions and “Results” for explanation).

B, separation of high affinity HL into ER and Golgi forms. Following β-ricin chromatography (see “Experimental Procedures”), the high mannose (ER) HL form was recovered in the flow-through, whereas the complex (Golgi) form was eluted with 0.2 M galactose. Aliquots of the two forms were subjected to endo H digestion and Western blotting. Due to the extremely small amount of the complex form located intracellularly, this form could not be detected in the galactose eluate unless the film was exposed for 1 h. C, specific activity of active HL located in the ER is similar to that of secreted HL. Aliquots of similar enzymatic activity from the β-ricin flow-through, HL secreted from CHO cells, and HL secreted from Lec1 cells were subjected to Western blotting. HL mass was calculated by densitometric scanning, and the specific activity of HL secreted from CHO cells was arbitrarily assigned a value of 1.0. *mu*, milliunits.
than that secreted from Cx-treated cells (compare dark circles and triangles). Thus, it appeared that Cst did not affect the maturation of the preexisting inactive HL pool. After 2 h, however, the inhibitory effect of Cst gradually increased. When the gain in activity derived from maturation of the precursor (i.e. activity secreted by Cx-treated cells; see open squares) was subtracted from the activity secreted by Cst and control cells, the inhibitory effect of Cst was significant, with less than 30% activity remaining. Thus, when glucose trimming of newly synthesized HL was prevented, maturation of this form was significantly compromised.

The inhibition of glucose trimming during Cst incubation was clearly evident when HL protein from these cells was subjected to Western blot analysis (Fig. 5B). The presence of three glucose residues on the four glycan chains of human HL caused a marked shift in the migration of the protein, so that the appearance of the newly synthesized, glucosylated HL form was distinct from the gradual disappearance of the preexisting HL pool. Taken together, these results suggest that there are two separate maturation patterns in the presence of Cst: one of the preexisting precursor, which was unaffected, and that of newly synthesized HL, which was severely impaired.

The unperturbed maturation of the preexisting HL precursor in the presence of Cst was confirmed when Cx-treated cells were incubated for 6 h in the absence or presence of Cst (Fig. 5C). The increase in activity due to HL maturation was similar under both conditions. Thus, glucose trimming must be crucial in the initial co- or post-translational stages but has no apparent effect on later stages of HL maturation.

Robust Interaction of HL with Calnexin—The requirement of newly synthesized HL to undergo glucose trimming for acquisition of enzymatic activity supported the notion that HL associates with the lectin chaperone calnexin (Cnx). However, although LPL is also dependent on glucose trimming for acquisition of an active conformation (35), we were unable to demonstrate a significant LPL-calnexin association unless the cells were under stress (22). It was of interest, therefore, to compare side by side the binding ability of the two lipases to calnexin. To show that association to calnexin was lipase-specific, PL was included in the analysis as a negative control, since this lipase is not dependent on mammalian calnexin for full expression of activity (36). Indeed, as shown in Fig. 6A, when comparable amounts of lipase protein were immunoprecipitated with anti-Cnx antibody, only HL associated in appreciable amounts to the chaperone. The robust binding of HL, but not of LPL, to Cnx is in agreement with the data shown above, suggesting that HL alone is undergoing slow maturation, presenting at steady state nonnative conformations that are associated with the chaperone.

The binding of HL to Cnx was strictly dependent on the trimming of the outer glucose residues from nascent glycan chains. Thus, when cells were incubated for 3 h with castanospermine, none of the predominant HL form, bearing Glc3Man9GlcNAc2 glycans, associated with Cnx (Fig. 6B). However, HL existing in the cell prior to the addition of Cst was found associated with the chaperone, again indicating that this form is undergoing chaperone-assisted folding or degradation.

The heterogeneous sedimentation of nonnative HL into increasingly larger molecular forms (Figs. 1 and 2) raised the question of whether HL associated to Cnx in solitary heterodimers (i.e. a single HL and a single Cnx molecule) or whether the two molecules also coexisted in higher molecular forms. To address this question, HL levels in calnexin co-immunoprecipitates were determined across the gradient fractions and expressed relative to molecular weight (Fig. 6C). The majority of HL in the Cnx immunoprecipitates sedimented at a relatively discrete peak centered at ~110 kDa, closely corresponding to a 1:1 HL-Cnx association (53 + 66 kDa (37), respectively). In addition, HL was associated with Cnx in higher molecular forms, namely in the broad “shoulder” (~150 kDa) descending from the main peak, in the 250-kDa region, and also in the largest aggregate at the bottom of the gradient. Thus, although most HL was bound to Cnx in solitary homodimers, Cnx was also a component of some of the larger HL-containing complexes.

HL Is a Component of Distinct Heterogeneous Complexes in the ER—The HL-Cnx association in complexes of relatively discrete sizes suggested that the broad distribution of immature HL in density gradients (Figs. 1 and 2) represented HL in an array of distinct, increasingly larger complexes. This was confirmed when the cells were treated with DSP, a membrane-permeable, thiol-cleavable cross-linker. As shown in Fig. 7 (upper panel), when lysates of DSP-treated cells were subjected to density centrifugation and analyzed in nonreduced SDS gels,
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DISCUSSION

The homology of HL and LPL to PL, whose crystal structure is known, implies that these lipases share a common folding pattern. The similarity in the folding pattern was assumed to extend also to a common maturation pattern. Specifically, it was suggested that HL and LPL require transport to the Golgi compartment, where subtle modifications transform an inactive lipase into the active form (38). However, we have recently shown that LPL becomes fully functional in the ER (22). In the present study, we show that HL also acquires full enzymatic activity in the ER, and quality control ensures that only this properly folded and assembled fraction is allowed access to the Golgi en route to secretion. However, the spatial similarity between HL and LPL maturation does not extend to a temporal similarity. We show that, unlike LPL, HL maturation is slow, measured in hours, resulting in the presence of large stores of inactive HL protein associated in an array of complexes within the ER.

The significantly lower specific activity of intracellular HL compared with the secreted fraction has been previously recognized (18, 38, 39). However, the relevance of the low specific activity to slow HL maturation was best understood when HL was compared with PL and LPL in terms of intracellular inactive mass and specific activity (Fig. 2).

PL expressed in CHO cells displays very high activity levels and a complete correlation in the sedimentation profile between mass and activity, suggesting that PL becomes fully active very soon after its synthesis. Moreover, PL displays full enzymatic activity even when expressed in lower eukaryotes, such as insect and yeast cells (36, 40). Additionally, PL activity is not affected by the murine cld/cld mutation, which virtually abolishes the activity of LPL and HL (35, 41). Thus, PL must acquire full catalytic activity with little assistance from the chaperones and folding factors employed by HL and LPL. On the other hand, the maturation of LPL is more discriminatory. Even in mammalian cells, whereas part of newly synthesized LPL protein acquires a functional conformation, the remainder is associated into misfolded aggregates that are degraded in the ER (22) (Fig. 2B). In insect cells, the expression of LPL activity, which is very low, increases only if it is co-expressed with calreticulin and, to a lesser degree, calnexin (42). Clearly, maturation of LPL requires the assistance of mammalian chaperones, and even in their presence the degree of successful folding and assembly is not complete. The maturation of HL appears to be even more complex. In all mammalian cell lines tested as well as in primary hepatocytes (38), only negligible amounts of intracellular HL protein were enzymatically active, whereas the majority of the lipase lacked activity and was improperly assembled (Figs. 1 and 2).

Slow Maturation of an Inactive HL Precursor in the ER—

About one-half of inactive HL protein in the ER was slowly converted to an active form, which was recovered in the medium (Fig. 3). We found that HL maturation, and not secretion, was the rate-limiting step in this process ($t_{1/2} = 2$ h). Indeed, depending on the rate of folding, newly synthesized glycoproteins bind to ER chaperones for periods ranging from a few minutes to several hours (43). Most likely, HL belongs to the latter case. On the other hand, most glycoproteins spend about the same length of time in the Golgi, usually 5–15 min (43). These arguments, along with the fact that the ER, and not the Golgi, is the site of rigorous quality control (44), oppose the contention that HL maturation supposedly occurs in the Golgi.

Fig. 6. HL co-immunoprecipitates with calnexin. A, among three lipases tested, HL interacts most robustly with calnexin. Cell lysates containing similar amounts of lipase protein were immunoprecipitated with anti-V5 antibody for detection of the lipase with other proteins, most likely chaperones, such as calnexin.
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FIG. 7. HL is a component of distinct heterogeneous complexes. CHO cells stably transfected with HL were suspended in PBS and incubated for 1 h on ice in the presence or absence of the thiol-labile cross-linking reagent DSP (2 mM). The reaction was quenched with 50 mM glycine and 40 mM NEM, maintaining the cells on ice for an additional 15 min. Cells were lysed by sonication in the presence of 0.2% sodium deoxycholate, and the lysates were subjected to sucrose gradient centrifugation. The fractions obtained were analyzed by PAGE in the absence of reducer, using either 3–8% Tris acetate gradient gels (DSP-treated cells; upper panel) or 7% Tris-glycine gels (control cells; lower panel).

away from numerous chaperones that may block its full activation (38).

Not All Inactive HL Becomes Functional—The precursor-product relationship between the inactive HL in the cell and the active lipase that was secreted did not extend to the entire intracellular HL pool. As shown in Fig. 3C, the fraction that became active and was recovered in the medium was 52% of the total inactive HL pool. The remainder disappeared from the cells at a rate that was similar to the rate of maturation (t1/2 = 2 h). Since all inactive HL contained a high mannose glycan structure (Fig. 4, A and B), it follows that the HL fraction that did not attain a native conformation underwent ER degradation. The liability of the inactive HL fraction contrasted to the stability of active HL, since little decrease was seen in the enzymatic activity of cells and medium over the course of 8 h in Cx (see graph of HL activity in cells and medium, Fig. 3, A or C). Thus, inactive HL followed the fate of other noncompliant proteins that fail to reach a native state and are targeted to ER-associated protein degradation (ERAD; see Ref. 45).

We suggest that all newly synthesized HL molecules have the opportunity to acquire a native, functional conformation, aided and monitored by the chaperones and folding enzymes in the ER. Only those molecules that fail to fold are eventually degraded. It is tempting to speculate that the sorting of active and inactive HL toward secretion or degradation occurs through the action of ER α-mannosidase I, the slowly acting enzyme that removes a mannose residue from the middle branch of the glycan chain to form the ManαGlcNAc2 isomer Man8B (46). This enzyme has been proposed to act as a “timer” of ER residency (47). If a glycoprotein reaches the Man8B form in its native state, it is recognized by the mannose-binding protein ERGIC53 and transported to the Golgi (48). Conversely, the Man8B isomer linked to a nonnative glycoprotein solicits its degradation in the ER (49).

HL-Calnexin Association: Robust and Specific—The detection of a robust amount of HL protein in association with calnexin provided additional evidence of the slow maturation of this lipase. Indeed, the rapid maturation of LPL did not allow detection of a similar LPL-Cnx complex (Fig. 6A), although calnexin has also been resolutely implicated in the maturation of this protein.

Calnexin and calreticulin interact with the glycan moieties of substrate glycoproteins only after they have been trimmed by glucosidases I and II to the monoglucosylated form (Glcα_Manα_GlcNAc2). Indeed, in the presence of the glucosidase inhibitor Cst, we demonstrated that HL maturation was severely impeded. However, interaction with calnexin was essential only in the early HL maturation stages (Fig. 5A). At steady state, cells contain mostly forms that, although immature, have passed the critical stage of calnexin association, and employ “later,” glucose-independent chaperones or folding factors.

It has been postulated that if there is a glycosylation site within the first 50 amino acids of a nascent glycoprotein, interaction with calnexin or calreticulin begins cotranslationally (50). Although the conserved N-linked glycosylation site in the amino-terminal region of human HL is slightly downstream, at position 57, preventing glycosylation at this locus caused a 75% reduction in HL activity. Interestingly, when, in addition to this locus, glycosylation at the earlier, nonconserved N-linked locus at position 20 was also abolished, HL activity was virtually nonexistent (14). These findings suggest that HL indeed belongs to the category of proteins that bind to calnexin at co-translational stages. Likewise, in LPL, the conserved N-linked glycosylation site at position 43 was essential for expression of activity, implying that LPL also belongs to this category. On the other hand, human PL has one glycosylation site at position 167, far beyond the critical initial 50 amino acids, making it almost implicit that calnexin/calreticulin are not utilized for PL maturation.

HL binding to calnexin appears to occur specifically through the monoglucosylated glycan chain, although association of proteins through polypeptide-based interactions has also been reported (51). Thus, when abolition of glucosidase activity by Cst retained newly synthesized HL in the Glcα-Manα_GlcNAc2 form, Cnx bound only to the preexisting HL protein (Fig. 6B). The PAGE migration of this preexisting form, including its subpopulation precipitated by calnexin, was slightly slower than HL from untreated cells (compare lane 2 and lower band in lane 4 to lanes 1 and 3). We attribute the slower migration of this form to a difference in the degree of glycosylation. Specifically, in untreated cells, Cnx binds primarily to the monoglucosylated glycan at asparagine 57. Folding proceeds through release and binding triggered by deglucosylation/reglucosylation carried out by glucosidase II and UDP-glucose:glycoprotein glucosyltransferase (52). Since the other three glycan chains of HL are not essential for proper folding (14), they are poorer substrates for UDP-glucose:glycoprotein glucosyltransferase and presumably remain glucose-free after initial deglucosylation. However, in the presence of Cst, the preexisting unfolded protein lingers in the ER, and as glucosidases I and II are inhibited, all four chains may be reglucosylated by UDP-glucose:glycoprotein glucosyltransferase to the Glcα_Manα_GlcNAc2 form.

The robust calnexin binding to the preexisting HL fraction during incubation with Cst (Fig. 6B, lane 2) is in apparent contradiction to the fact that its maturation proceeds unaffected (Fig. 5, A and C). Specifically, when glucosidase II is
inactive HL release from calnexin through deglucosylation cannot occur, and the protein presumably remains trapped by the chaperone. If so, how could this Cnx-bound fraction become active and then be secreted?

We propose that, in the presence of Cst, the majority of Cnx-trapped HL (Fig. 6B, lane 2) represents the inactive fraction destined for degradation. Recent findings, which establish the active role of Cnx in ERAD, support this hypothesis. Specifically, besides its role in protein folding, Cnx also exists in complex with EDEM (ER degradation-enhancing a-mannosidase-like protein), the recently discovered receptor responsible for directing malformed proteins toward degradation (53). EDEM extracts misfolded glycoproteins from calnexin, an action that requires the activity of glucosidase II. Thus, in the presence of Cst, the glycoprotein substrate remains attached to Cnx, and its transfer to EDEM is impeded. Taken together, we suggest that in untreated cells, HL associated to Cnx represents mostly newly synthesized lipase on its way to maturation, whereas in the presence of HL, Cst bound to Cnx represents predominately the fraction destined for degradation.

**HL Forms Multifunctional Complexes in the ER**—Despite the use of detergent for cell lysis, the sedimentation pattern of HL in density gradients clearly suggested its association into progressively larger molecular forms (Figs. 1 and 2). Thus, under these conditions, HL remained bound to other ER proteins, such as, for instance, calnexin. Although the majority of the calnexin-bound HL in the cells appeared as a complex of a single calnexin and a single HL molecule, some higher molecular weight complexes were also present (Fig. 6C). Based on the unique organization and mode of function of the lectin chaperones, the additional components of these higher complexes might be inferred. Specifically, the peak trailing the HL-calnexin heterodimer might represent a ternary complex with EDEM (ER degradation-enhancing a-mannosidase-like protein), the recently discovered receptor responsible for directing malformed proteins toward degradation (53). EDEM extracts misfolded glycoproteins from calnexin, an action that requires the activity of glucosidase II. Thus, in the presence of Cst, the glycoprotein substrate remains attached to Cnx, and its transfer to EDEM is impeded. Taken together, we suggest that in untreated cells, HL associated to Cnx represents mostly newly synthesized lipase on its way to maturation, whereas in the presence of HL, Cst bound to Cnx represents predominately the fraction destined for degradation.

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