

Role of N-linked carbohydrate processing and calnexin in human hepatic lipase secretion

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Abstract The addition and endoplasmic reticulum (ER) glucosidase processing of N-linked glycans is essential for the secretion of rat hepatic lipase (HL). Human HL is distinct from rat HL by the presence of four as opposed to two N-linked carbohydrate side chains. We examined the role of N-linked glycosylation and calnexin interaction in human HL secretion from Chinese hamster ovary (CHO) cells stably expressing a human HL cDNA. Steady-state and pulse-chase labeling experiments established that human HL was synthesized as an ER-associated precursor containing high mannose N-linked glycans. Secreted HL had a molecular mass of ~65 kDa and contained mature N-linked sugars. Inhibition of N-linked glycosylation with tunicamycin (TM) prevented secretion of HL enzyme activity and protein mass. In contrast, incubation of cells with the ER glucosidase inhibitor, castanospermine (CST), decreased human HL protein secretion by 60%, but allowed 40% of fully active HL to be secreted. HL protein mass and enzyme activity were also recovered from the media of a CHO-derivative cell line genetically deficient in ER glucosidase I activity (Lec23) that was transiently transfected with a human HL cDNA. Co-immunoprecipitation experiments demonstrated that newly synthesized human HL bound to the lectin-like ER chaperone, calnexin, and that this interaction was inhibited by TM and CST. These results suggest that under normal conditions calnexin may increase the efficiency of HL export from the ER. Whereas a significant proportion of human HL can attain activity and become secreted in the absence of glucose trimming and calnexin association, these interrelated processes are nevertheless essential for the expression of full HL activity.—Boedeker, J. C., M. Doolittle, S. Santamarina-Fojo, and A. L. White. **Role of N-linked carbohydrate processing and calnexin in human hepatic lipase secretion.** *J. Lipid Res.* 1999. 40: 1627–1635.

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Human hepatic lipase (HL) is a ~65 kDa glycoprotein that plays an important role in the hydrolysis of triglyceride and phospholipid components of circulating lipoproteins. HL mediates the conversion of intermediate density

lipoproteins (IDL) to low density lipoproteins (LDL), and of high density lipoprotein (HDL)₂ to HDL₃. In addition, HL may directly participate in the hepatic uptake of very low density lipoprotein (VLDL) and chylomicron remnant particles (1). HL is secreted by liver parenchymal cells and is active on the hepatocyte cell surface and on endothelial cells of liver, adrenal gland, and ovaries (2–4). Cell surface binding of HL is mediated by heparin sulfate proteoglycans (5–7). Post-heparin plasma HL activity levels are inversely correlated with plasma HDL concentrations (8–10). Because low plasma HDL concentrations are associated with an increased risk of coronary artery disease (CAD) (11), high HL levels may increase the risk of CAD, although other studies have suggested an anti-atherogenic role for HL (12, 13). The mechanisms that determine the rate of HL secretion from the liver are therefore of considerable interest.

HL is a member of a lipase gene family that also includes lipoprotein lipase (LPL) and pancreatic lipase (PL). HL and LPL are particularly closely related (14). Two N-linked glycosylation sites are conserved in HL and LPL of all species examined, one in the N-terminal region of the molecule (at amino acids 56 and 43 in human HL and human LPL, respectively), and one in the C-terminal region (at amino acids 375 and 359 in human HL and LPL, respectively). Human HL is distinct from LPL and from HL in other species in that it contains two additional N-linked glycans, at positions 20 and 340 of the mature protein (15–17).

N-linked glycans are added to secretory proteins co-translationally in the endoplasmic reticulum (ER) lumen,

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO, Chinese hamster ovary cells; CHX, cycloheximide; CST, castanospermine; endoH, endo-B-N-glucosaminidase H; ER, endoplasmic reticulum; HDL, high density lipoprotein; HL, hepatic lipase; IDL, intermediate density lipoprotein; LPL, lipoprotein lipase; PL, pancreatic lipase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TM, tunicamycin; VLDL, very low density lipoprotein.

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as GlcNac₂Man₉Glc₃ precursors (where GlcNac is N-acetylglucosamine, Man is mannose, and Glc is glucose). While still in the ER, the outer and two inner glucose residues are removed by ER glucosidases I and II, respectively (18). Addition of N-linked carbohydrate is often necessary to maintain protein solubility in the ER (19), and trimming of glucose residues is required for interaction with the lectin-like ER chaperone proteins, calnexin and calreticulin (20, 21).

Studies in primary rat hepatocytes demonstrated that addition and ER processing of N-linked glycans play an important role in HL secretion. Rat HL is synthesized as a 51–55 kDa ER-associated precursor containing endoglycosidase (endo) H-sensitive N-linked glycans. After transport from the ER, Golgi-specific maturation of N-linked glycans results in an increase in the molecular mass of the secreted enzyme to 53–59 kDa (3, 22, 23). Inhibition of N-linked glycosylation with tunicamycin (TM), or inhibition of glucose trimming with ER glucosidase inhibitors, results in intracellular retention of inactive rat HL (22, 24–26). Inhibition of later steps in N-linked glycan processing affects neither HL secretion nor acquisition of enzyme activity (24).

The extent of dependence on N-linked glycan addition and processing for secretion may vary for different members of the lipase family. Most in vitro studies support an essential role for N-linked glycosylation in LPL secretion (27,28). However, although processing of N-linked carbohydrate is clearly required for maximal secretion of LPL, inhibition of ER glucosidase activity allows secretion of 25% of LPL as fully active enzyme (29). Site-directed mutagenesis studies also indicate that the different N-linked glycan chains play somewhat different roles in secretion of human LPL, rat HL, and human HL (30). Deletion of the conserved N-terminal glycosylation site completely prevents LPL secretion, whereas ~25% of mutant rat and human HL are secreted as fully active enzymes. Mutation of the conserved C-terminal glycosylation site does not affect human LPL or rat HL secretion, but reduces secretion of human HL by ~60% (30). Together, these studies suggest that folding of LPL and HL in the ER may depend to different extents on the presence and processing of N-linked glycans and on interaction with calnexin and/or calreticulin. The role of these ER chaperone proteins in HL and LPL secretion has not been previously addressed.

In the current study we analyzed the role of N-linked carbohydrate processing and calnexin interaction in human HL secretion from stably transfected Chinese hamster ovary (CHO) cells. Our results demonstrate that trimming of N-linked carbohydrate side chains and calnexin interaction are essential for the full expression of human HL activity. However, a substantial portion (~40%) of catalytically active HL is secreted in the absence of either process.

MATERIALS AND METHODS

Materials

Tunicamycin, insulin, BSA/linoleate, and heparin were from Sigma. Protease inhibitors (Complete[®]), catanospermine and FuGene transfection reagent were from Roche Molecular Bio-

chemicals. Expre³⁵S³⁵S label was from DuPont NEN. DMEM was from Life Technologies. Protein A Sepharose was from Repligen Corp. Anti-calnexin antibody was from SressGen. All other reagents were of analytical grade.

Cell lines

Pro5, Lec1, and Lec23 cells (31, 32) were kindly provided by P. Stanley. Chinese hamster ovary (CHO) cells stably expressing high levels of human HL were as described (33). All cells were maintained in DMEM containing 10% FBS in a humidified atmosphere of 5% CO₂.

Production of anti-human HL polyclonal antibody

Rabbit antibodies to human HL were produced using an expression plasmid encoding the full-length human HL cDNA (33), kindly provided by H. Wong, essentially as described (34). Briefly, 500 µg of plasmid DNA resuspended in water was injected into the spleen of a New Zealand White rabbit. Subsequently, 500-µg aliquots of plasmid DNA were injected intramuscularly at 2-week intervals. Anti-HL antibody production was detected 4 weeks after the initial inoculation.

Metabolic labeling and immunoprecipitation

For overnight labeling, cells were incubated in DMEM containing one-tenth the normal concentration of methionine and cysteine, supplemented with insulin (5 µg/ml), BSA/linoleate (0.5 mg/ml, 0.79% linoleic acid), heparin (10 U/ml), and 125 µCi/ml Expre³⁵S³⁵S label. For pulse-chase studies, cells were pre-incubated in methionine- and cysteine-free DMEM plus insulin and BSA/linoleate for 1 h, labeled in the same medium for 15 min with 125 µCi/ml Expre³⁵S³⁵S label, and chased in DMEM containing 10% FBS and 10 U/ml heparin. Media were collected, clarified by centrifugation at 2000 *g* for 5 min, and then protease inhibitors were added. Cells were lysed in extraction buffer (EB: 50 mM Tris, pH 9.0, 100 mM NaCl, 1% Triton X-100, 0.3% CHAPS, 1% sodium deoxycholate). To attempt quantitative recovery of HL, samples were immunoprecipitated by two successive overnight incubations at 4°C with rabbit anti-human HL antiserum plus protein A Sepharose (40 µl of a 50% suspension) to which monoclonal anti-human HL antibody 7B4 (kindly provided by L. Curtiss) was pre-bound. Pre-binding was performed for 1 h at room temperature in high salt buffer (0.75 M glycine, 1.5 M NaCl, pH 8.9). Protein A pellets were washed 3× with EB, then eluted at 100°C with SDS-PAGE sample buffer containing 2% SDS and 10% 2-mercaptoethanol. Eluents from successive rounds of precipitation were combined and analyzed by 4–10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography (35).

For co-immunoprecipitation experiments, cells were lysed in ice-cold buffer containing 0.1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4. Anti-calnexin antibody and protein A Sepharose (40 µl of a 50% suspension) were added and the samples were shaken at 4°C for 3 h. Protein A pellets were then washed 3 times for 5 min at 4°C in the same buffer. Co-precipitated proteins were eluted with 50 µl of EB + 10 mM EDTA for 30 min at 37°C; 1 ml of EB was added and the protein A pellets were removed by centrifugation. HL was then immunoprecipitated from the samples as described above.

To quantify results, autoradiographs were scanned using an Arcus II desktop scanner (Agfa). Integrated band intensities were determined using Scanalytics ONE-Dscan software. Analysis of serially diluted samples demonstrated that this method was quantitative over at least a 16-fold range.

Endoglycosidase digests

Digestion of HL immunoprecipitates with endo-B-N-acetylglucosaminidase H (endoH), peptide N-(N-acetylglucosaminyl) as-

paragine amidase (N-glycanase), acyl neuraminyl hydrolase (neuraminidase), and endo- α -N-acetylgalactosaminidase (O-glycanase) was performed exactly as described (35).

Transfection

A construct expressing a full-length human HL cDNA (36) was transiently transfected into Pro5, Lec23, and Lec 1 cells using FuGene[®] transfection reagent according to the manufacturer's instructions. For 35-mm and 60-mm dishes of cells, 2 and 6 μ g of DNA were used, respectively. Experiments were performed 48 h post-transfection.

HL activity assay

HL triacylglycerol hydrolase activity in conditioned media was assayed using as substrate an emulsion of radiolabeled triolein prepared by sonication, as described (37). Samples were assayed in duplicate or triplicate and values obtained were normalized either to total cell protein determined by BCA assay (Pierce) or to the relative mass of HL protein in the media, determined by immunoprecipitation of overnight labeled samples, as described above. Results are presented as mU of activity (nmoles of free fatty acid hydrolyzed per minute) per mg cell protein, or per unit of HL mass. Addition of anti-HL antibodies to the samples prior to enzyme assay inhibited lipase activity by 100%, confirming that all of the activity was attributable to HL (data not shown). Inhibitors used in the experiments described (CHX, TM, CST) did not inhibit HL activity when added to conditioned media (data not shown).

Cytotoxicity assay

Lactate dehydrogenase (LDH) activity in media and cell samples was assayed with a commercial kit (Roche Molecular Biochemicals), according to the manufacturer's instructions.

Statistical analyses

Comparisons were made using the Student's *t*-test.

RESULTS

Synthesis and secretion of human HL in stably transfected CHO cell cultures

To document the synthesis and secretion of human HL from CHO cells stably expressing a human HL cDNA, cultures were labeled overnight with [³⁵S]methionine and [³⁵S]cysteine in the presence of 10 U/ml heparin. HL was then immunoprecipitated from media and cell lysates and analyzed by SDS-PAGE (Fig. 1A).

A protein with heterogeneous mobility on SDS-PAGE was recovered from media samples incubated with the rabbit anti-HL antibody, but not from samples incubated with pre-immune serum (Fig. 1A). This protein had a maximum molecular mass of ~66 kDa, consistent with the expected size for glycosylated human HL (15, 17). Barely detectable amounts of this protein were observed in the cell lysates. However, an additional form of HL with a greater electrophoretic mobility was recovered from inside the cells (Fig. 1A).

To analyze the relationship between the two forms of HL, pulse-chase experiments were performed (Fig. 1B). At 0 and 15-min of chase after a 15 min pulse, only the smaller form of HL could be seen in the cell lysates. At 30 min of chase, trace amounts of the larger form of the protein appeared in the cells and medium. The amount of

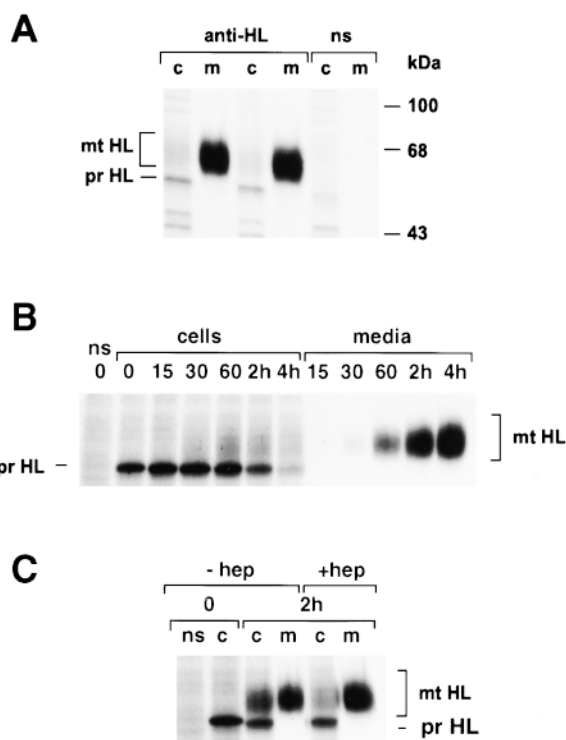


Fig. 1. Synthesis and secretion of human HL from stably transfected CHO cells. A: CHO cells expressing a human HL cDNA were labeled for 16 h with [³⁵S]methionine and [³⁵S]cysteine in the presence of 10 U/ml heparin. Cells (c) and media (m) were immunoprecipitated with rabbit anti-HL polyclonal antibody (duplicate dishes of cells) or with pre-immune serum (ns) and analyzed by 4–10% gradient SDS-PAGE, as described in Materials and Methods. The positions of the precursor (pr) and mature (mt) forms of HL and molecular mass standards are indicated. B: CHO cells were labeled for 15 min and then chased for between 0 min and 4 h in medium containing heparin. HL was analyzed as described in A. C: Cells were labeled for 15 min, then chased for 0 min or 2 h in the presence or absence of heparin. HL in cells and media was analyzed as in A.

HL in the medium continued to increase with increasing chase times, and by 4 h of chase virtually no radiolabeled HL remained inside the cells (Fig. 1B). Thus, the smaller intracellular form of human HL represents a precursor of the larger, secreted protein.

Substantially more (2.8-fold) radiolabeled HL was recovered from the medium at 4 h of chase than was ever observed in the cell lysates (Fig. 1B). Re-immunoprecipitation of samples failed to recover any additional HL from the culture medium. However, as much as 25% more HL was recovered from the cell lysates (data not shown). Using a variety of conditions, we were unable to quantitatively immunoprecipitate HL from the cells. This could be due to masking of HL epitopes in cell lysates or to decreased reactivity of the antibodies against the HL precursor.

In rat hepatocyte cultures, heparin inhibits binding of newly synthesized HL to the hepatocyte surface and increases the secretion of HL protein mass (38). Omission of heparin from the media in pulse-chase experiments resulted in a modest reduction in HL secretion from our

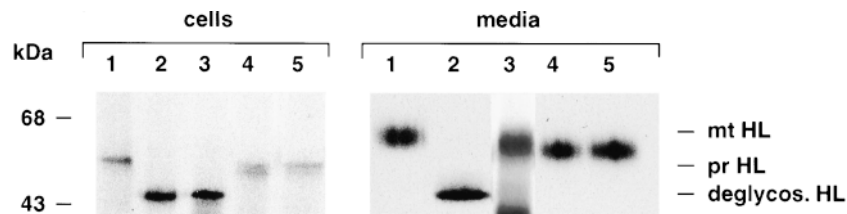


Fig. 2. Endoglycosidase digests of the precursor and mature forms of human HL. HL was immunoprecipitated from stably transfected CHO cells labeled for 30 min (cells) or from medium collected from cells after a 16-h label, as described in Materials and Methods. The HL was then digested with various endoglycosidase enzymes; lanes 1–5, undigested control, N-glycanase, endoH, neuraminidase, and neuraminidase + O-glycanase digested samples, respectively. The positions of molecular mass standards and of the precursor (pr), mature (mt), and deglycosylated (deglycos.) forms of HL are indicated. Some proteolytic degradation of the endoH-digested medium sample was observed (media lane 2). A longer exposure of this sample is therefore presented.

CHO cell cultures over a 2-h chase period, and an increase in the amount of mature HL associated with the cells. Total mature HL (cells + media) remained unchanged (Fig. 1C).

Analysis of HL carbohydrate

To confirm the structural relationship between the precursor and secreted forms of HL, the proteins were digested with various endoglycosidase enzymes (Fig. 2). The intracellular HL precursor was sensitive to digestion with both N-glycanase, which removes all N-linked glycans from proteins, and with endoH, which cleaves high mannose N-linked sugars only (Fig. 2, cell lanes 2 and 3). Resistance to endoH is acquired in the media Golgi apparatus (18). These results suggest that the HL precursor represents a pre-medial Golgi form of the enzyme. Consistent with this, the HL precursor was resistant to digestion with neuraminidase, which removes sialic acid residues, and with O-glycanase, which is specific for O-linked glycans (Fig. 2, cell lanes 4 and 5).

Secreted HL was also sensitive to digestion with N-glycanase (Fig. 2, media lane 2). The digested protein had an electrophoretic mobility indistinguishable from that of the N-glycanase-digested HL precursor, demonstrating that the difference in molecular mass of the two forms of HL was due to the structure of their N-linked glycans. Deglycosylated secreted HL ran as a much tighter band on SDS-PAGE than the undigested protein, suggesting that the heterogeneity in the electrophoretic mobility of secreted HL is due to heterogeneity in the structure of its N-linked glycans. Secreted HL was resistant to endoH digestion (Fig. 2, media lane 3). A small shift in HL molecular mass was observed after treatment with neuraminidase (Fig. 2, media lane 4), consistent with the presence of some sialic acid residues on the mature N-linked glycans. However, no evidence of O-glycosylation of HL was found (Fig. 2, media lane 5).

N-linked glycosylation is essential for HL secretion

Nascent N-linked glycan chains (GlcNac₂Man₉Glc₂; where Nac is N-acetylglucosamine, Man is mannose, and Glc is glucose) are added en bloc to secretory proteins as

they are co-translationally translocated across the ER membrane. Inhibition of glycosylation by treatment of cells with tunicamycin (TM) prevents secretion of rat HL (22, 24–26). To determine the role of N-linked glycans in human HL secretion, pulse-chase experiments were performed in the presence of TM (Fig. 3A).

In TM-treated cells, the molecular mass of the HL precursor was significantly decreased compared to that in control cells, due to the absence of N-linked glycans (Fig. 3A). The amount of labeled HL recovered from TM-treated cells after a 15-min pulse was less than that recovered from control cells. Careful titration of TM to levels where glycosylation was inhibited without significant inhibition of protein synthesis did not increase the amount of radiolabeled HL recovered (data not shown). TM can induce the aggregation of newly synthesized proteins in the ER (19). We suspect that the decreased recovery of HL from our TM-treated cells was due to the inability of the anti-HL antibodies to recognize aggregated HL. TM drastically reduced HL secretion; by 4 h of chase, virtually no radiolabeled HL had been secreted, and the majority remained inside the cell (Fig. 3A).

We also examined the effect of TM on secretion of HL activity (Fig. 3B). TM reduced the amount of HL activity secreted over a 4-h period to $55 \pm 10\%$ of that secreted from control cells (Fig. 3B). This was similar to the amount of activity released from cells incubated with the protein synthesis inhibitor, cycloheximide (CHX) ($56 \pm 8\%$ of control; Fig. 3C). Pulse-chase experiments demonstrated that a prolonged time period is required for newly synthesized HL to be secreted (Fig. 1B). The HL activity secreted from CHX-treated cells thus presumably reflects secretion of HL synthesized before addition of the inhibitor (24). Lipase activity detected in the presence of CHX was not due to release of intracellular HL resulting from CHX cytotoxicity, as $<1\%$ of total cellular LDH activity was released into the medium under all conditions analyzed (data not shown). As the effect of TM was almost identical to that of CHX, TM appeared to completely block secretion of HL activity.

These results suggest that addition of N-linked glycans is essential for the secretion of human HL activity and protein mass.

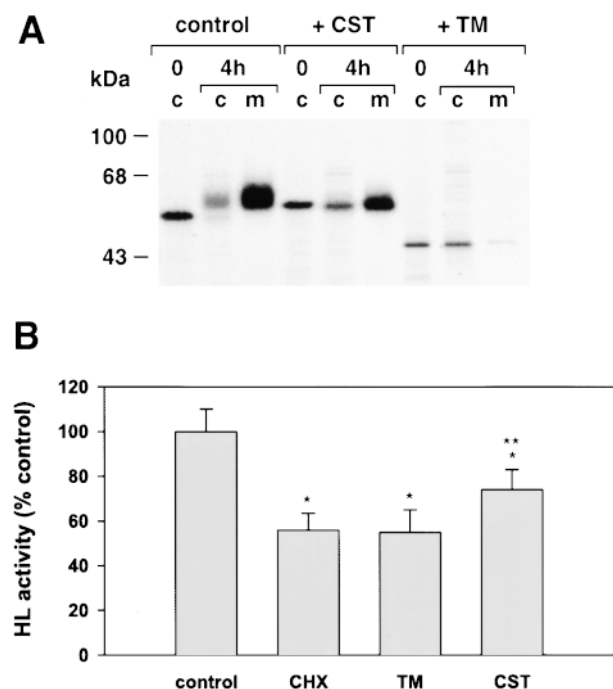


Fig. 3. Influence of castanospermine (CST) and tunicamycin (TM) on human HL secretion. **A:** CHO cells stably expressing human HL were pre-incubated, labeled for 15 min, and then chased for 4 h under control conditions or in the presence of 1 mM CST or 5 μ g/ml TM, as described in Materials and Methods. HL in cells (c) and media (m) was analyzed as in Fig. 1. The positions of molecular mass markers are indicated. **B:** Cells were incubated for 4 h in medium containing no addition (control), 500 μ M cycloheximide (CHX), 5 μ g/ml TM, or 1 mM CST. HL activity in the media was assayed as described in Materials and Methods. Results are expressed as percentage of control values and represent the mean \pm SD; $n = 13$ for each condition (combined results of three independent experiments), except CHX, where $n = 6$. For control cells, 10.19 ± 1.76 (mean \pm SD) mU HL activity was secreted per mg of cell protein. * $P < 1 \times 10^{-5}$ compared to control. ** $P < 1 \times 10^{-7}$ versus control, $P < 0.02$ versus CHX and $P < 1 \times 10^{-4}$ versus TM.

ER glucosidase activity is not essential for human HL secretion

Immediately after addition of nascent N-linked glycans to proteins in the ER, the outer and two inner glucose residues are removed by ER glucosidases I and II, respectively (18). Inhibition of ER glucosidase activity with castanospermine (CST) prevents rat HL secretion (24). To examine the role of ER glucosidase activity in human HL secretion we examined the effect of CST on secretion of HL protein mass and activity from the stably transfected CHO cells (Fig. 3).

In pulse-chase experiments, the HL precursor synthesized in the presence of CST had a slower mobility on SDS-PAGE than that synthesized under control conditions (Fig. 3A), consistent with the retention of glucose residues on the HL N-linked glycans. The amount of precursor recovered from control and CST-treated cells immediately after the pulse was very similar (ratio of control: CST-treated cells = 1.2:1). By 4 h of chase a significant amount of radiolabeled HL was secreted into the culture medium

of CST-treated cells. The majority of this HL did not show an increase in molecular mass on secretion, presumably because maturation of its N-linked glycans was inhibited in the presence of the untrimmed glucose residues (Fig. 3A). However, some maturation may have been permitted through the action of Golgi α -mannosidase that will remove Glc_3Man from N-linked glycans (18). Over four independent experiments, the amount of radiolabeled HL recovered in the media of CST-treated cells at 4 h of chase was $43 \pm 10\%$ (mean \pm SD) of that for control cells ($P < 0.001$). Higher concentrations of CST (up to 5 mM) did not further reduce HL secretion (data not shown). Re-immunoprecipitation of media from CST-treated cells did not recover any additional HL protein (data not shown).

CST significantly decreased secretion of HL activity from CHO cells over a 4-h period, to $74 \pm 9\%$ of control values (Fig. 3B). However, this amount was significantly greater than that secreted from TM- or CHX-treated cultures (Fig. 3B). Subtraction of the amount of HL activity secreted in the presence of CHX from the values obtained for control and CST-treated cells allowed us to estimate that CST reduced secretion of HL activity to 44% of control. These values agree very well with the effect of CST on HL protein secretion (Fig. 3A), and suggest that the specific activity of HL secreted in the presence of CST was very similar to that secreted from untreated cells.

These results suggest that processing of N-linked glycans by ER glucosidases is not required for human HL secretion, although the efficiency of its secretion was significantly reduced in the presence of CST.

Human HL is secreted from cells deficient in ER glucosidase I activity

To further examine the role of glucose trimming in human HL secretion, we transiently expressed HL in cells genetically deficient in ER glucosidase I activity (Lec23 cells) (31). As controls, we transfected HL into the parent cell line, CHO-Pro5, and a cell line deficient in N-acetylglucosaminyltransferase I activity (Lec1 cells) (32).

Immunoprecipitation of labeled cells and media demonstrated that HL was secreted from all three cell lines (Fig. 4). The molecular mass of HL in cell lysates and media from Lec23 cells was similar to that in CST-treated cells (Fig. 3), consistent with the absence of glucose trimming in the ER. In Lec1 cells, the HL precursor had identical electrophoretic mobility as that in control cells. However, a decrease in HL molecular weight was observed on secretion from Lec1 cells (Fig. 4). This presumably reflects the normal trimming of mannose residues from N-linked glycans in the Golgi apparatus. Unlike control cells, however, subsequent addition of complex glycans is prevented in Lec1 cells.

Transient transfection made it difficult to compare the efficiency of HL secretion from the different cell types. However, over three experiments, the amount of HL recovered from the media of Lec23 cells was $53 \pm 25\%$ (mean \pm SD) of that for control cells. For Lec1 cells the value was $58 \pm 31\%$.

We also examined secretion of HL activity from Pro5,

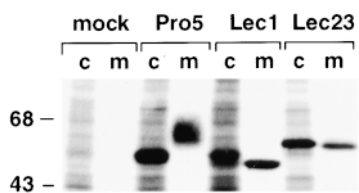


Fig. 4. Secretion of human HL from glycosylation defective cell lines. CHO-Pro5, Lec1, and Lec23 cells transiently expressing human HL, and mock-transfected Pro5 cells (mock) were labeled for 16 h as described in Materials and Methods. HL was immunoprecipitated from the cells (c) and media (m) and analyzed as described in the legend to Fig. 1. The positions of molecular mass standards are indicated.

Lec1, and Lec23 cells. No HL activity was observed in the media of untransfected cells (Table 1). In contrast, HL activity was clearly secreted into the media after transfection of all three cell types. The amount of activity secreted from Lec1 cells was significantly higher than that secreted from control cells, whereas secretion of HL activity from Lec23 cells was significantly lower than from the other cell types. However, when normalized to HL protein mass assessed by immunoprecipitation of radiolabeled HL, the specific activity of HL secreted from Lec 1 and Lec23 cells was similar (185 and 122%, respectively) to that secreted from Pro5 cells (Table 1).

These results unequivocally demonstrate that ER glucosidase activity and Golgi-specific maturation of N-linked glycans are not required for secretion of active human HL.

HL interacts with calnexin

The ER chaperone calnexin interacts with the majority of its substrate proteins through binding to monoglucosylated N-linked glycans. After removal of the three glucose residues from nascent N-linked glycans in the ER, unfolded proteins are recognized by the ER resident enzyme UDP-glucose:glycoprotein glucosyl transferase (GT) and a

single glucose residue is re-attached (39). The ER chaperones calnexin and calreticulin bind to such monoglucosylated proteins and help them to fold (20, 21). The re-attached glucose residue is then removed by glucosidase II. Depending on the folded state of the protein, it will then either continue down the secretory pathway or will enter a reglucosylation/deglucosylation cycle involving cyclic interactions with calnexin and calreticulin, until it is fully folded and can be secreted (20). Inhibition of glucose trimming with CST, or inhibition of N-linked glycosylation with TM, inhibits the interaction of calnexin and calreticulin with their substrate proteins. As the efficiency of HL secretion was reduced in the absence of ER glucosidase activity, this suggested that calnexin and/or calreticulin may normally play a role in HL intracellular processing.

We investigated a potential role for calnexin in HL secretion by a co-immunoprecipitation in pulse-chase experiments (Fig. 5A). When pre-immune serum was substituted for anti-calnexin antibodies (Fig. 5A), no HL was recovered in the co-immunoprecipitates. However, a small portion of HL did co-precipitate with calnexin at all time points analyzed, up to 120 min of chase (Fig. 5A; note that a longer autoradiographic exposure is presented for co-precipitated HL than for HL in the supernatants). Thus, HL appeared to interact with calnexin throughout its time in the ER.

The interaction of HL with calnexin was dependent on the presence of monoglucosylated side chains, as the interaction of HL with calnexin was abolished in cells treated with CST or TM (Fig. 5B). Because trimming of glucose residues from nascent N-linked glycans occurs very rapidly, CST added only to the chase medium can be used to trap radiolabeled proteins in their monoglucosylated form (40). Under these conditions, a 2.2-fold increase in association of HL with calnexin was observed compared to control cells (Fig. 5B). However, the portion of HL detected in association with calnexin under these conditions was still very small.

These results suggest a role for calnexin in the processing of newly synthesized HL in the ER. Although a portion of HL can be secreted in the absence of calnexin association, glucose trimming and calnexin interaction appear to be required for the full expression of HL activity.

TABLE 1. Active HL is secreted from Pro5, Lec1, and Lec23 cells

Cell Type	HL Activity ^a	Activity/HL
	(mean ± SD)	Mass ^b
	<i>mU/mg</i>	<i>% of control</i>
Pro5		
Mock	0	
Transfected	22.90 ± 0.45	100
Lec1		
Mock	0	
Transfected	42.93 ± 2.18	185
Lec23		
Mock	0	
Transfected	17.88 ± 0.73	122

HL activity secreted from mock-transfected cells or cells transfected with a human HL cDNA was assayed as described in Materials and Methods. Results are presented from a typical experiment performed in triplicate.

^a FFA hydrolyzed, nmol per mg total cell protein per 16 h.

^b Activity normalized to HL mass as determined by immunoprecipitation of radiolabeled protein (see Materials and Methods).

DISCUSSION

Most previous studies of HL synthesis and secretion have analyzed rat HL. Human HL differs from the rat enzyme in the presence of four, compared to two, N-linked glycans (14). In the current study we examined the role of N-linked glycosylation in human HL secretion using Chinese hamster ovary cells stably expressing a human HL cDNA. Similar to rat HL, we found that co-translational addition of N-linked glycans was essential for human HL secretion. However, although required for full expression of human HL activity, in contrast to rat HL, trimming of glucose residues from N-linked glycans was not essential for secretion of human HL mass or activity.

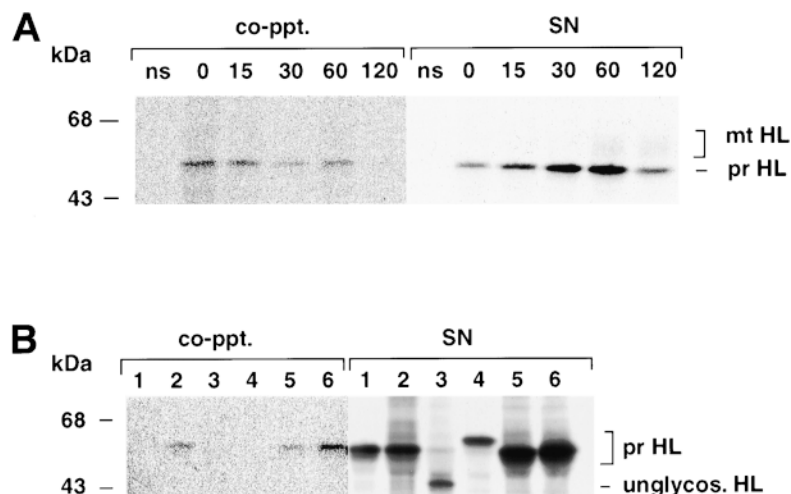


Fig. 5. Human HL interacts with calnexin. A: CHO cells stably expressing human HL were labeled for 10 min and chased for up to 120 min, as indicated. Cell lysates were immunoprecipitated with anti-calnexin antibody or with pre-immune rabbit antiserum (ns). Co-precipitated HL (co-ppt.) and HL that remained in the supernatants (SN) was then analyzed by immunoprecipitation and SDS-PAGE. For co-ppt. samples, ns = pre-immune serum co-precipitate immunoprecipitated with anti-HL. For SN samples, ns = pre-immune serum SN immunoprecipitated with pre-immune serum. The positions of the precursor (pr) and mature (mt) forms of HL are indicated. B: Cells were pre-incubated for 1 h and labeled for 5 min as follows: lanes 1 and 2, control conditions; lanes 3, + 5 $\mu\text{g/ml}$ TM; lanes 4, + 1 mM CST; lanes 5 and 6, control conditions, then chased for 20 min + 5 $\mu\text{g/ml}$ TM (lanes 5) or 1 mM CST (lanes 6). Samples were analyzed as in A. In lanes 1, pre-immune serum was substituted for anti-calnexin antibody. The positions of the HL precursor (pr) and unglycosylated HL (unglycos.) are indicated. In panels A and B, a longer autoradiographic exposure is presented for the co-ppt. samples than for the SN samples.

A combination of pulse-chase experiments and endoglycosidase digests revealed that human HL was synthesized as a lower molecular weight, pre-medial Golgi-associated precursor containing high mannose N-linked glycans that was processed to a higher molecular mass form containing mature N-linked glycans before secretion. Processing of human HL from its precursor to mature form took an extended time period (up to 4 h), suggesting that HL has a prolonged residence time in the ER compared to the majority of secretory proteins. This presumably reflects the time required for HL folding and dimerization in the ER. Based upon the time course of secretion of HL activity in the presence of the protein synthesis inhibitor, cycloheximide, secretion of human HL from HepG2 cells shows similar characteristics (41). Of interest, secretion of HL from rat hepatocytes occurs much more rapidly (23, 24), which may suggest differential time requirements for human and rat HL processing in the ER. Alternatively, the different results may reflect the different cell systems used (primary hepatocytes versus transformed cell lines).

Studies in primary rat hepatocytes demonstrated a large increase in HL secretion in the presence of heparin that was accompanied by decreased degradation of HL from the cell surface (38). Deletion of heparin from the media of our CHO cell cultures resulted in a decrease in HL secretion in pulse-chase experiments. However, total mature HL was unchanged, suggesting insignificant uptake and degradation of HL from the cell surface in these short-term experiments. In contrast, after an extended labeling period (16 h), we did observe a large difference in the total amount of mature HL between control and heparin-treated cultures (data not shown). This may reflect a long turnover time for HL at the cell surface in CHO cells or stabilization of HL in the medium by heparin.

To examine the requirement for N-linked glycosylation in human HL secretion, we utilized the glycosylation inhibitor, TM. Consistent with previous studies on both LPL and HL, we found that human HL secretion was almost completely prevented in the absence of N-linked glycosylation. This was most likely due to misfolding of unglycosylated HL in the ER (19). Indeed, our anti-HL antibodies showed reduced recognition of HL in TM-treated cells. However, the portion of radiolabeled HL that we were able to immunoprecipitate from the TM-treated cell lysates appeared stable throughout the 4-h chase time. A pathway for degradation of misfolded, ER-associated proteins has recently been elucidated that involves translocation across the ER membrane into the cytosol and subsequent degradation by the proteasome (42–44). Further studies involving longer chase times will be required to determine whether unglycosylated HL is a substrate for proteasome-mediated degradation.

In contrast to studies on rat HL, we found that the human HL protein was secreted in the presence of the ER glucosidase inhibitor, CST, albeit at reduced efficiency (43% of secretion from control cells). Secretion of HL enzyme activity was reduced to a similar extent in the presence of CST (to 44% of control levels), suggesting that HL secreted in the absence of glucose trimming was fully active. Active HL was also secreted from Lec23 cells that are deficient in ER glucosidase I activity.


Our results with human HL are similar to those reported for human LPL, where CST allowed secretion of 25% of fully active LPL protein (30). In contrast, very recent studies by Verhoeven et al. (41) demonstrated that secretion of endogenous human HL (both protein mass and activity) from the hepatoma cell line, HepG2, was prevented by CST. The reason for the discrepancy between their results and those presented here is not immediately

clear. The study by Verhoeven et al. (41) analyzed secretion of HL protein mass using an ELISA assay. This assay was unable to detect HL protein mass in cell lysates (41). Thus, it is possible that the assay would not detect the unprocessed form of HL secreted in the presence of CST. However, this would not explain the absence of HL activity secreted from HepG2 cells in the presence of CST. It is possible that the requirements for HL secretion may be different in the different cell lines used (HepG2 versus CHO cells). Alternatively, the different results may reflect the low level of endogenous HL expression in HepG2 cells, compared to the relatively high levels of human HL expression achieved in the current study. Whatever the explanation for the different results, the current study unequivocally demonstrates that catalytically active human HL protein can be secreted in the absence of processing by ER glucosidase enzymes.

ER glucosidase enzymes play an important role in regulating the interaction of newly synthesized proteins with the ER chaperones, calnexin and calreticulin. These chaperones bind only to proteins with monoglucosylated N-linked carbohydrate side chains (20, 21). Inhibition of ER glucosidase activity can prevent interaction of calnexin and calreticulin with their substrate proteins (45). Misfolding of LPL in Lec23 cells suggests an important role for these chaperones in LPL folding (M. H. Doolittle, submitted for publication). The differential requirement for glucosidase activity in human LPL and rat and human HL secretion may reflect differential dependence of these proteins on the lectin chaperones for folding.

We examined the interaction of newly synthesized human HL with calnexin in co-immunoprecipitation experiments. We found that HL associated with calnexin under control conditions, but not when N-linked glycosylation or ER glucosidase activity were prevented by pre-incubation with TM or CST, respectively. Association of HL with calnexin was enhanced 2-fold when CST was added after HL synthesis to trap HL in its monoglucosylated form. However, the portion of HL associated with calnexin was still very small. This may reflect a very transient association of HL with the chaperone or interaction of only a subpopulation of HL with calnexin. Alternatively, we may have underestimated the extent of interaction due to disruption of the calnexin-HL complex after cell lysis. The 60% reduction in HL secretion after pre-incubation with CST indicates that calnexin may play an important role in HL folding and processing in the ER. However, as a portion of HL was secreted under these conditions, it is clear that calnexin interaction is not required for secretion of HL protein or acquisition of enzyme activity.

In conclusion, the current study demonstrates that human HL secretion is dependent on the presence of N-linked glycans and normally involves interaction with the ER chaperone, calnexin. ER glucosidase processing and interaction with calnexin are essential for full expression of human HL activity, but are not absolutely required for secretion of catalytically active enzyme. These studies suggest distinct requirements for secretion of human HL compared to rat HL. Future studies will be designed to

elucidate the precise mechanisms involved in maturation and secretion of human HL. 

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