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Intracellular Activation of Rat Hepatic Lipase Requires Transport to the Golgi Compartment and Is Associated with a Decrease in Sedimentation Velocity*

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Hepatic lipase (HL) is an *N*-glycoprotein that acquires triglyceridase activity somewhere during maturation and secretion. To determine where and how HL becomes activated, the effect of drugs that interfere with maturation and intracellular transport of HL protein was studied using freshly isolated rat hepatocytes. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), castanospermine, monensin, and colchicin all inhibited secretion of HL without affecting its specific enzyme activity. The specific enzyme activity of intracellular HL was decreased by 25–50% upon incubation with CCCP or castanospermine, and increased 2-fold with monensin and colchicin. Glucose trimming of HL protein was not affected by CCCP, as indicated by digestion of immunoprecipitates with jack bean α-mannosidase. Pulse labeling experiments with [^{35}S]methionine indicated that conversion of the 53-kDa precursor to the 58-kDa form, nor the development of endoglycosidase H-resistance, were essential for acquisition of enzyme activity. In sucrose gradients, HL protein from secretion media sedimented as a homogeneous band of about 5.8 S, whereas HL protein from the cell lysates migrated as a broad band extending from 5.8 S to more than 8 S. With both sources, HL activity was exclusively associated with the 5.8 S HL protein form. We conclude that glucose trimming of HL protein in the endoplasmic reticulum is not sufficient for activation; full activation occurs during or after transport from the endoplasmic reticulum to the Golgi and is associated with a decrease in sedimentation velocity.

Hepatic lipase (HL)[1] is an extracellular enzyme present in the liver of most vertebrates. The enzyme is synthesized and secreted by liver parenchymal cells, and subsequently bound in the space of Disse, where it plays an important role in plasma lipoprotein metabolism (1–3). HL hydrolyzes phospholipids and triacylglycerols present in high and intermediate density lipoproteins, and facilitates the hepatic uptake of remnant particles (4–6) and of cholesterol(esters) carried in high density lipoproteins (7, 8). In addition, HL may act as a ligand protein for remnant binding to the liver (9). In humans, a low HL activity is associated with an increased atherosclerotic risk (10, 11). When expressed in transgenic mice, human HL was shown to markedly reduce the accumulation of aortic cholesterol (12). HL may protect against development of premature atherosclerosis by contributing to reverse cholesterol transport and reducing the number of atherogenic remnants in the circulation. Expression of HL in the liver is under hormonal and dietary control, which may be exerted at the level of synthesis, intracellular processing, secretion, extracellular binding, and internalization.

When studying the post-translational control of HL expression in suspensions of freshly isolated rat hepatocytes, we noted that newly synthesized HL acquires catalytic activity toward triacylglycerols somewhere along the secretory pathway (13). First, the specific enzyme activity of intracellular HL was 3–5-fold lower than that of secreted HL. Second, HL activity secreted by hepatocytes in the absence of protein *de novo* synthesis was 5-fold higher than was accounted for by the fall in the intracellular HL activity. Such an apparent activation was also observed for human HL in the HepG2 hepatoma cell line (14). HL is a glycoprotein bearing two (rat) to four (human) asparagine-linked glycans (15–17). For the synthesis and secretion of fully active HL, *N*-glycosylation is a prerequisite (18, 19). When glycosylation is prevented, either by tunicamycin or by site-directed mutagenesis, inactive HL protein accumulates intracellularly (16, 20). Along the secretory pathway, the *N*-linked oligosaccharide chains are extensively processed. In rat hepatocytes treated with castanospermine, a selective RER glucosidase inhibitor which prevents secretion of newly synthesized HL, inactive HL was present; upon removal of the inhibitor, the HL protein acquired catalytic activity and was secreted (13). These observations show that newly synthesized HL protein becomes catalytically active during oligosaccharide processing after the terminal glucose residues have been removed by the glucosidases in the RER, and suggest that activation may be intimately linked to the glycosylation state of the HL protein.

The presence of terminal glucoses on HL protein itself may prevent the acquisition of catalytic activity. However, the glucose residues on *N*-glycoproteins have recently been implicated in the protein folding and quality control system of the RER, which prevents malfolded proteins from reaching the Golgi (21, 22). It is possible therefore, that glucose trimming is only required for transport of the newly synthesized HL out of the RER and that activation occurs subsequently in a distal compartment of the secretory pathway. In line with this, inhibition of the Golgi mannosidase I with 1-deoxymannojirimycin has no effect on either activation or secretion of HL in rat hepatocytes

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[1] The abbreviations used are: HL, hepatic lipase; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Endo-H, endo-β-*N*-acetylglucosaminidase H; JBAM, jack bean α-D-mannoside mannohydrolase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RER, rough endoplasmic reticulum; LPL, lipoprotein lipase; MOPS, 4-morpholinepropanesulfonic acid; CSP, castanospermine.

(13, 19). This suggests that once the glucose residues have been removed, activation and subsequent secretion proceed independently of further oligosaccharide processing.

If glucose trimming in the RER is necessary for activation of HL protein itself rather than for transport of newly synthesized HL protein out of the RER, one would expect that inhibition of the transport process leads to the intracellular accumulation of active HL protein. The present study was performed to test this possibility. We determined in which intracellular compartment HL protein is activated, by using inhibitors that primarily affect vesicular transport in the secretory pathway. CCCP, monensin, and colchicin inhibit transport of glycoproteins from the RER to the Golgi (23, 24), from medial- to trans-Golgi (25) and between the Golgi and the plasma membrane (26, 27), respectively. Our data show that active HL accumulates in monensin- and colchicin-treated hepatocytes, but not in cells treated with CCCP. Hence, glucose trimming alone does not activate HL but is necessary for translocation of HL protein to the Golgi compartment, where the protein apparently acquires its triglyceridase activity.

EXPERIMENTAL PROCEDURES

Materials—Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), monensin, and ketoconazole were purchased from Calbiochem (La Jolla, CA), whereas colchicin was from Merck (Darmstadt, Germany). Castanospermine, 1-deoxymannojirimycin, jack bean α-D-mannoside mannohydrolase (α-mannosidase), and CHAPS were from Roche Molecular Biochemicals (Germany). Endo-β-*N*-acetylglucosaminidase H was from Genzyme (Boston, MA). Protease inhibitors were from Sigma, except for Trasylol which was from Bayer (Mijdrecht, Holland). Tran^{35}S-label (1100 Ci/mmol) was obtained from ICN (Costa Mesa, CA), and glycerol [1-^{14}C]trioleate (50–80 mCi/mmol) was from Amersham Pharmacia Biotech. Ham's F-10 and methionine-free minimal essential medium were purchased from Life Technologies, Inc. (Breda, Holland), whereas bovine serum was from BioTrading (Wilnis, Holland). Heparin was from Leo Pharmaceuticals (Weesp, Holland). Goat and rabbit anti-HL antisera were raised against rat HL purified from liver heparin perfusates according to Jensen and Bensadoun (28); from the antisera partly purified IgG fractions were prepared by precipitation in 50% saturated ammonium sulfate followed by 17% (w/v) Na_2SO_4, as described previously (13). Alkaline phosphatase-conjugated goat anti-rabbit IgG was obtained from Tago (Burlingame, CA), and *p*-nitrophenol phosphate was from Merck. Broad-range protein size markers were from Bio-Rad. All other chemicals were from Sigma. Polystyrene 96-well EIA plates (code 3590) were from Costar (Cambridge, MA).

Hepatocyte Isolation and Incubation—Hepatocytes were isolated from male Wistar rats (200–250 g body weight) by collagenase perfusion: non-parenchymal cells were removed by differential centrifugation (29). Cell viability was determined by trypan blue exclusion and ranged from 85 to 90%. The cells were suspended at a density of 4×10^6 cells/ml in minimal essential medium containing 25 units/ml heparin and 20% of dialyzed, heat-inactivated bovine serum (30). Cell suspensions were incubated at 37 °C under an atmosphere of 5% CO_2, 95% O_2 in a shaking water bath. The incubations were started with the addition of inhibitors. CCCP, monensin, and colchicin were added from 1000-fold stock solutions in ethanol; other inhibitors were added from 100-fold stocks in PBS. At the times indicated, samples of the cell suspension were collected on ice. The cells were separated from the medium by centrifugation for 5 s at 10,000 × *g*. The cells were washed once in PBS and then resuspended at 15×10^6 cells/ml in a 40 mM NH_4OH buffer, pH 8.1 (31), containing 25 units/ml heparin and a mixture of protease inhibitors (1 mM EDTA, 10 units/ml Trasylol, 0.1 mM benzamidine, and 2 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin). After 30 min on ice, the lysates were sonicated for 15 s (MSE Soniprep 150, amplitude 14 μm) and centrifuged for 10 min at 10,000 × *g* and 4 °C. The supernatants were used for analysis of intracellular HL. Cell-free media and cleared lysates were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

Hepatic Lipase Activity—Hepatic lipase activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl using a gum acacia-stabilized glycerol [^{14}C]trioleate emulsion as substrate (19). Assays were performed for 30 min at 30 °C. Activities were expressed as milliunits (nanomoles of free fatty acids released per min). In a total assay volume of 125 μl, release of free fatty acids was linear with time and sample volume up to 50 μl for the cell-free media and 10 μl for the cell lysates.

In immuno-inhibition assays, 40 μl of the cell-free media or 10 μl of the cell lysates were preincubated for 1 h on ice in a total volume of 50 μl with either 100 μg of goat non-immune IgGs or anti-rat HL IgGs. Thereafter, 75 μl of substrate was added to the supernatant, and the residual immunoresistant triglyceridase activity was determined. The lipase activity in the extracellular media was completely inhibited by the anti-HL IgGs whereas 85–95% of the lipase activity in the cell lysates was sensitive to immuno-inhibition.

Hepatic Lipase Mass—The amount of HL protein was determined by a solid-phase enzyme-linked immunosorbent assay in which the antigen was sandwiched between goat and rabbit polyclonal anti-HL IgGs. EIA plate wells were coated with 20 μg of goat anti-HL IgGs. After blocking with 1% bovine serum albumin in PBS, the wells were incubated successively with: (i) sample, either 50 μl of cell-free medium or 5 μl of cell lysate; (ii) 3 μg/ml rabbit anti-HL IgGs in PBS, and (iii) alkaline phosphatase-conjugated goat anti-rabbit IgG at a 1:1500 dilution in PBS. Finally, the presence of alkaline phosphatase was detected with *p*-nitrophenol phosphate as substrate. Color development was stopped with NaOH (1 M, final concentration), and the absorbance at 405 nm was measured in a Molecular Devices microplate reader. Absorbances were read against a standard curve prepared for each plate by serial dilutions of rat HL partly purified from liver heparin perfusates by affinity chromatography on Sepharose-heparin; HL activity was eluted from the column by a linear salt gradient and the peak fractions were pooled. After adding bovine serum albumin to a final concentration of 1%, aliquots were frozen in liquid nitrogen and stored at −80 °C until use.

Pulse Labeling with [^{35}S]Methionine—Freshly isolated rat hepatocytes were incubated in methionine-free minimal essential medium in the absence or presence of inhibitors, as described above. After 1 h, 80 μCi of Tran^{35}S-label was added per ml of cell suspension, and the incubation was continued for the time indicated. The incubations were stopped on ice, and the cells and media were separated by centrifugation (5 s, 10,000 × *g*). The cell-free medium was collected in vials containing cold methionine (final concentration 1 mM) and the mixture of protease inhibitors described above. After washing twice with cold PBS, the cells were lysed in cold PBS containing 1% Triton X-100, 1% sodium deoxycholate, 0.25% SDS, 1 mM methionine, 25 units/ml heparin, 10 mM HEPES, pH 7.4, and the mixture of protease inhibitors. After 30 min on ice, the lysates were centrifuged for 10 min at 10,000 × *g* and 4 °C, and the supernatants were used for further analysis.

Immunoprecipitations and Glycosidase Digestions—HL protein was immunoprecipitated from cell-free media and cell lysates by overnight incubation at 4 °C with 50 μl of a 50% slurry of goat anti-HL IgGs immobilized onto Sepharose (13). The beads were collected by centrifugation (20 s, 10,000 × *g*), and washed twice in successively: (i) 1% Triton X-100 in PBS, (ii) 1 M NaCl in PBS, and (iii) PBS (all containing 1 mM phenylmethylsulfonyl fluoride). For digestion with jack bean α-mannosidase (JBAM), the beads were resuspended in 100 μl of a 50 mM sodium acetate buffer, pH 5.0, containing 5 mM Zn^{2+}, and then incubated overnight with or without 50 μg of JBAM. Thereafter, the immunoprecipitated proteins were released by boiling for 5 min in Laemmli's sample buffer without 2-mercaptoethanol, and the beads were removed by centrifugation. After treating with 2-mercaptoethanol, the released proteins were separated by SDS-PAGE using 7.5% gels. For digestion with Endo-H, the immunoprecipitates were resuspended in 50 mM NaP_i, pH 6.0, containing 0.5% SDS, and the proteins were released from the beads by boiling for 5 min. The eluate was diluted in 50 mM NaP_i, pH 6.0, to reduce the concentration of SDS to 0.2%, and then incubated overnight at 37 °C in the presence or absence of 40 milliunits/ml of Endo-H. After addition of Laemmli's sample buffer and boiling for 5 min, the proteins were separated by SDS-PAGE using 7.5% gels.

After SDS-PAGE, the gels were Coomassie-stained for estimation of molecular sizes. The ^{35}S-labeled proteins were visualized, and their radioactivity quantified, by exposure of the dried gels to a phosphor screen (Bio-Rad GS-363 Molecular Imager System, Hercules, CA). Sensitivity to JBAM or Endo-H was indicated by an increase in electrophoretic mobility.

Overall Protein de Novo Synthesis—Incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material was taken as a measure for overall protein *de novo* synthesis. Incubations were performed as described above. Of the cell-free media and lysates, 5-μl aliquots were spotted in duplicate onto Whatman 3MM filters, and trichloroacetic acid precipitation was performed as described previously (19). The radioactivity on the filters was measured using the Molecular Imager

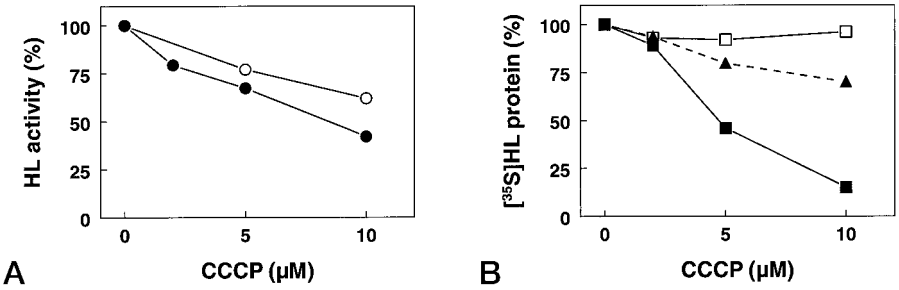

FIG. 1. **Effect of CCCP on synthesis and secretion of HL.** Freshly isolated rat hepatocytes were incubated for 3 h in the presence of different concentrations of CCCP. At the end of the incubation, the HL activity in the cell-free media (●) and cell lysates (○) was measured (*panel A*). Data are expressed as percentage of the activity found for the control incubation, which was 9.1 and 4.3 milliunits/ml in the cell-free medium and cell lysate, respectively. In parallel incubations, 80 μCi/ml Tran^{35}S-label was added after 1 h of preincubation with CCCP (*panel B*). The incubation was continued for an additional hour and then cell-free media (■) and cell lysates (□) were prepared. HL protein was immunoprecipitated by overnight incubation with goat anti-HL IgGs coupled to Sepharose. The immunoprecipitated proteins were separated by SDS-PAGE and the radioactivity in the immunoreactive bands at the 53–58-kDa position in the gels were quantified by PhosphorImaging. The sum of the radioactivity in the bands from the lysate and cell-free medium was taken as a measure for total synthesis of HL protein (▲). Data are expressed as percentage of the radioactivity present in the corresponding bands from control media and lysate. The results are representative for two similar experiments.

System. The duplicate measurements, which never differed by more than 5%, were averaged. The data were corrected for the trichloroacetic acid-precipitable material in the media and lysates of a control cell suspension that was put on ice before addition of Tran^{35}S-label.

Sucrose Velocity Gradient Centrifugation—Hepatocytes were incubated for 3 h with heparin, and cell-free medium was prepared as described above. Cells were lysed in PBS containing 8 mM CHAPS, 25 units/ml heparin and the mixture of protease inhibitors. After 30 min on ice, the lysates were cleared by centrifugation (10 min, 10,000 × *g*, 4 °C). Of the secretion media and cleared lysates, 200-μl aliquots were layered on top of linear 5–20% (w/w) sucrose gradients (4.0 ml) in 2 mM MOPS buffer, pH 8.0, 4 mM CHAPS, and 25 units/ml heparin. The gradients were run at 40,000 rpm for 15 h at 4 °C in a Beckman SW60 rotor (Beckman Instruments). Gradients were collected in 0.32-ml fractions by aspiration from the bottom of the tubes. Total recovery of HL activity from the sucrose gradients was 95 ± 10 and 135 ± 25% (means ± S.D., $n = 5$) for secretion media and cell lysates, respectively. The sedimentation markers bovine albumin ($s_{20,w}$ = 4.3 S) and rabbit IgG ($s_{20,w}$ = 7 S) were run in parallel; their position in the sucrose gradients was determined by Coomassie staining (32).

Statistics—All data are expressed as mean ± S.D. Differences were tested statistically by one-way analysis of variance followed by the Student-Newman-Keuls test, and considered significant at $p < 0.05$ (33).

RESULTS

Effect of CCCP on HL Synthesis and Secretion—When freshly isolated rat hepatocytes were incubated for 3 h in the presence of heparin, HL activity in the cell lysates remained almost constant at 4.3 ± 1.3 milliunits/ml ($n = 6$), which equals 0.27 ± 0.08 milliunits/10^6 cells. During this incubation, HL activity in the extracellular medium increased from 0.4 ± 0.1 to 9.1 ± 2.6 milliunits/ml, which corresponds to 2.3 ± 0.6 milliunits/10^6 cells. In the presence of increasing concentrations of CCCP, the extracellular appearance of HL activity gradually fell (Fig. 1*A*). Complete inhibition was obtained with 20 μM CCCP and above. Intracellular HL activity decreased to 63 ± 10% ($n = 3$) of controls when cells were incubated with 10 μM CCCP; a further increase in the CCCP concentration did not have an additional effect on intracellular HL activity. To study the effect of CCCP on *de novo* synthesis of HL protein, [^{35}S]methionine was included during the last hour of incubation. Fig. 1*B* shows that CCCP induced a dose-dependent reduction in the ^{35}S radioactivity of HL protein immunoprecipitated from the media plus lysate (53 + 58 kDa bands; see below). This parallelled the effect of the inhibitor on incorporation of ^{35}S radioactivity into trichloroacetic acid-precipitable material, and hence on overall protein synthesis (not shown). With 20 μM CCCP and above, HL and overall protein synthesis were completely blocked. When incubated with CCCP up till 10 μM, [^{35}S]HL in immunoprecipitates from the cell lysates was hardly affected, whereas [^{35}S]HL in the extracellular media was

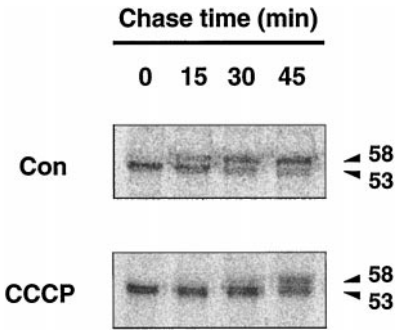

FIG. 2. **Effect of CCCP on HL maturation.** Hepatocytes were preincubated for 45 min with or without 10 μM CCCP. The cells were pulsed for 5 min with [^{35}S]methionine, and after washing, the cells were chased in control medium (*Con*) or in medium containing 10 μM CCCP, respectively. At the times indicated, samples were withdrawn and put on ice. HL was immunoprecipitated from the whole cell suspensions and analyzed by SDS-PAGE and PhosphorImaging. The apparent molecular weight of the bands is indicated in kDa.

highly sensitive to inhibition. With 10 μM CCCP, where HL synthesis was reduced by approximately 30%, the newly synthesized HL protein was no longer secreted into the extracellular medium but remained in the cells.

Pulse-chase experiments were performed to study the effect of CCCP on maturation of newly synthesized HL protein. After a 5-min pulse with [^{35}S]methionine, ^{35}S-labeled HL migrated as a 53-kDa protein (Fig. 2). During the subsequent chase of control cells, the 53-kDa protein was converted into a 58-kDa protein with a half-life of approximately 20 min. Pulse labeling of cells that had been preincubated with 10 μM CCCP also resulted in a ^{35}S-labeled HL protein with apparent molecular mass of 53 kDa. During the chase in the presence of CCCP, the 53-kDa HL protein matured into the 58-kDa form but at a much lower rate. The 58-kDa form appeared only after 30 min chase; approximately 50% of the 53-kDa form had matured into the 58-kDa form after 45 min of chase. The 53-kDa band was sensitive to digestion with Endo-H, whereas the 58-kDa band was Endo-H resistant (see below). Hence, CCCP retarded the maturation of the 53 kDa, high-mannose type precursor form into the 58-kDa complex-type form of HL. Taken together, the effects of CCCP on HL maturation and secretion are in agreement with its proposed action as inhibitor of the RER-to-Golgi transport.

Comparison between Effects of CCCP and Castanospermine—The effect of 10 μM CCCP on HL expression was compared with that of 100 μg/ml castanospermine, which inhibits RER-to-Golgi transport of *N*-glycoproteins by interfering with oligosaccharide processing. After 3 h of incubation, both HL

TABLE I

Effect of castanospermine and CCCP on intracellular and extracellular HL

Freshly isolated rat hepatocytes were incubated for 3 h in the presence of heparin without further additions (control), or with 10 μM CCCP or 100 μg/ml castanospermine (CSP). Then, cell-free media and cell lysates were assayed for HL activity and HL protein. Data are expressed as mean ± S.D. for three–five independent experiments.

	HL activity		HL protein		Specific activity	
	Milliunits/ml	%	Microgram/ml	%	Milliunits/μg	%
Cell-free media						
Control	10.4 ± 1.9	100	0.20 ± 0.06	100	42.8 ± 8.4	100
CCCP	1.4 ± 1.1[a]	14	0.03 ± 0.02[a]	15	40.1 ± 15.4	94
CSP	3.4 ± 0.6[a]	33	0.07 ± 0.02[a]	37	44.5 ± 12.8	104
Cell lysates						
Control	4.8 ± 2.1	100	0.22 ± 0.07	100	23.1 ± 10.5	100
CCCP	3.0 ± 0.2[b]	63	0.21 ± 0.10	94	17.7 ± 1.3[b]	76
CSP	1.8 ± 0.5[b]	36	0.13 ± 0.04[b]	61	10.9 ± 3.4[b]	47

[a] Statistically significant difference from control media ($p < 0.05$).
[b] Statistically significant difference from control lysates ($p < 0.05$).

activity and HL protein were reduced in the extracellular medium of CCCP-treated cells by approximately 85% compared with controls, whereas with castanospermine, both parameters were decreased in parallel by 65% (Table I). The specific enzyme activity of secreted HL was about 45 milliunits/μg, which was not significantly affected by either treatment. Under the conditions used, the specific enzyme activity of HL in the control cell lysates was only 50% of that in the cell-free media. Upon treating the cells with CCCP, the amount of intracellular HL protein was hardly affected, although simultaneously, HL activity decreased by approximately 35% (Table I). Hence, the specific enzyme activity of intracellular HL was 25% lower in CCCP-treated cells than in control cells. In the presence of CSP, both HL protein and HL activity in the cells were significantly reduced compared with controls, but the effect on HL activity was stronger than on HL protein. As a result, the specific enzyme activity of residual HL fell by approximately 50%. In parallel incubations, the effect of the inhibitors on overall protein *de novo* synthesis was determined. Incorporation of [^{35}S]methionine into total trichloroacetic acid-precipitable material was reduced to 52.5 ± 24.4 and 71.2 ± 8.1% of control by CCCP and CSP, respectively ($n = 3$).

To test whether CCCP also interferes with oligosaccharide processing in the RER, the glucose trimming status of the glycan chains on newly synthesized HL protein was evaluated with JBAM (34, 35). Digestion of high mannose-type glycoproteins with this exomannosidase will remove five mannose residues from oligosaccharides bearing terminal glucose residues ($Glc_{1-3}Man_9GlcNAc_2$), but eight mannoses from completely glucose-trimmed glycan chains ($Man_9GlcNAc_2$). The mobility of the ^{35}S-labeled 58-kDa HL protein band present in control cells and secretion media, was not affected by incubation with JBAM (Fig. 3), which agrees with the conclusion that this protein reflects mature HL bearing complex-type oligosaccharides. Upon digestion with JBAM, the 53-kDa band present in the control cells was converted into two higher mobility bands with apparent molecular masses of 52 and 49 kDa, which may reflect HL protein with incompletely and fully glucose-trimmed glycans, respectively. A similar digestion pattern was observed for the 53-kDa ^{35}S-labeled HL protein present in CCCP-treated cells. In contrast, [^{35}S]HL that is retained in CSP-treated cells predominantly migrated as a 55-kDa band whose mobility was increased to approximately 52 kDa upon treatment with JBAM. These observations suggest that CCCP inhibits transport of *de novo* synthesized HL protein out of the RER without interfering with glucose trimming.

Effect of Monensin and Colchicin—In the presence of 50 μM monensin, secretion of HL activity was instantaneously and almost completely inhibited (Fig. 4*A*). After 2 h of incubation,

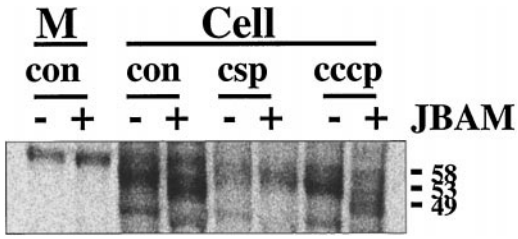

FIG. 3. **Evaluation of glucose trimming status of HL protein by digestion with jack bean α-mannosidase.** Hepatocytes were preincubated for 45 min in the absence (*Con*) or presence of 10 μM CCCP or 100 μg/ml CSP, and then labeled for 30 min with [^{35}S]methionine. After immunoprecipitation from the cell free media (*M*) and from the cell lysates (*Cell*), HL protein was incubated overnight without (−) or with (+) JBAM. The proteins were then resolved by SDS-PAGE and analyzed by PhosphorImaging. The apparent molecular weight of the radioactive bands is indicated in kDa.

HL activity in the extracellular medium was 14 ± 4% ($n = 4$) of parallel controls. Simultaneously, the intracellular HL activity increased linearly with time to 250 ± 44% ($n = 4$) of control cells (Fig. 4*B*). Under these conditions, [^{35}S]methionine incorporation into total protein was 44.2 ± 13.1% of control ($n = 3$). Half-maximal effects of monensin on extracellular and intracellular HL activity were obtained with 5 ± 2 μM ($n = 3$), which is much higher than the concentrations that inhibit transport across the Golgi in other cell types. At this concentration, secretion of HL activity was almost completely inhibited during the first 60 min of incubation, but thereafter, secretion proceeded at a rate similar to that in untreated control suspensions. With other concentrations of monensin, secretion of HL activity also started after an initial inhibitory period; this lag time increased with the amount of monensin used. Apparently, in our suspensions of freshly isolated rat hepatocytes, monensin loses its efficacy in a time- and dose-dependent manner. Rapid detoxification of monensin has been shown to occur in freshly isolated rat liver microsomes by the cytochrome P-450 3A system, where it can be inhibited by competing substrates such as ketoconazole (36). When we co-incubated intact rat hepatocytes with 25 μM ketoconazole, the dose-response curve for monensin was shifted to markedly lower concentrations (data not shown); half-maximal effects on HL secretion and intracellular HL activity were now obtained with 0.3 ± 0.1 μM monensin ($n = 3$). This effective dose suggests that monensin affects HL secretion and intracellular HL activity through its inhibitory effect on Golgi function. Due to its rapid detoxification in freshly isolated rat hepatocytes, much higher concentrations of monensin are needed to maintain an effective dose throughout the 2–3-h incubation period.

In the presence of 50 μM colchicin, the secretion rate of HL

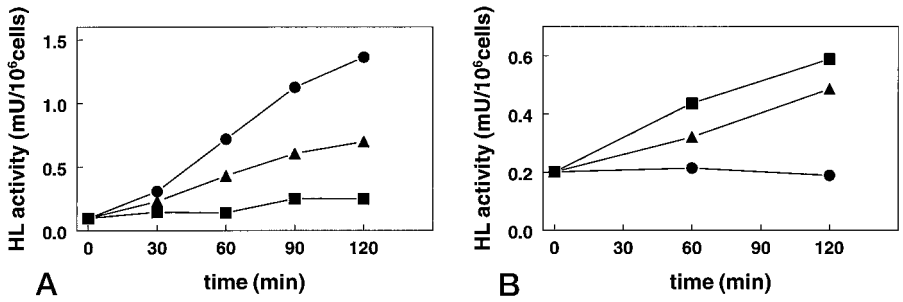

FIG. 4. **Effect of monensin and colchicin on extracellular and intracellular HL activity.** Freshly isolated rat hepatocytes were incubated in the absence (●) or presence of 50 μM monensin (■) or 50 μM colchicin (▲). At the indicated times, aliquots of the cell suspension were collected from the incubation, and HL activity was measured in the cell-free media (*panel A*) and cell lysates (*panel B*). Data are representative for two similar experiments.

activity was reduced to 45 ± 9% of controls (Fig. 4*A*), whereas intracellular HL activity gradually increased to 236 ± 34% of controls ($n = 4$) after 2 h of incubation (Fig. 4*B*). Protein *de novo* synthesis was reduced to 71.7 ± 16.6% of control ($n = 3$). Immuno-inhibition assays using anti-HL IgGs confirmed that HL was responsible for the observed changes in intracellular and secreted triglyceridase activity.

After 2 h of incubation with 50 μM monensin or colchicin, the amount of HL protein in the extracellular medium was reduced in parallel with HL activity, similar to the effects observed with castanospermine and CCCP (Fig. 5*A*). In the cell lysates, the amount of HL protein increased to 136 ± 21 and 137 ± 22% of control values ($n = 4$) with monensin and colchicin, respectively (Fig. 5*B*). These increases in intracellular HL protein were significantly less than the concomitant change in intracellular HL activity ($p < 0.05$; $n = 4$). As a result, the specific enzyme activity of intracellular HL was increased with both inhibitors.

Specific Triglyceridase Activity of HL—The effects of the different agents on the specific enzyme activity of HL are summarized in Fig. 5*C*. Under all conditions, the specific enzyme activity of secreted HL remained constant at approximately 45 milliunits/μg of HL. In control cells, the specific enzyme activity of intracellular HL was much lower than of secreted HL. The specific activity was further reduced by 25 and 50% upon treating the cells with CCCP and castanospermine, respectively. In monensin- and colchicin-treated cells, the specific activity was increased to levels close to that of secreted HL. In the presence of 1 mM 1-deoxymannojirimycin, an inhibitor of Golgi mannosidase I, neither secretion of HL (Fig. 5*A*) nor intracellular HL (Fig. 5*B*) were altered. Hence, the specific enzyme activity of intracellular and secreted HL was not affected (Fig. 5*C*) despite changes in the glycosylation state of secreted HL (see below). Taken together, these observations suggest that the catalytic activity of HL increases upon transport from the RER to the Golgi compartment.

Activation and Apparent Molecular Mass of HL—Hepatocytes were incubated for 3 h with the various inhibitors, and [^{35}S]methionine was present during the last 2 h. [^{35}S]HL immunoprecipitated from control media migrated as a single band of approximately 58 kDa (Fig. 6*A*). The ^{35}S-labeled HL secreted by CCCP- and colchicin-treated cells also migrated at the position of 58 kDa, but the radioactivity was reduced to 35 and 17% of control, respectively. In the presence of monensin, secretion of [^{35}S]HL was decreased to 3% of control, and the radioactive band migrated at a slightly higher mobility than the 58-kDa band in the other secretion media (Fig. 6*A*). [^{35}S]HL immunoprecipitated from control lysates appeared as two bands on SDS-PAGE, a band of approximately 53 kDa, and a band of 58 kDa that co-migrated with [^{35}S]HL from the cell-free media (Fig. 6*B*). The total ^{35}S radioactivity in HL immunoprecipitated from CCCP-treated cells was similar to control cells, whereas in monensin- and colchicin-treated cells total ^{35}S-labeled HL was 2–2.5-fold higher. In lysates prepared from CCCP-treated cells, the radioactivity of the 53-kDa band was increased whereas that of the 58-kDa band was decreased (Fig.

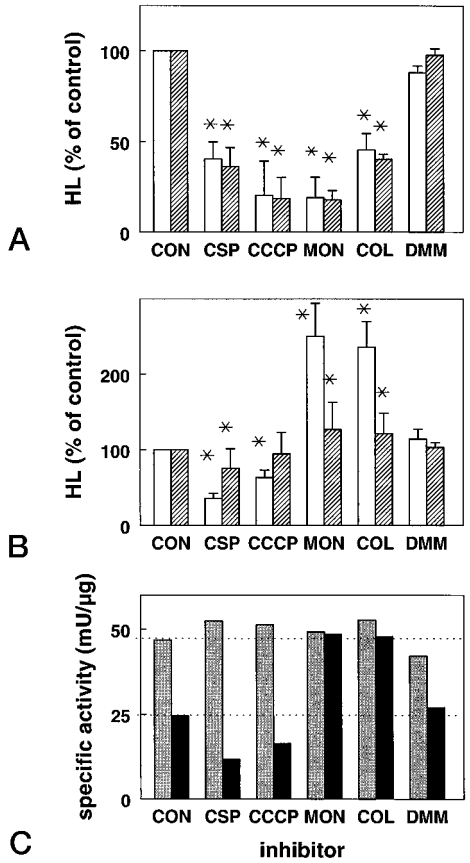

FIG. 5. **Effect of various inhibitors on intracellular and extracellular HL activity and amount of HL protein.** Hepatocytes were incubated for 3 h in the absence (*CON*) or presence of 100 μg/ml CSP, 10 μM CCCP, 50 μM monensin (*MON*), 50 μM colchicin (*COL*), or 1 mM 1-deoxymannojirimycin (*DMM*). At the end of the incubation, cell-free media (*panel A*) and cell lysates (*panel B*) were prepared and HL activity (*open bars*) and the amount of HL protein (*hatched bars*) were measured. Data are mean ± S.D. for three to six experiments and are expressed as percentage of control, which was 8.8 ± 3.0 and 4.3 ± 1.3 milliunits/ml for secreted and intracellular HL activity, and 0.19 ± 0.05 and 0.18 ± 0.03 μg/ml for secreted and intracellular HL protein, respectively. Statistically significant differences from the corresponding controls are indicated by *asterisks* ($p < 0.05$). In *panel C*, the specific enzyme activity of HL in the extracellular medium (*gray bars*) and cell lysate (*closed bars*) was calculated from the mean HL activity and HL protein found for each condition in the medium and cell lysate, respectively.

6*B*). In colchicin-treated cells, the ^{35}S label in the 53- and 58-kDa bands were increased in parallel. In contrast, the accumulation of [^{35}S]HL radioactivity in the monensin-treated cells only occurred in the 53-kDa band; in addition, the largest band migrated at a mobility that was slightly higher than the 58-kDa band found in the other cells. Comparison of the data in Fig. 6 with the effect of the inhibitors on intracellular and secreted HL activity indicated that HL activity varied in parallel with the expression of the 58-kDa protein form, except for

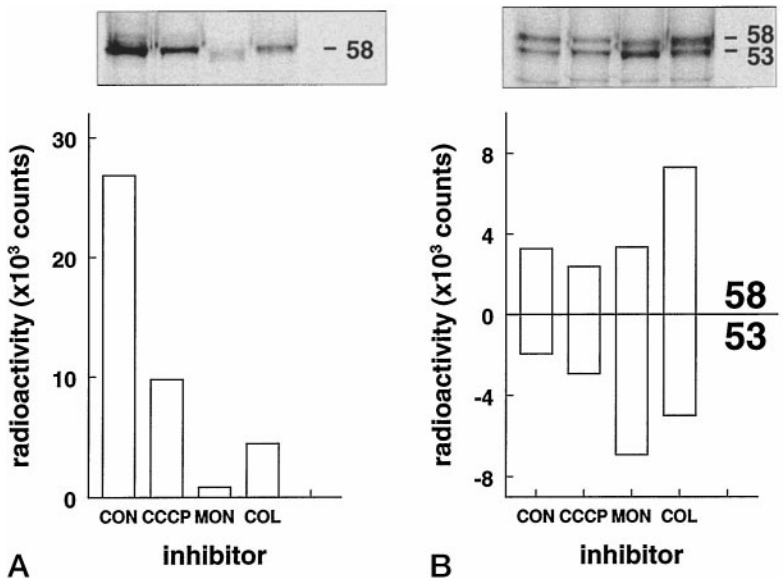

FIG. 6. **Effect of various inhibitors on the electrophoretic mobility of HL.** Cells were incubated for 3 h in the absence (*CON*) or presence of 10 μM CCCP (*CCCP*), 50 μM monensin (*MON*), or 50 μM colchicin (*COL*). During the last 2 h of the incubation, 80 μCi/ml Tran^{35}S-label was present. At the end of the incubation, HL protein was immunoprecipitated from the cell-free media and cell lysates, and then analyzed by SDS-PAGE and PhosphorImaging. The radioactivity in the immunoreactive bands was quantified. *Panels A* and *B* show part of the PhosphorImage and the quantitative results for the cell-free media and cell lysates, respectively. The positions of the 58- and 53-kDa bands are indicated. In *panel B*, the quantitative data for the 58- and 53-kDa bands are given as *upward* and *downward bars*, respectively. The results are representative for three similar experiments.

the monensin-treated cells, where intracellular HL activity increased in parallel with the 53-kDa protein form.

Activation and Resistance to Endoglycosidase H —The electrophoretic mobility of [^{35}S]HL secreted in the absence or presence of CCCP, monensin, or colchicin was not affected by overnight incubation with Endo-H (Fig. 7*A*). Hence, secreted HL was completely Endo-H resistant. As a reference, we used [^{35}S]HL that was secreted by cells in the presence of 1-deoxymannojirimycin, which prevents the maturation of the *N*-glycans from high-mannose to complex-type. This HL migrated as a single 53-kDa band, the mobility of which was almost completely shifted to 47 kDa corresponding to deglycosylated HL upon digestion with Endo-H (Fig. 7*A*). Of the two HL bands immunoprecipitated from control cell lysates, the 58-kDa band was Endo-H resistant whereas the 53-kDa band was sensitive to Endo-H (Fig. 7*B*). Upon digestion, the 53-kDa band was completely shifted to a higher mobility, partly to the position of 47 kDa, and partly to the position of a 51-kDa band. The mobility of the latter band was not altered upon prolonged incubation with additional Endo-H (not shown), and may reflect HL with one Endo-H-resistant and one Endo-H-sensitive oligosaccharide. Similar results were obtained with [^{35}S]HL in lysates from CCCP- and colchicin-treated cells. With monensin-treated cells, however, part of the [^{35}S]HL migrating at the 53-kDa position was not shifted to a higher mobility upon digestion with Endo-H and thus appeared to be Endo-H resistant. These data suggest that the accumulation of intracellular HL activity observed in monensin-treated cells coincides with the production of a 53-kDa, mainly Endo-H resistant form of HL.

Oligomeric Structure and Catalytic Activity of HL—Since we could not attribute the acquisition of catalytic activity to a particular change in the glycosylation state of HL protein, we attempted to correlate activation with possible noncovalent modifications of HL. Recent reports have indicated that catalytically active rat and human HL exist predominantly as noncovalent homodimers (37, 38). We therefore determined the oligomeric state of intracellular and secreted HL by monitoring the sedimentation profile in sucrose gradients. Aliquots of cell lysates and secretion media were loaded onto linear sucrose gradients which were subjected to overnight ultracentrifugation. Preliminary experiments showed that heparin had to be present throughout the gradients to prevent formation of large HL containing aggregates with the secretion media, whereas both heparin and CHAPS (4 mM) were necessary to prevent aggregate formation with the cell lysates. When secretion media were run under these conditions, HL activity and HL protein sedimentated as a homogeneous band (Fig. 8, *lower panel*) at a position intermediate between the sedimentation markers albumin (4.3 S) and IgG (7 S). Partly purified HL from rat liver perfusates ran at the same position (data not shown). The sedimentation coefficient of this band was rougly estimated at 5.8 S, which is consistent with the dimeric structure of catalytically active HL. No indications were found for the existence of a separate population of HL protein in the 3 to 4 S region corresponding to monomeric HL. The sedimentation profile of HL activity from cell lysates was similar to that of secreted HL (Fig. 8, *upper panel*), with a major band of about 5.8 S. In contrast, HL protein sedimentated as a broad band that extended from the 5.8 S position to more than 8 S. HL activity coincided with the slow-migrating part of HL protein, whereas the fast-migrating part of HL protein was associated with very low triglyceridase activity. The specific enzyme activity of the 5.8 S protein form in the cell lysate was approximately 40 milliunits/μg, compared with 50 milliunits/μg for the 5.8 S form in the secretion media. The specific enzyme activity of the faster-migrating protein forms in the cell lysate (fractions 4 to 6) ranged from 10 to 16 milliunits/μg. Also in the cell lysates,

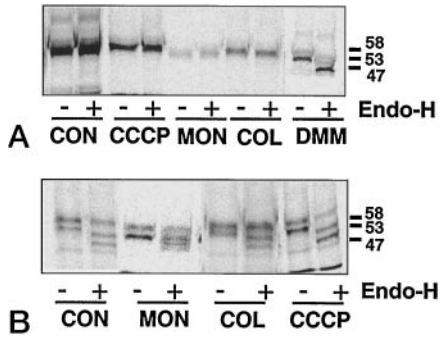

FIG. 7. **Effect of various inhibitors on the Endo-H sensitivity of HL.** Experiments were performed as described in the legend to Fig. 6, except that prior to electrophoretic separation, the immunoprecipitated proteins were incubated overnight at 37 °C without (−) or with (+) Endo-H. As a positive control for Endo-H activity, a secretion medium from a 3-h incubation of rat hepatocytes with 1 mM 1-deoxymannojirimycin (*DMM*) was included. *Panel A* and *B* show the PhosphorImages of the gels obtained with cell-free media and cell lysates, respectively. The positions of the 58- and 53-kDa HL protein forms, as well as the 47-kDa deglycosylated form are indicated. Data are representative for two similar experiments.

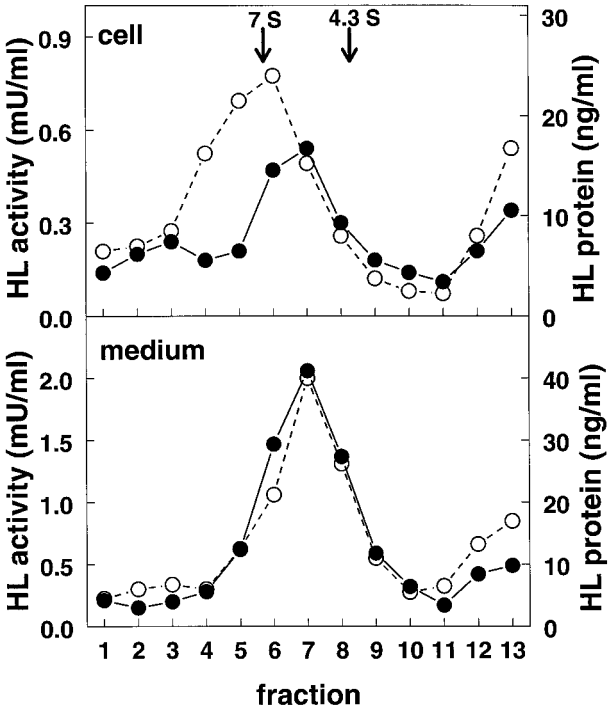

FIG. 8. **Sucrose gradient sedimentation profiles of intracellular and secreted HL.** Hepatocytes were incubated for 3 h in the presence of 25 units/ml heparin and 20% serum, and then cell-free media and cell lysates were prepared. Aliquots of the cell-free media (*lower panel*) and cell lysates (*upper panel*) were layered on top of a linear 5–20% (w/w) sucrose gradient containing 25 units/ml heparin and 4 mM CHAPS. After centrifugation, 13 fractions of the gradient were recovered by aspiration from the bottom of the tube. HL activity (*closed symbols*) and HL protein (*open symbols*) was measured for each fraction. The position of bovine albumin (4.3 S) and rabbit IgG (7 S) in parallel gradients is indicated by the *arrows*. The data are representative for three independent experiments.

the existence of a separate population of HL protein in the 3 to 4 S region corresponding to monomeric HL was not evident.

DISCUSSION

The data presented here confirm our previous report that newly synthesized HL protein is apparently activated during maturation and secretion in rat hepatocytes (13). This was also observed for human HL in HepG2 cells (14). This activation was prevented by treating the cells with inhibitors of RER glucosidases, which interfere with proper oligosaccharide processing of the newly synthesized HL (13). Upon incubation with castanospermine the specific enzyme activity of intracellular HL decreased, which may suggest that activation is closely coupled to glucose trimming in the RER. However, we show here that a fall in the specific activity of intracellular HL was also induced with CCCP, which interferes with transport of glycoproteins out of the RER to the Golgi, but leaves oligosaccharide processing in the RER essentially unaffected. Hence, glucose trimming alone appears not to be sufficient for activation of HL, but is necessary for transport of HL out of the RER; HL then matures into a catalytically active protein in a vesicular compartment distal from the RER. The RER glucosidases have been recently proposed to assist in the folding of newly synthesized glycoproteins thereby making them transport competent (21). In contrast to CCCP and castanospermine, a marked increase in the specific enzyme activity of intracellular HL was induced by treating the cells with monensin, which interferes with intra-Golgi vesicular transport (25), as well as with colchicin, which interferes with post-Golgi transport in rat hepatocytes (26, 27). This finding clearly demonstrates that HL has acquired full catalytic activity when accumulating in the Golgi compartment. Our data are best explained by the model that newly synthesized HL acquires catalytic activity during or after transport out of the RER.

Lipoprotein lipase, which is closely related to hepatic lipase, has also been shown to acquire catalytic activity after the glucose residues of the glycan side chains have been removed. Studies with monensin in mouse brown fat adipocytes (39) and CCCP in 3T3-L1 adipocytes (40) led to the conclusion that LPL is activated after transport of the protein from the RER into the Golgi. This was supported by the observation that incubation of adipocytes with brefeldin A, which induces the fusion of the RER with the Golgi compartment, results in the intracellular accumulation of fully active LPL (41). In our studies using freshly isolated rat hepatocytes, maturation and secretion of HL was not affected by brefeldin A (data not shown), possibly due to the rapid detoxification of the drug by these cells (42). In HepG2 cells, brefeldin A induced the intracellular accumulation of catalytically active HL (14); moreover, the inactive HL that accumulated in castanospermine-treated HepG2 cells was converted to fully active HL upon co-incubation with brefeldin A (14). These combined data suggest that both HL and LPL require transport to the Golgi compartment to become catalytically active. In contrast, Ben-Zeev *et al.* (43) reported that LPL accumulated intracellularly as a fully active enzyme when expressed as a hybrid with a C-terminal KDEL sequence. As this sequence was thought to function as an RER retention signal, the authors concluded that activation of LPL does occur before the protein reaches the Golgi compartment. Recent studies have demonstrated, however, that the KDEL sequence may function as a retrieval signal; KDEL-bearing proteins are cycled back into the RER from the Golgi or even beyond (44–46). Therefore, the catalytically active LPL-KDEL hybrid that accumulates in the RER may have been activated in the Golgi before being cycled back into the RER.

Our data indicate that catalytic activity co-varies with the presence of the 58-kDa Endo-H resistant form of HL in CCCP and colchicin-treated cells, and with the 53 kDa, mainly Endo-H resistant form in monensin-treated cells. *N*-Glycoproteins are processed from an Endo-H sensitive into an Endo-H-resistant form upon trimming of mannose residues by Golgi mannosidases. These observations suggest therefore, that the activation of HL is closely linked to, or occurs only after, some of the mannoses on the glycan chains have been trimmed off by the Golgi mannosidases. However, cells incubated with 1-deoxymannojirimycin secrete a 53 kDa, fully Endo-H sensitive form of HL whose specific enzyme activity is virtually identical to that of control HL (Fig. 7; Ref. 19). Therefore, trimming of the oligosaccharides on HL by the Golgi mannosidases is not crucial for the acquisition of triglyceridase activity. Taken together, these data demonstrate that activation of HL cannot be attributed to a change in its glycosylation state detectable by SDS-PAGE or Endo-H sensitivity.

Our observation that intracellular HL protein is present in complexes with various sedimentation velocities, and that the catalytic activity is associated with the slow-sedimenting protein forms suggests that activation of HL coincides with changes in the oligomeric state of HL protein. Catalytically active rat and human HL has recently been shown to exist predominantly as noncovalent homodimers (37, 38). HL activity in both secretion media and cell lysates was linked to protein forms that sedimented as approximately 5.8 S particles, which is consistent with the dimeric structure. We did not find evidence for the existence of (inactive) HL in the 3–4 S range corresponding to the monomeric protein. Instead, cell lysates but not secretion media contained HL protein with low

or no catalytic activity that predominantly sedimentated as particles of 7 S and higher. This shows that (inactive) HL is present in multimeric complexes, either as homo-oligomers or with other proteins. Together with our previous observations that inactive, intracellular HL protein can be secreted as active HL in the absence of protein *de novo* synthesis (13, 14), these findings suggest that activation of HL involves release of the dimeric form from the larger complexes. Although the intracellular localization of these multimer complexes is unknown at present, it is tempting to speculate that they are present in the RER. It is well established now that virtually all newly synthesized *N*-glycoproteins temporarily associate with one or more chaperone proteins that are abundant in the RER and assist in their proper folding (21, 22, 47). CCCP blocks release of endoplasmic reticulum proteins from their chaperones probably by depleting endoplasmic reticulum-ATP levels (48). Castanospermine interferes with proper glycoprotein folding (21, 22), thereby favoring formation of complexes with itself or with other endoplasmic reticulum proteins. Transport of HL proteins out of the RER will move them away from the numerous RER chaperones that may block their full activation. Further studies are required to establish the composition and intracellular localization of the multimer complexes, and the precursor-product relationship with the active HL dimers.

In conclusion, we have shown here that activation of newly synthesized HL protein is associated with a change in the oligomeric state of HL that occurs during or after transport from the RER to the Golgi compartment. The necessity to exit the RER in order to become activated is not unique to lipases, but has recently also been reported for two membrane-bound *N*-glycoproteins, the macrophage mannose receptor (49) and the trans-Golgi network protease furin (50). The modification that causes activation of these proteins has not been identified, but was shown not to depend on oligosaccharide processing *per se*. It may involve a rather subtle covalent modification not detected by the rather course methods of SDS-PAGE and Endo-H digestion. Alternatively, activation of these proteins may be due to noncovalent changes in their structure, similar to HL. Hence, our observations on the role of the oligomeric state in activation of newly synthesized HL may bear relevance to other proteins as well.

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