Identification of lipoprotein-binding proteins in rat liver Golgi apparatus membranes

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Abstract The low density lipoprotein (LDL) receptor has been shown to be a plasma membrane glycoprotein responsible for the cellular binding and endocytosis of plasma lipoproteins. Inasmuch as the Golgi apparatus has been shown to participate in glycoprotein processing and in the assembly of plasma lipoproteins by hepatic and intestinal epithelial cells, the present studies were designed to test the hypothesis that lipoprotein receptors are present within Golgi membranes. Utilizing ligand blotting with a variety of iodinated lipoproteins, several lipoprotein-binding proteins were identified in rat liver Golgi membranes at apparent molecular weights (M_r) 200,000, 160,000, 130,000, 120,000, 100,000, 80,000, and 70,000. The 130,000 protein was the most prominent and was identified as the mature LDL receptor by its binding characteristics and an M_r characteristic of the plasma membrane receptor. Enzymatic deglycosylation studies suggested that the 120,000 and 100,000 proteins were LDL receptor precursors lacking sialic acid. Antibody to the LDL receptor recognized all the bands on immunoblots except the 70,000 protein, with the 130,000 protein being the most prominent. Isolation of the Golgi fractions in the presence of protease inhibitors did not eliminate any of the proteins recognized by the antibody but did result in sharper bands on the blots. Additionally, we investigated the hypothesis that conditions that regulate plasma membrane LDL receptors also cause detectable changes in receptors in Golgi membranes. All the binding proteins were increased in Golgi membranes from rats treated with 17-α-ethynylestradiol. Colchicine caused an accumulation of 120,000 M_r protein, suggesting blockage of final sialylation in the *trans* Golgi. When protein synthesis was inhibited by cycloheximide, there was no reduction of mature LDL receptors in Golgi membranes, consistent with recycling of receptors through this organelle.—**Harrison, J. C., L. L. Swift, and V. S. LeQuire.** Identification of lipoprotein-binding proteins in rat liver Golgi apparatus membranes. *J. Lipid Res.* 1988. **29:** 1439–1449.

Supplementary key words low density lipoprotein • LDL receptor • receptor recycling

The intracellular pathways that lipoprotein receptors follow during their synthesis, processing, transport to the cell surface, and recycling from endosomes have been the topic of much discussion but little direct investigation. The possibility of regulation of plasma membrane receptor density by intervention at one or more points along these intracellular routes has also not been addressed experimentally. Several lines of evidence indicate that the Golgi apparatus plays an important role in the processing, transport, and recycling of lipoprotein receptors. The LDL receptor is an integral membrane glycoprotein containing N- and O-linked oligosaccharides that are processed by the Golgi apparatus following synthesis of the receptor peptide in the endoplasmic reticulum (1, 2). Once terminally glycosylated by sialyltransferase in the *trans* Golgi, the receptors are transported to the cell surface by Golgi membrane vesicles, possibly the same secretory vesicles that transport newly synthesized lipoproteins to their point of exocytosis into the space of Disse (3). Once receptors have reached the plasma membrane, moved to coated pits, and been endocytosed back into the cell, some or all of them may pass through the Golgi apparatus during recycling, possibly to be refurbished before their return to the cell surface (4, 5).

To investigate the role of the Golgi apparatus in lipoprotein receptor processing, transport, and regulation, we have explored two hypotheses. The first to be tested was that lipoprotein receptors can be detected on Golgi apparatus membranes. The second hypothesis was that conditions that regulate liver plasma membrane LDL receptors cause detectable changes in receptors at the level of the Golgi apparatus.

To detect lipoprotein receptors, octylglucoside extracts of rat hepatic Golgi membranes were studied by ligand blotting techniques using iodinated lipoproteins and by immunoblotting. We chose to study rat liver Golgi apparatus membranes for the following reasons. Rat liver contains at least two distinct lipo-

Abbreviations: LDL, low density lipoprotein; SDS, sodium dodecyl sulfate; β-VLDL, β-migrating very low density lipoproteins; Endo H, endo-β-N-acetylglucosaminidase H; EE, 17α-ethynylestradiol; apo, apolipoprotein; MVB, multivesicular body.

protein receptors, the LDL receptor (6–8) and the chylomicron remnant receptor (9). Additionally, the Golgi apparatus preparation in rat liver has been extensively characterized (10–12). To alter hepatic lipoprotein receptor expression, rats were treated with 17α-ethynylestradiol which has been shown to increase the number of lipoprotein receptors in rat liver (6, 13–15).

The results indicate that the LDL receptor is present in Golgi membranes in a mature form and in a precursor form lacking sialic acid and resistant to endo-β-N-acetylglucosaminidase. In addition, other lipoprotein-binding proteins were identified in the Golgi membranes. The results underline the importance of the Golgi apparatus in the processing and transport of lipoprotein receptors in rat liver.

MATERIALS AND METHODS

Animals and drugs

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN and Sasco Inc., St. Louis, MO) were used for all experiments. Rats to be treated with EE weighed 225–250 g and were maintained under conditions of constant temperature and humidity with food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, IL) and water ad libitum. Control animals received 1.0 ml propylene glycol (1,2-propanediol, Kodak) per kg body weight subcutaneously each day for 4 days. 17α-Ethynylestradiol (EE, Sigma Chemical Co., St. Louis, MO) was dissolved in propylene glycol at 5 mg/ml and administered subcutaneously at a dosage of 5 mg/kg body weight on the same time schedule. Typical experimental groups contained six treated animals and six control animals. In some experiments, rats received only one dose of EE as above and were killed 24 hr later. Additionally, 4 hr prior to being killed, the 1-day EE-treated rats were given intraperitoneally 0.125 μmol of colchicine (Sigma, dissolved in 0.15 M NaCl just prior to injection) per 100 g body weight (16). Cycloheximide (Sigma) was given intraperitoneally in phosphate-buffered saline at a dosage of 20 mg/kg body weight, and animals were killed 2 hr later when protein synthesis was maximally blocked (17). In studies to label the endocytic compartment of the liver, human ^{125}I-labeled LDL (0.5 ml, 2.18 mg protein/ml, 85 μCi/mg) was injected into the jugular bulb of ether-anesthetized rats 15 min before they were killed (12). Rats (80–100 g) were made hypercholesterolemic by feeding a diet of Wayne Lab-Blox supplemented with 5% lard, 0.1% 6-N-propyl-2-thiouracil (Sigma), 0.3% sodium taurocholate, and 1% cholesterol (ICN Nutritional Biochemicals, Cleveland, OH) for 21 days. Rabbits were made hypercholesterolemic by feeding Wayne Rabbit Ration supplemented with 1% cholesterol and 10% corn oil for 6 weeks.

Analytical methods

Total serum cholesterol and triglycerides were assayed by the method of Babson, Shapiro, and Phillips (18) and Van Handel and Zilversmit (19). Protein was estimated by the procedure of Lowry et al. (20). UDP-galactose:N-acetylglucosamine galactosyl transferase was assayed according to Morré, Merlin, and Kennan (21).

Lipoprotein isolation and labeling

All lipoproteins were isolated from plasma by ultracentrifugation at 4°C using Beckman 60-Ti and 40 rotors and a Beckman L3-50 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Human LDL were isolated between the densities 1.019 and 1.063 g/ml. Rat and rabbit β-VLDL were isolated from the plasma of hypercholesterolemic animals at d 1.006 g/ml. All lipoprotein fractions were washed one time at the upper density limit and dialyzed against 0.15 M NaCl, 0.01% EDTA, pH 7.4. Labeling of lipoproteins with ^{125}I was accomplished as described by Goldstein, Basu, and Brown (22). Specific activities averaged 300 cpm/ng lipoprotein protein. Trichloroacetic acid precipitation and lipid extraction revealed less than 5% of the label in the lipid portion of LDL and about 40–50% of ^{125}I in the lipid portion of β-VLDL.

Preparation of membranes

A rat liver cell membrane fraction was prepared according to Kovanen, Brown and Goldstein (15) as an 8000–100,000 *g* pellet. Rat liver Golgi apparatus was isolated intact as previously described (11). In some experiments the Golgi apparatus fraction retrieved from the sucrose gradient was diluted in distilled water instead of saline, and a more gentle final centrifugation (5000 rpm, 20 min) was used to pellet the Golgi apparatus as described by Hornick et al. (12). This fraction was designated Golgi fraction I and the standard fraction pelleting at 20,000 rpm for 30 min as described by Swift et al. (11) was designated Golgi fraction II. Additional purification of Golgi fraction I was achieved on 5–15% (w/w) linear Ficoll gradients after the manner of Taylor et al. (23). Intact Golgi apparatus pellets and cell membranes were solubilized in octylglucoside (n-octyl-β-D-glucopyranoside, Sigma) by the method of Schneider et al. (24). In one experiment cell and Golgi fraction I membranes were isolated and solubilized in the presence of 100 μM leupeptin (Boehringer Mannheim Biochemicals, Indianapolis, IN) and aprotinin (500 KIU/ml, Sigma).

Electron microscopy

Golgi apparatus fractions were negatively stained on carbon stabilized Formvar-coated nickel grids using 2% aqueous phosphotungstic acid, pH 6.5, and viewed using a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Polyacrylamide gel electrophoresis

Protein extracts were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels by a modification of the method of O'Farrell (25). The running gel was 7.5% acrylamide in a 0.375 M Tris-HCl buffer, pH 8.8, containing 0.1% SDS. The stacking gel was 3.0% acrylamide in 0.125 M Tris-HCl, pH 6.8, containing 0.1% SDS. The running buffer was 0.025 M Tris-HCl, pH 8.3, containing 0.192 M glycine and 0.1% SDS. Membrane protein extracts, 10–100 μg protein in 70 μl of 0.125 M Tris-maleate, pH 6.0, 1 mM $CaCl_2$, 0.15 M NaCl, and 40 mM octylglucoside were brought to a final volume of 80 μl and final concentrations of 0.5% SDS, 0.001% bromphenol blue, and 13% sucrose. In some experiments, 5% (v/v) 2-mercaptoethanol was included in the sample buffer to abolish receptor binding. Electrophoresis was carried out at 20°C, 10 mA per slab until the dye entered the resolving gel and 20 mA per slab until the dye ran out of the gel.

Electrophoretic transfer of proteins to nitrocellulose

Proteins were transferred from SDS slab gels to nitrocellulose paper (Bio-Rad) according to Burnette (26) using a Trans-Blot cell (Bio-Rad) and 25 mM Tris, pH 8.3, 192 mM glycine, and 20% (v/v) methanol as the blotting buffer. Electrophoresis was conducted at 200 mA for 16 hr in a 4°C cold room.

Ligand blotting and immunoblotting

Incubation of the nitrocellulose with iodinated lipoproteins was accomplished by the method of Kroon, Thompson, and Chao (27) with minor modifications. To block nonspecific protein binding sites, the nitrocellulose paper was first soaked in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5 mM $CaCl_2$ (buffer A) containing 50 mg of bovine serum albumin (Fraction V, Sigma)/ml, at 37°C for 30 min. The paper was then transferred to a Ziploc plastic bag (Dow) containing 40 ml of buffer A with 10 mg bovine serum albumin/ml and 4×10^6 cpm of iodinated lipoprotein, and incubated for 90 min at room temperature on a rocking platform. The paper was washed three times with buffer A containing 10 mg bovine serum albumin/ml, 200 ml buffer/wash, for 10 min, 20 min and 20 min, all at room temperature on a rocking platform. Finally the nitrocellulose was pressed between paper towels to remove excess moisture and dried between filter papers at 37°C. Autoradiography was performed with Kodak XR film and a Cronex Lightening Plus Enhance screen (E. I. DuPont) at −70°C for 1–48 hr. Autoradiograms were scanned on an LKB 2202 Ultrascan Laser Densitometer with Recording Integrator (LKB Instruments, Bromma, Sweden). To determine EDTA sensitivity of binding, calcium was omitted from buffer A and 10 mM EDTA was added to all incubations and washes.

For immunoblotting, nitrocellulose paper strips were washed in 0.01 M phosphate, pH 7.0, 0.15 M NaCl, containing 0.03% Tween 20, and incubated for 2 hr at room temperature with 1 μl/ml of rabbit polyclonal antisera to the rat hepatic LDL receptor (kindly provided by Dr. Allen Cooper, Research Institute, Palo Alto Medical Foundation, Palo Alto, CA). The blots were washed again and incubated for 1 hr with horseradish peroxidase-conjugated goat anti-rabbit IgG (H and L chain specific, Cappel, Cooper Biochemical, West Chester, PA). Bands were visualized by exposing the blots to 80 μM *o*-dianisidine in 3×10^{-3}% H_2O_2.

Enzyme treatment of membrane proteins

Solubilized membrane proteins were diluted eightfold and centrifuged at 100,000 *g* for 1 hr, as described by Schneider et al. (24). The pellets were resuspended in the appropriate buffer for neuraminidase or Endo H treatment and divided into three equal aliquots, each containing 100 μg of membrane protein. Endo H (Miles Labs, gift from Dr. Ann Soderquist, Vanderbilt University) digestion was conducted according to Soderquist and Carpenter (28) except that boiling and reducing agents were omitted. Neuraminidase (Worthington Biochemical, Freehold, NH) treatment was with 0.2 U neuraminidase in 0.1 M sodium acetate buffer, pH 5.5, 1 mM phenylmethylsulfonyl fluoride (Sigma), for 2 hr at 37°C and 16 hr at 4°C. For the Endo H experiment, one control aliquot was incubated identically except that the enzyme was omitted, and the other was frozen in electrophoresis buffer overnight.

RESULTS

Animals

EE treatment for 4 days resulted in a 5% loss in body weight while control rats experienced a 10% weight gain over the same period. EE treatment produced a decrease in serum cholesterol and triglycerides (22 ± 9 mg/dl and 9 ± 5 mg/dl) compared with control rats (108 ± 27 mg/dl and 73 ± 20 mg/dl). Rats

subjected to 1-day EE and colchicine treatment had a total serum cholesterol of 63 ± 15 mg/dl.

Golgi apparatus preparation

By negative contrast electron microscopy, the isolated, intact hepatic Golgi apparatus from control and EE-treated rats exhibited the morphology previously described for Golgi apparatus from control rats (10, 11). Negative contrast electron micrographs showed plate-like cisternae continuous with anastomosing tubules and secretory vesicles filled with newly synthesized lipoproteins. Lysosomes, mitochondria, and rough endoplasmic reticulum were not identified by negative contrast or thin-section electron microscopy of Golgi fractions II, I, and Ficoll Golgi fractions from control and EE-treated rats. Multivesicular bodies (MVB) were observed in Golgi fraction II from EE-treated rats but not in Golgi fraction I or the Golgi Ficoll fractions from EE animals or in any of the control rat liver Golgi fractions.

The Golgi membrane fractions prepared from control rat livers were enriched approximately 70- to 100-fold in galactosyltransferase compared with homogenates (**Table 1**). Further enrichment of galactosyltransferase in Golgi fractions could be produced by decreasing the *g* forces used to obtain the final Golgi pellet. However, total recovery of galactosyltransferase was concomitantly decreased. Ficoll gradient centrifugation of the Golgi I fraction produced a further 20–50% enrichment in galactosyltransferase (data not shown).

To estimate MVB contamination in our Golgi membrane preparation, we labeled the endocytic compartment of the liver cells by injecting human ^{125}I-labeled LDL 15 min prior to killing the animals. Less than 1% of liver homogenate ^{125}I was recovered in the Golgi fractions from control (I, 0.08%, II, 0.21%) and EE-treated (I, 0.35%, II, 0.65%) rats. Radioactivity recovered with Golgi membranes from EE-treated animals was approximately three- to fourfold more than that recovered with Golgi membranes from control animals. The amount of radioactivity recovered could be decreased in preparations from both control and EE rats by a more gentle centrifugation to obtain the final Golgi pellet (Golgi fraction I).

TABLE 1. Enzyme activity in homogenate and isolated fractions

Fraction	Galactosyltransferase		
	Specific Activity	Fold Enrichment	% Total Activity
Homogenate	3.45 ± 0.73 (5)		100
Cell membranes	19.8	5.7	
Golgi fraction I	325 ± 57 (5)	94	15 ± 4
Golgi fraction II	228 ± 12 (2)	66	20

Values given for specific activity are mean ± SD; numbers in parentheses represent number of preparations analyzed. Specific activity is expressed as nanomoles galactose transferred/mg of protein per hr. The membrane fractions were prepared as described in Materials and Methods, with Golgi fraction I being pelleted at 5,000 rpm for 20 min and Golgi fraction II at 20,000 rpm for 30 min in an SW-27 rotor.

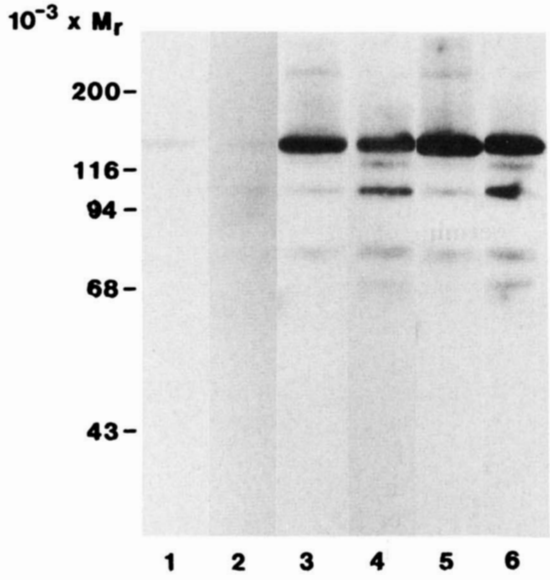

Fig. 1. Ligand blotting of cell and Golgi membranes from livers of 4-day EE-treated rats: comparison of three lipoprotein ligands. Solubilized cell and Golgi fraction II membrane proteins from livers of EE-treated rats were subjected to electrophoresis and transferred to nitrocellulose paper as described in Materials and Methods. Lanes 1, 3, and 5 contain 112 μg of cell membrane protein/lane and lanes 2, 4, and 6 contain 48 μg of Golgi fraction II membrane protein/lane. The nitrocellulose strips were incubated with the following lipoproteins: lanes 1 and 2, human ^{125}I-labeled LDL, 6.8 × 10^6 cpm, 6 μg protein/ml; lanes 3 and 4, rabbit ^{125}I-labeled β-VLDL, 4 × 10^6 cpm, 2 μg protein/ml; lanes 5 and 6, rat ^{125}I-labeled β-VLDL, 4 × 10^6 cpm, 0.7 μg protein/ml. Lipoprotein incubations, washing, and autoradiography were performed as described in Materials and Methods. Autoradiograms of lanes 1 and 2 were developed for 6 hr and of lanes 3–6 for 4 hr.

Ligand blotting

Ligand blotting experiments using Golgi fraction II and cell membranes from EE-treated rats revealed seven lipoprotein binding proteins with the following apparent molecular weights (M_r): 200,000, 160,000, 130,000, 120,000, 100,000, 80,000, and 70,000 (**Fig. 1**). Human ^{125}I-labeled LDL bound to a protein of M_r 130,000 in both cell and Golgi fraction II membranes (Fig. 1). ^{125}I-Labeled β-VLDL from cholesterol-fed rabbits and rats bound the same protein of apparent M_r 130,000 as well as several other proteins but with higher affinity than human ^{125}I-labeled LDL. This higher affinity resulted in more ^{125}I-labeled β-VLDL than ^{125}I-labeled LDL being bound at subsaturating ligand concentrations. The greater radioactivity bound

with ^{125}I-labeled β-VLDL allowed visualization of binding protein bands on autoradiograms with shorter exposure times than for ^{125}I-labeled LDL (Fig. 1). In our studies rat ^{125}I-labeled β-VLDL remained an effective probe for up to 1 month following iodination. Rabbit β-VLDL was less stable; therefore, subsequent studies utilized rat β-VLDL.

To determine whether lipoprotein binding proteins could be detected on membranes from control rats, membranes from control and EE-treated rats were blotted and incubated with rat ^{125}I-labeled β-VLDL. In addition, equal amounts of membrane protein were applied to each lane of the gel. **Fig. 2** shows that lipoprotein-binding proteins were present in cell and Golgi fraction II membranes from control rats, but the intensity of the bands was increased by EE treatment. With longer developing times, all of the bands observed in fractions from EE-treated rats could be visualized in cell and Golgi fraction II membranes from control rats. This indicated that the number of bands and their apparent molecular weights were not changed by EE treatment. Golgi II and cell membrane fractions from both groups of animals had the same number of lipoprotein binding bands with the same apparent molecular weights.

To investigate the possibility that lipoproteins might be binding to contaminating organelles in Golgi fractions, the Golgi fraction was purified further and the endocytic compartment was marked with human ^{125}I-labeled LDL. To purify the fraction, a lower *g* force centrifugation was used to obtain Golgi fraction I. Golgi fraction I was further purified on a Ficoll gradient to produce the Golgi Ficoll fraction. Both of these steps have been shown by other investigators to increase the purity of the Golgi fraction as monitored by enrichment in galactosyltransferase (12, 23, 29). As detailed above, we found increasing enrichment of galactosyltransferase and decreasing morphologic evidence of contamination in moving from Golgi fraction II to Golgi fraction I to the Golgi Ficoll fraction. Lipoprotein blotting with ^{125}I-labeled β-VLDL revealed the same pattern of lipoprotein binding proteins in all three Golgi fractions (**Fig. 3** and Fig. 6, Ficoll fraction data not shown). Smaller amounts of protein were recovered in the more highly purified fractions from control rats, with correspondingly fainter bands on the autoradiograms. However, at longer developing times, all the major lipoprotein-binding proteins were observed in all three Golgi fractions from control and EE-treated animals.

Marking of the endocytic compartment was accomplished by injecting rats with human ^{125}I-labeled LDL.

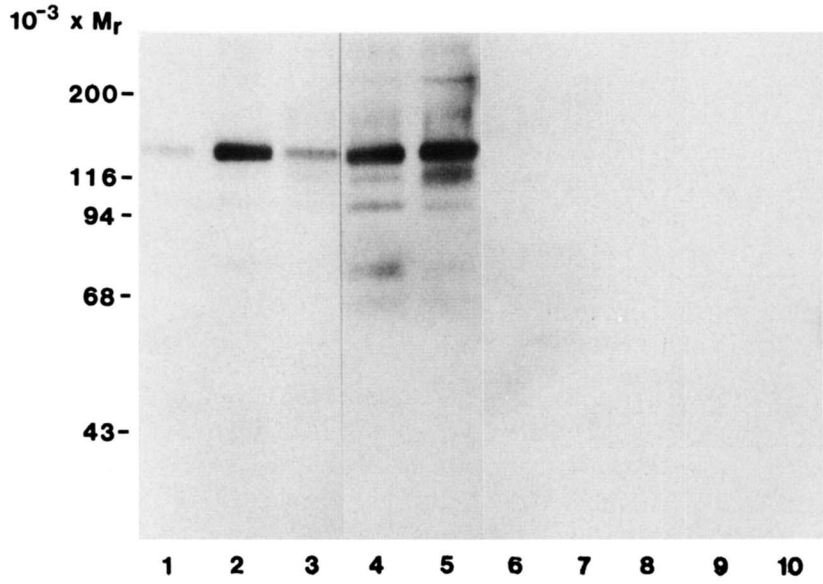

Fig. 2. Ligand blotting with rat ^{125}I-labeled β-VLDL: comparison of lipoprotein binding proteins in liver cell and Golgi membranes from control and 4-day EE-treated rats and Golgi membranes from 1-day EE/colchicine-treated rats. Solubilized cell and Golgi fraction II membrane proteins (43 μg protein/lane) were subjected to electrophoresis and transferred to nitrocellulose paper as described in Materials and Methods. The nitrocellulose was incubated with rat ^{125}I-labeled β-VLDL, 4 × 10^6 cpm, 0.7 μg protein/ml, in the absence (lanes 1–5) or presence (lanes 6–10) of 10 mM EDTA. The following liver membrane proteins were present: lanes 1 and 6, control cell; lanes 2 and 7, 4-day EE cell; lanes 3 and 8, control Golgi; lanes 4 and 9, 4-day EE Golgi; lanes 5 and 10, 1-day EE/colchicine Golgi. Lipoprotein incubations, washing, and autoradiography (4 hr) were performed as described in Materials and Methods.

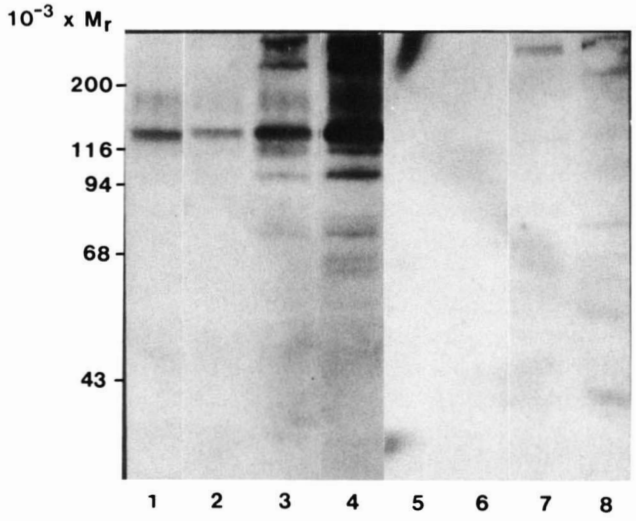

Fig. 3. Ligand blotting of liver Golgi membranes from rats injected with human ^{125}I-labeled LDL. Control rats and rats treated for 4 days with EE were injected with human ^{125}I-labeled LDL 15 min before being killed and liver Golgi fractions I and II were isolated as described in Materials and Methods and the legend for Table I. Solubilized Golgi membrane proteins (43 μg protein/lane) were subjected to electrophoresis and transferred to nitrocellulose paper as described in Materials and Methods. The nitrocellulose was incubated with rat ^{125}I-labeled β-VLDL, 4 × 10^6 cpm, 0.2 μg/ml, in the absence (lanes 1–4) or presence (lanes 5–8) of 10 mM EDTA. The following liver Golgi membrane proteins were present: lanes 1 and 5, control Golgi fraction I; lanes 2 and 6, control Golgi fraction II; lanes 3 and 7, 4-day EE Golgi fraction I; lanes 4 and 8, 4-day EE Golgi fraction II. Lipoprotein incubations, washing, and autoradiography (4 hr) were performed as described in Materials and Methods.

As discussed above, total radioactivity in the Golgi fractions was monitored as a means of assessing trace endocytic contamination of Golgi fractions and was less than 1%. Golgi fraction I contained about one-half the activity of Golgi fraction II. Autoradiograms of membrane fractions from rats injected with human ^{125}I-labeled LDL provided yet another means of evaluating contamination. The ^{125}I-labeled apoB-100 of the injected human LDL appeared as the highest M_r band on the autoradiogram (Fig. 3). The density of the band reflected the presence of endocytosed material as well as nonspecific trapping of ^{125}I-labeled LDL in the membrane preparations. The apoB-100 band was plainly visible in both Golgi fractions I and II from EE-treated rats, where EE induction of LDL receptors had increased the endocytosis of injected human ^{125}I-labeled LDL. However, in Golgi fractions from control rats, the same band was only faintly visible and could not be detected from background film density by laser densitometric scanning of the autoradiogram.

In an effort to increase receptor density in Golgi membranes, rats were injected with EE and 24 hr later they were injected with colchicine. Four hours after colchicine injection, the animals were killed and hepatic Golgi fraction II membranes were prepared. Ligand blotting experiments on these membranes using rat ^{125}I-labeled β-VLDL demonstrated an increased intensity and broadening of the band at an apparent M_r of 120,000 compared with Golgi fraction II membranes from rats treated with EE for 4 days (Fig. 2).

Figs. 2 and 3 show that the binding of rat ^{125}I-labeled β-FLDL to Golgi and cell membranes was dependent upon a divalent cation, in that binding was abolished by the presence of EDTA. The binding of human ^{125}I-labeled LDL was also EDTA-sensitive (data not shown). A band of apparent M_r 44,000 that was not EDTA-sensitive was observed inconsistently. Reduction of disulfide bonds by inclusion of 2-mercaptoethanol in the electrophoresis samples abolished binding to all bands (**Fig. 4**). Unfortunately, lipoprotein binding was observed in only a fraction of the width of the control lane (lane 1) because of the diffusion of 2-mercaptoethanol from lane 2 during electrophoresis.

To examine the carbohydrate portion of the lipoprotein-binding proteins, enzymatic deglycosylation was used. When Golgi fraction II membrane proteins were treated with Endo H prior to electrophoresis, there was no change in the apparent M_r of any of the

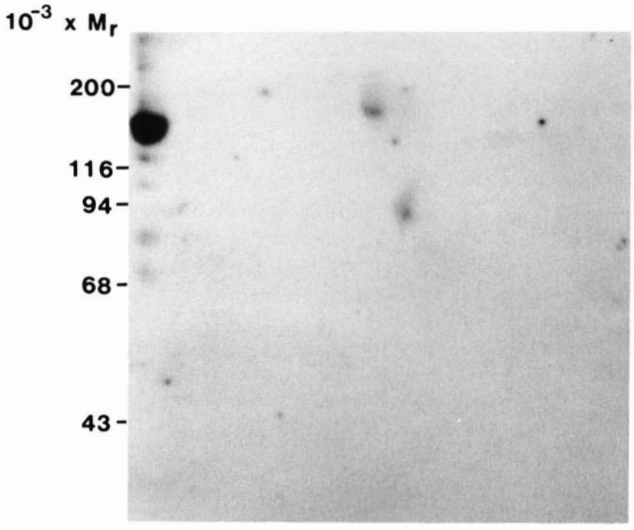

Fig. 4. Ligand blotting of rat liver cell and Golgi membranes: effect of 2-mercaptoethanol. Solubilized cell and Golgi membrane proteins (10–112 μg protein/lane) were subjected to electrophoresis and transferred to nitrocellulose paper as described in Materials and Methods, with 5% (v/v) 2-mercaptoethanol included in the electrophoresis sample buffer in all except the first lane. The nitrocellulose was incubated with rat ^{125}I-labeled β-VLDL, 4 × 10^6 cpm, 0.2 mg protein/ml. The first lane contained 112 μg of liver Golgi membrane proteins from 4-day EE-treated rats and the remaining lanes contained the same cell and Golgi membrane samples from control and cycloheximide-treated rats described in Fig. 6, lanes 1–6. Lipoprotein incubations, washing, and autoradiography (6 hr) were performed as described in Materials and Methods.

bands detected by ligand blotting (**Fig. 5**). Neuraminidase treatment of Golgi membrane proteins caused a decrease in the apparent M_r of the major lipoprotein binding protein from 130,000 to 100,000. The apparent M_r of the protein at 120,000 also decreased to apparent M_r 100,000 and a wide band suggesting the presence of partial desialylation products appeared from 130,000 to 120,000 (Fig. 5). No changes were noted in the apparent M_r 100,000, 80,000, and 70,000 proteins, indicating their lack of sialic acid. The 160,000 and 200,000 proteins were not visible in these experiments, leaving their carbohydrate nature in question.

The effect of cycloheximide treatment of rats on the expression of lipoprotein binding proteins in Golgi fraction I and II and cell membranes was also explored.

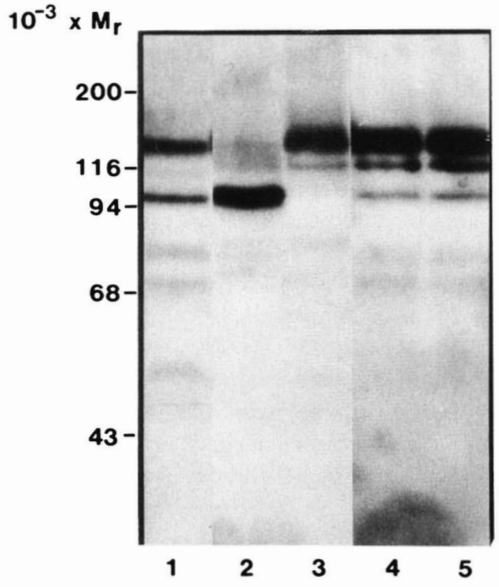

Fig. 5. Ligand blotting of Golgi membrane proteins: effect of pretreatment with neuraminidase and endoglycosidase H. Solubilized Golgi fraction II membrane proteins from 4-day EE-treated rats were precipitated and incubated with enzymes as described in Materials and Methods. Neuraminidase, 0.2 U in 50 μl of 0.1 M sodium acetate buffer, pH 5.5, 1 mM phenylmethylsulfonyl fluoride, was incubated with 100 μg of protein for 2 hr at 37°C and 16 hr at 4°C. Endo H, 5 mU, in 50 μl of 0.1 M sodium citrate buffer, pH 6.0, 0.1% SDS, was incubated with 100 μg of protein for 18 hr at 37°C. The controls shown were incubated identically except that enzyme was replaced with an equal volume of buffer. Following enzyme incubation the samples were adjusted to 0.5% SDS, 0.001% bromphenol blue, and 13% sucrose in 80 μl, subjected to electrophoresis, and transferred to nitrocellulose paper as described in Materials and Methods. The nitrocellulose was incubated with rat ^{125}I-labeled β-VLDL, 4 × 10^6 cpm, 0.3 μg protein/ml. The 4-day EE Golgi fraction II membrane samples are as follows: lane 1, octylglucoside solubilized, no precipitation or enzyme treatment; lane 2, neuraminidase-treated; lane 3, incubated as lane 2 but in absence of neuraminidase; lane 4, Endo H-treated; lane 5, incubated as lane 4 but in absence of Endo H. Lipoprotein incubations, washing, and autoradiography (6 hr) were performed as described in Materials and Methods.

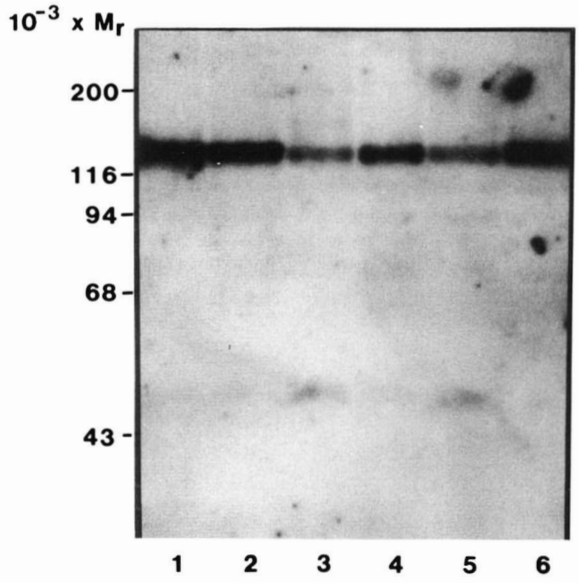

Fig. 6. Ligand blotting of liver cell and Golgi membranes from control rats and rats treated with cycloheximide. Animals were given cycloheximide (20 mg/kg body wt), killed 2 hr later, and liver cell and Golgi fraction I and II membranes were prepared as in Materials and Methods. Solubilized membrane proteins were subjected to electrophoresis and transferred to nitrocellulose paper. The nitrocellulose was incubated with rat ^{125}I-labeled β-VLDL, 4 × 10^6 cpm, 0.2 μg protein/ml. The following liver membrane proteins were present: lane 1, control cell, 50 μg; lane 2, cycloheximide cell, 50 μg; lane 3, control Golgi I, 10 μg; lane 4, cycloheximide Golgi fraction I, 10 μg; lane 5, control Golgi fraction II, 25 μg; lane 6, cycloheximide Golgi fraction II, 25 μg. Lipoprotein incubations, washing, and autoradiography (10 hr) were performed as described in Materials and Methods.

The dose and time of cycloheximide administration were such that protein synthesis by the rat liver was totally inhibited, but further processing and transport of proteins were not affected (17). As seen in **Fig. 6,** cycloheximide treatment did not decrease the intensity of the major lipoprotein binding bands on the autoradiogram. With longer development the bands at lower apparent M_r were also visible.

The nature of the two proteins of apparent M_r 80,000 and 70,000 that bind rat ^{125}I-labeled β-VLDL was further probed. Ligand blots using human ^{125}I-labeled LDL with only apoB showed binding to only the proteins of apparent M_r 130,000 and 100,000 when developed to the limits of the film background intensity (data not shown). In contrast, when rabbit ^{125}I-labeled LDL that contained no immunologically detectable apoE was used to detect LDL binding sites by ligand blotting, the bands of apparent M_r 80,000 and 70,000 were visible (data not shown).

Immunoblotting

Results of immunoblotting experiments are shown in **Fig. 7**. The 130,000 M_r protein was the most prom-

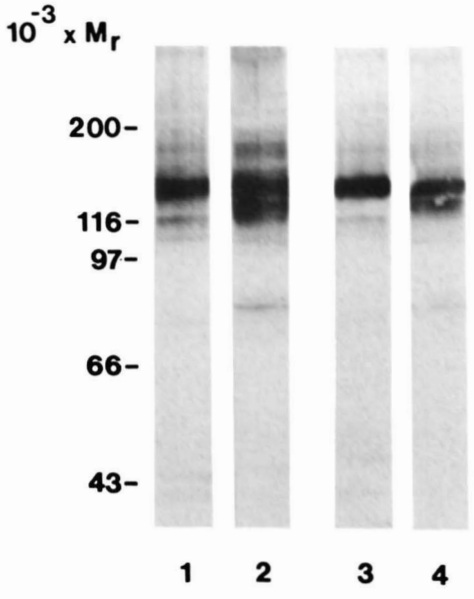

Fig. 7. Immunoblot of Golgi and cell membranes from 4-day EE-treated rats. Golgi and cell membranes were isolated and solubilized in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of 100 μM leupeptin and 500 KIU/ml aprotinin. The samples (56 μg/lane) were subjected to electrophoresis and transferred to nitrocellulose membranes as described in Materials and Methods. The nitrocellulose was incubated with antibody to the LDL receptor (1 μl/ml), and bound antibody was visualized with HRP-conjugated goat anti-rabbit IgG. Lanes 1 and 3, Golgi membranes; lanes 2 and 4, cell membranes.

inent protein recognized in Golgi and cell membranes by the antibody, consistent with its identity as the LDL receptor. The antibody recognized a number of other proteins in Golgi membranes, including the 120,000, 100,000, and 80,000 M_r proteins. Very faint bands were observed in the 200,000 and 160,000 M_r region of the blots. Isolation and solubilization of Golgi and cell membranes in the presence of protease inhibitors did not eliminate any of the proteins recognized by the antibody in the Golgi membranes but did eliminate a protein of M_r 75,000 observed in cell membrane fractions. However, samples prepared in the presence of protease inhibitors appeared to give sharper bands on the immunoblots compared with samples prepared without the inhibitors.

DISCUSSION

The mature LDL receptor identified in these studies in rat liver Golgi membranes as a 130,000 M_r protein exhibited the binding characteristics typical for the plasma membrane LDL receptor (6, 13, 15, 30, 31). First, its affinity for lipoproteins containing apoE, such as rat and rabbit β-VLDL, was much greater than its affinity for lipoproteins containing only apoB, such as human and rabbit LDL (Fig. 1 and data not shown). Second, lipoprotein binding was abolished in the presence of EDTA and when 2-mercaptoethanol was included in the electrophoresis sample buffer (Figs. 2–4). Third, the β-VLDL binding was increased in Golgi membranes from livers of rats treated with EE compared with those from untreated animals (Fig. 2). Finally, the receptor was resistant to Endo H digestion and sensitive to neuraminidase treatment. This is consistent with the carbohydrate nature of the mature LDL receptor. Further evidence confirming the identity of the LDL receptor in Golgi membranes was provided by immunoblotting experiments. Cooper, Nutik, and Chen (8) have prepared and characterized monoclonal and polyclonal antibodies to the LDL receptor from livers of EE-treated rats. The 130,000 M_r protein in our Golgi membranes was the most prominent protein recognized by the polyclonal antibody of Cooper et al. (8).

The lipoprotein-binding proteins at M_r 100,000 and 120,000 correspond to those generated when the mature receptor was treated with neuraminidase (Fig. 5). Both proteins were resistant to Endo H digestion, consistent with their presence in the Golgi apparatus membranes and processing of their N-linked oligosaccharides as far as the $GlcNAcMan_3GlcNAc_2$ form. The M_r 120,000 protein accumulated in Golgi membranes from rats treated with both EE and colchicine (Fig. 2, lane 5). Both the 120,000 and 100,000 proteins were recognized by the antibody to the LDL receptor (Fig. 7). These data suggest that these proteins represent precursors to the mature form of the receptor. It is possible that they could be either newly synthesized receptors, which have not been completely sialylated, or recycling receptors, which have lost sialic acid during endocytosis and are being refurbished by the Golgi apparatus.

The lipoprotein-binding proteins of apparent M_r 80,000 and 70,000 represent either proteolytic fragments of the mature LDL receptor or totally different lipoprotein receptors. The apparent M_r of these proteins was less than the protein mass of the human adrenal LDL receptor (M_r 93,102) (1). Antibody to the LDL receptor recognized the 80,000 M_r protein in Golgi membranes, suggesting that this protein is a fragment of the mature receptor. Furthermore, isolation and solubilization of Golgi membranes in the presence of protease inhibitors did not eliminate the 80,000 M_r protein, but did appear to reduce its relative staining intensity. A proteolytic product of the purified bovine adrenal LDL receptor with an apparent M_r of 80,000 has been reported (32). The fact that the 70,000 M_r protein was not detected on immunoblots in the Golgi membranes suggests that it is unrelated

to the mature receptor. On the other hand, it may not have been present in sufficient amounts to be detected, or it may be a fragment of the receptor which does not contain the antibody recognition site. Neither the 80,000 nor the 70,000 M_r protein was observed in cell membranes on the immunoblots, which may be related to the amount of protein present on the blots. We do not believe the 80,000 and 70,000 proteins are related to the apoE receptor, reported to have an M_r of 56,000 in dog and human liver membranes (33), because of differences in EE induction (9), octylglucoside solubility, and ability to bind rabbit LDL containing no detectable apoE. Further studies will be needed to identify these proteins.

The higher apparent M_r binding proteins of 200,000 and 160,000 were similar to several other lipoprotein-binding proteins reported in the literature (27, 34). However, they were barely visible in the immunoblots. Whether they represent aggregates of the receptor or abnormal forms of the receptor remains to be determined.

Several lines of evidence strongly suggest that our Golgi membrane fractions were not significantly contaminated with membrane material from endosomes, multivesicular bodies (MBVs), plasma membranes, or endoplasmic reticulum, and that the lipoprotein-binding proteins we report were indeed on the Golgi apparatus membranes. First, the Golgi fractions were highly enriched in galactosyltransferase. Further purification by modifications in centrifugation force and/or Ficoll gradient increased the enrichment in galactosyltransferase. However, such steps did not reduce the relative amounts or number of lipoprotein-binding protein bands observed on ligand blots. Second, we did not observe by electron microscopic (EM) techniques contaminating organelles or MVB-like structures in the Golgi fractions from control rats. We did see evidence of MVB-like structures in Golgi fraction II from EE-treated rats, which have markedly increased endocytosis. However, these structures were not observed in Golgi fraction I from EE rats and the Golgi Ficoll fraction from EE-treated rats. Thus, in the EE-induced state, when endocytosis was maximal and the potential for endocytic contamination of the Golgi preparation was great, Golgi fractions with no observable endocytic contamination were prepared. The MVB-like structures that were identified in less pure fractions appeared to be markedly reduced by our final purification steps. However, we again emphasize that this further purification did not change the binding proteins observed. In the control animals, where initial endocytosis by LDL receptors is presumably three- to tenfold lower than with EE treatment (15), the potential for contamination is even less. Third, injection of ^{125}I-labeled LDL to mark the endocytic compartment revealed little or no contamination by two different monitoring parameters. No ^{125}I-labeled apoB-100 was detected in control Golgi fractions by laser densitometric scanning of autoradiograms although the band was very faintly visible. Further, the total radioactivity recovered in the Golgi fractions was much less than 1% and much less than that reported by Hornick et al. (12). It is doubtful that all of the ^{125}I-labeled LDL in the fractions was truly in endosomes or MVBs, as these structures were not visible on EM in control Golgi I and II as discussed above. Particles of ^{125}I-labeled LDL may be nonspecifically trapped in Golgi fractions during the isolation procedure. As discussed elsewhere by Hamilton et al. (29), dilution and gentle centrifugation free MVBs from the intact Golgi apparatus and eliminate much of the 1.6% ^{125}I recovery in Golgi from EE-treated rats which they consider "appreciable" (12) or "significant" (29). Fourth, lipoprotein particles released from the Golgi fractions have been found to have the composition characteristic of nascent Golgi lipoproteins, not of endocytosed plasma lipoproteins (11, 35). Finally, the absence of any Endo H-sensitive lipoprotein-binding proteins (Fig. 5) supported the absence of endoplasmic reticulum in Golgi fraction II. It is our opinion that contamination was not sufficient to account for the binding activity demonstrated in these fractions even if endocytic membranes were greatly enriched in receptors.

Identification of lipoprotein receptors in the Golgi apparatus membranes confirmed their presence in an organelle through which they are believed to pass during the processing of a cell surface receptor. The presence of N- and O-linked oligosaccharides on the LDL receptor, an integral membrane glycoprotein (1, 2), and the role of the Golgi apparatus in modifying and attaching these sugar chains are both well established. In addition to processing newly synthesized receptors, the Golgi apparatus may also participate as one site in the recycling pathway of some or all endocytosed cell surface lipoprotein receptors. LDL receptor recycling has been studied indirectly in fibroblasts (36, 37), and although the degradation pathway of labeled LDL has been followed (14, 38, 39), the exact recycling route of the receptor itself is unknown. A pathway through the Golgi apparatus for "reprocessing" (4) or "purifying" (5) of the receptor has been mentioned. In our studies, the amount of LDL receptor (M_r 130,000) detected in Golgi membranes by ligand blotting was not decreased in membranes from cycloheximide-treated rats relative to controls (Fig. 6) suggesting that LDL receptors may recycle through the Golgi apparatus of rat hepatocytes.

The participation of the Golgi apparatus in all phases

of the life cycle of the LDL receptor continues to suggest its importance in regulation of liver LDL receptor expression, and therefore, in the regulation of plasma lipoprotein levels. Further investigation of lipoprotein receptors in the Golgi apparatus and other subcellular compartments promises to enhance our understanding of the basic cell biology of receptor transport and regulation.

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