papers on methodology

Measurement of total hepatic low density lipoprotein receptor levels in the hamster[1]

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Abstract The ability to measure the total concentration of low density lipoprotein (LDL) receptors in hepatic tissues is of crucial importance to understanding changes in hepatic cholesterol metabolism. Such measurements can be made in conjunction with estimates of LDL receptor transcriptional activity, cell surface LDL receptor number, and rates of hepatic LDL uptake to evaluate the mechanisms controlling cellular LDL receptor expression. Current methods for assessing hepatic LDL receptor levels use microsomes as a source of LDL receptor, and thus rely on consistent contamination of the microsomal preparation with LDL receptor-containing plasma membranes, endocytic vesicles, and/or secretory vesicles. Because this contamination is variable, and may vary with alterations in either the distribution of LDL receptors among the various cellular membrane fractions or in the composition of the intracellular membranes, measurement of LDL receptor concentration in microsomal fractions may not accurately reflect the total compliment of LDL receptors within the cell. We have developed the methodology for isolating the full complement of hepatic LDL receptor containing membranes by discontinuous sucrose density gradient centrifugation, and for quantitating LDL receptor concentration using a Western immunoblotting procedure that uses an anti-C-terminal LDL receptor peptide polyclonal antiserum and assesses the intensity of color formation by reflectance densitometry. Using this methodology, we observed a 126 kDa immunoreactive band for the bovine adrenal cortex LDL receptor that also exhibited LDL binding activity as visualized by biotinylated LDL-ligand blotting, and a doublet of 140 kDa for the hamster liver LDL receptor. These bands were not observed when ligand blotting was conducted in the presence of either 10 mM EDTA or a 5-fold excess of unlabeled LDL, or when immunoblotting was conducted using either preimmune serum or antiserum that had been preabsorbed with LDL receptor peptide. The intensity of color formation was a linear function of the amount of membrane extract separated by electrophoresis. Intra-assay variation averaged 7%, and inter-animal variation averaged 20%. Cholestyramine, tiqueside, CP-88488, 17α-ethinyl estradiol, mevinolin, and the combination of cholestyramine plus mevinolin, pharmacological interventions known to increase LDL receptor activity in experimental animals, produced the predicted increases in hamster total hepatic LDL receptor concentration that were highly correlated with concomitant increases in HMG-CoA reductase activity and reductions in serum cholesterol. ■ This method thus represents a reliable method for measuring the full compliment of LDL receptors in the liver in both the steady state and after pharmacological intervention and should be useful in combination with measurements of LDL receptor transcriptional activity and hepatic LDL uptake to further understand the mechanisms regulating LDL receptor expression in the liver.—**Cosgrove, P. G., B. J. Gaynor, and H. J. Harwood, Jr.** Measurement of total hepatic low density lipoprotein receptor levels in the hamster. *J. Lipid Res.* 1993. **34:** 1983–2003.

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Receptors that bind plasma low density lipoprotein (LDL) are found on the surface of most animal cells (1). These receptors play an important role in the metabolism of cholesterol in humans and animals by binding LDL and facilitating its cellular uptake by receptor-mediated endocytosis and its degradation in lysosomes (1). This process supplies cholesterol to cells for diverse purposes including membrane synthesis (2), steroid hormone formation in the adrenal cortex (3–5), ovaries (5, 6), and testes (6), and bile acid production (7) and lipoprotein synthesis (8) in the liver.

In each tissue, the number of LDL receptors can be regulated both by unique mechanisms that occur in specialized cells (3–6) and by common mechanisms that occur in most cells, such as rates of cell growth and variations in intracellular demands for cholesterol (9, 10). Thus, the adrenal cortex and the liver, which have greater requirements for cholesterol than do other body tissues, express much larger numbers of LDL receptors (11). The adrenal gland has the greatest concentration of LDL receptors per mass of tissue (3, 11, 12), whereas, the liver expresses the largest total number of receptors per organ

Abbreviations: LDL, low density lipoprotein; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

[1]Portions of these studies were presented at the 1990 Annual Meeting of the Federation of American Societies for Experimental Biology (83).

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(11–14). In addition to normal physiological changes in LDL receptor transcription and cell surface activity, LDL receptor expression can also be modulated by dietary manipulations (15–18) and through pharmacologic interventions using agents such as the bile acid sequestrants, cholestyramine (12, 19, 20) and CP-88488 (21–23), the cholesterol synthesis inhibitors, mevinolin (24) and ketoconazole (25, 26), the cholesterol absorption inhibitor, tiqueside (27), the calcium channel blockers, verapamil (28, 29), amlodipine (29, 30), and SIM 6080 (31), and other agents such as doxazosin (32), oncostatin M (33, 34), and 17α-ethinyl estradiol (35–37), all of which induce perturbations in cholesterol homeostasis in vivo.

Based on many observations of the uptake and clearance of LDL in cultured cells, experimental animals, and humans, it is generally believed that the majority of plasma LDL catabolism occurs by LDL receptor-mediated uptake in the liver (13, 14, 38). Indeed such studies in several species, including rats, hamsters, guinea pigs, mice, and rabbits suggest that the liver removes at least two-thirds of the LDL from the circulation daily and that the majority of this LDL is removed via LDL receptors (1, 9, 16, 39). Several lines of evidence also suggest that the activity of the LDL receptor in liver is important in controlling the level of circulating LDL (1, 9, 12). However, despite its implied importance in maintaining plasma LDL cholesterol concentration, much information regarding the factors and mechanisms that regulate or determine LDL receptor dependent uptake in the liver in vivo remain unknown. Thus, in order to better understand the regulatory mechanisms at play in controlling LDL receptor expression in vivo, it is important to evaluate not only modulations of LDL receptor transcription and cell surface LDL receptor activity, but also of other factors, such as total intracellular LDL receptor pool size, that may influence intracellular pool distribution and trafficking of functional LDL receptors within the intracellular compartment and to the plasma membrane.

A major difficulty in studying regulation of LDL receptor expression in the liver has been the lack of suitable methods for directly assessing total hepatic LDL receptor concentration. While measurement of LDL turnover (13, 14, 17, 36), LDL receptor mRNA concentration (15, 24, 35, 40), and rates of cell surface LDL binding and uptake (25, 28, 32–34, 41), have been effectively used to estimate rates of LDL receptor transcriptional and cell surface activities in vivo and in cultured cells, these methods do not estimate the total content of cellular LDL receptors. For example, in vivo LDL turnover studies and cell surface binding and uptake studies in isolated or cultured hepatocytes measure only the activity of the cell surface LDL receptor pool and thus do not take into account differences in the proportion of the total LDL receptor pool that is contained within the cell either in the process of migrating/recycling to the cell surface or within a regulatory pool and available in reserve for recruitment to the cell surface. In addition, observations made by several laboratories, suggesting the existence of post-translational regulation of LDL receptor gene expression (42–47), also question the validity of equating modulation in LDL receptor mRNA levels with changes in total cellular LDL receptor concentration. An accurate method for assessing the total intracellular concentration of LDL receptors is thus of crucial importance to relating transcriptional rates to cell surface LDL receptor concentrations and to assessing the role of intracellular trafficking and pool size to the emergence of functional LDL receptors at the cell surface.

Several methods are currently available to directly examine LDL receptor concentration in isolated hepatic membranes. These methods include measurement of saturable or EDTA-sensitive binding of ^{125}I-labeled LDL (48) or ^{111}In-labeled LDL (49) to isolated membranes, ligand blotting analysis of isolated membrane proteins after polyacrylamide gel electrophoresis using biotin-modified or ^{125}I-labeled lipoproteins (50–54), and immunoblotting (55–58) and ELISA (59) methodologies for analysis of isolated membrane proteins following polyacrylamide gel electrophoresis using specific anti-LDL receptor antisera. Although a useful technique in nonhepatic tissues, binding of ^{125}I-labeled LDL to isolated hepatic membranes is not well suited to measurement of hepatic LDL receptor content due to a high level of nonspecific binding of radiolabeled LDL to hepatic membranes (56), resulting in a signal to noise ratio of less than two (60, 61). In addition, although immunoblotting, ELISA, and ligand blotting analyses represent reliable methods for measuring LDL receptors in isolated membranes, all of these methods currently use microsomal membranes as a source of LDL receptors, and thus rely on a consistent contamination of the microsomal preparation with LDL receptor-containing plasma membranes, endocytotic vesicles and/or secretory vesicles. Because this contamination is variable, and may vary with alterations in either the distribution of LDL receptors among the various cellular membrane fractions or in the composition of the intracellular membranes, and because microsomal preparations contains only a portion of the LDL receptor-containing membranes, these methods do not accurately reflect the total content of LDL receptors within the cell.

In this report we describe the development of a method for isolating the entire complement of hepatic LDL receptor-containing membranes in a single fraction using sucrose gradients, and the visualization of these receptors, after Triton X-100 extraction, using a Western blotting enzyme immunoassay procedure that uses an anti-bovine LDL receptor C-terminal peptide antiserum. This method exhibits an inter-assay variability of approximately 7% and an inter-animal variability of approximately 20% and is capable of assessing increases in LDL receptor levels produced through a number of pharmacological

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manipulations known to derepress LDL receptor transcription. This method should be useful in combination with measurements of LDL transcriptional activity and hepatic LDL uptake to further our understanding of the mechanisms regulating LDL receptor expression in the liver.

MATERIALS AND METHODS

Chemicals

Leupeptin, TPCK (tosylamide-2-phenylethylchloromethyl ketone), TLCK (N-α-*p*-tosyl-L-lysine chloromethyl ketone), iodoacetamide, PMSF (phenylmethylsulfonyl fluoride), EDTA, EGTA, 17α-ethinyl estradiol, keyhole limpet hemocyanin, hydrogen peroxide, glucose-6-phosphate dehydrogenase, NaBr, $NaIO_4$, biotin hydrazide, sodium cyanoborohydride, and octylglucoside were from Sigma Chemical Co. (St. Louis, MO). Gelatin, Tween-20, 4-chloro-1-naphthol, and electrophoresis grade Tris, were from Bio-Rad Laboratories (Richmond, CA). $NADP^+$, glucose-6-phosphate, and dithiothreitol were from U.S. Biochemicals (Cleveland, OH). Suramin was from Mobay Chemical Co. (New York, NY). [3-^{14}C]HMG-CoA (57 mCi/mmol) and [5-^{3}H]mevalonolactone (24 Ci/mmol) were from New England Nuclear (Boston, MA). Goat anti-rabbit IgG-horseradish peroxidase conjugate (cat #AP132P) and anti-apolipoprotein A-I antiserum were from Chemicon (El Segundo, CA). Prestained molecular weight standards were from Bethesda Research Laboratories (Gaithersburg, MD). Precast 4–20% gradient polyacrylamide minigels were from Integrated Separations Systems (Hyde Park, MA). A streptavidin-horseradish peroxidase conjugate (cat #RPN 1231) was from Amersham Corp. (Arlington Heights, IL). Protein A-Sepharose, DEAE-cellulose, and CNBr-activated Sepharose 4B were from Pharmacia (Piscataway, NJ). BA-85 nitrocellulose sheets were from Schleicher and Schuell (Keene, NH). Cholestyramine resin (Questran) was from Mead Johnson & Co. (Evansville, IN). Mevinolin was a gift from Dr. Alfred W. Alberts (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ). Tiqueside and CP-88488 were from Pfizer Central Research (Groton, CT). A premixed, 37.5:1 (w/w), liquid preparation of acrylamide and bisacrylamide was obtained from Amersco (Solon, OH). A 15-amino acid peptide, corresponding to the sequence of the C-terminal tail of the bovine LDL receptor (Fig. 1) was prepared through custom synthesis by Cambridge Research Biochemicals Ltd. (Button End, Harston Cambridge CB2 5NX, England).

Buffers and solutions

LDL receptor Homogenization Buffer contained 20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 5 mM EDTA, 5 mM EGTA, 0.1 mM iodoacetamide, 1 mM PMSF, 0.3 mM leupeptin, 0.3 mM TPCK (added from a 0.5 M stock solution in DMSO), and 0.3 mM TLCK (added from a 0.5 M stock solution in DMSO). Solubilization Buffer I contained 250 mM Tris maleate (pH 6.0), 2 mM $CaCl_2$, 10 mM EDTA, 10 mM EGTA, 0.2 mM iodoacetamide, 1 mM PMSF, 0.6 mM leupeptin, 0.6 mM TPCK, and 0.6 mM TLCK. Solubilization Buffer II contained 2 mM $CaCl_2$, 0.32 M NaCl, and 1.5% (v/v) Triton X-100. Sucrose Buffer contained 20 mM Tris (pH 8.0), 1 mM $CaCl_2$, 0.1 mM iodoacetamide, 1 mM PMSF, 0.3 mM leupeptin, 0.3 mM TPCK, 0.3 mM TLCK, and the desired percentage (w/v) of sucrose. Tris-Buffered Saline (TBS) contained 20 mM Tris (pH 7.5) and 500 mM NaCl. LDL receptor Microsomal Isolation Buffer contained 20 mM Tris (pH 8.0), 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM PMSF. TEDK Buffer contained 50 mM Tris (pH 7.5), 1 mM EDTA, 5 mM dithiothreitol, and 50 mM KCl. Electrophoresis Sample Buffer contained 320 mM Tris (pH 6.8), 8% (w/v) SDS, and 0.8 M sucrose. Ligand Blotting Buffer I contained 50 mM Tris (pH 8.0), 2 mM $CaCl_2$, 90 mM NaCl and 5% BSA. Ligand Blotting Buffer II contained 50 mM Tris (pH 8.0), 2 mM $CaCl_2$, 90 mM NaCl and 0.5% BSA. DEAE-cellulose Equilibration Buffer contained 50 mM Tris maleate (pH 6.0), 2.0 mM $CaCl_2$, and 1% (v/v) Triton X-100. DEAE-cellulose Elution Buffer contained 50 mM Tris maleate (pH 6.0), 2.0 mM $CaCl_2$, and 40 mM octylglucoside. LDL-Sepharose Equilibration Buffer contained 25 mM Tris (pH 7.6), 50 mM NaCl, and 1 mM EDTA. LDL-Sepharose Elution Buffer contained 50 mM Tris (pH 6.0) and 3.5 mM suramin.

Preparation and biotinylation of LDL

Human LDL was isolated by sequential flotation essentially as described by Havel, Eder, and Bragdon (62). Briefly, plasma isolated from venous human blood that was obtained after a 14-h fast, was adjusted to 1.022 g/ml by addition of solid NaBr and centrifuged at 250,000 *g* for 16 h at 4°C. After centrifugation, the floating VLDL-containing lipid fraction was removed and the lower fraction was adjusted to 1.065 g/ml by addition of solid NaBr and recentrifuged as described above. The floating LDL-containing lipid fraction was removed, readjusted to the original plasma volume at 1.063 g/ml, and centrifuged at 150,000 *g* for 18 h at 4°C. After centrifugation the LDL-containing lipid fraction was removed, dialyzed against PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and stored at 4°C until needed.

Biotinylated-LDL was prepared by the method of Wade, Knight, and Soutar (50). Briefly, LDL isolated as described above, was first oxidized by incubation with 4 mM $NaIO_4$ for 30 min at 0°C. The resulting oxidized LDL was passed over a Sephadex G-25 column to remove unreacted $NaIO_4$ and then incubated with 2.5 mg biotin

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hydrazide per mg oxidized LDL for 30 min at room temperature. After incubation, sodium cyanoborohydride was added to the incubation mixture to a final concentration of 1.0 mM and the mixture was incubated at 4°C overnight. After incubation, the mixture was dialyzed against PBS, filtered sterilely at 4°C, and stored at 4°C until use.

Preparation of LDL-Sepharose 4B

LDL-Sepharose 4B was prepared from CNBr-activated Sepharose 4B according to manufacturer's instructions as described by Schneider et al. (63) using 30 mg LDL, isolated as described above, per gram of dried CNBr-activated Sepharose 4B. The resulting LDL-Sepharose 4B was stored at 4°C in LDL-Sepharose Equilibration Buffer containing 0.2% (w/v) NaN_3 and was used within 2 weeks of preparation.

Production of antisera

Polyclonal, monospecific antiserum prepared against the 15-amino acid peptide corresponding to the C-terminal sequence of the bovine LDL receptor (Fig. 1) was prepared through custom synthesis by Cambridge Research Biochemicals Ltd. (Button End, Harston Cambridge CB2 5NX, England) as previously described (64). Briefly, the C-terminal LDL receptor peptide (Cys-Tyr-Pro-Ser-Arg-Gln-Met-Val-Ser-Leu-Glu-Asp-Asp-Val-Ala) was conjugated to keyhole limpet hemocyanin using *m*-maleimidobenzoic acid N-hydroxylsuccinimide ester as a heterobifunctional crosslinking agent to produce an N-terminally bound peptide conjugate as described by Green et al. (65). Five female New Zealand White/Sandy Half Lop rabbits, weighing between 1.5 kg and 2.0 kg, were immunized by subcutaneous injections at multiple sites in their hindquarters with 100 μg of the peptide–hemocyanin conjugate in either Freund's complete adjuvant (injection 1) or Freund's incomplete adjuvant (injections 2–7) every 2 weeks for 16 weeks. Blood samples were collected prior to the start of the immunization schedule and 7 days after the second through seventh injections. Serum was separated and samples were stored at −20°C. All serum samples were assayed for the presence of antibodies against the LDL receptor peptide by ELISA using unimmunized rabbit sera and LDL receptor peptide absorbed antisera as controls. The immunoglobulins contained in serum samples possessing antibodies to the LDL receptor peptide were partially purified by caprylic acid precipitation and molecular exclusion chromatography and were stored at −80°C.

Animals

Male Sprague-Dawley rats (100–150 g) and male golden Syrian hamsters (100–120 g) were housed in reversed lighting cycle rooms and were administered Agway Prolab RMH 3200 laboratory chow and water ad libitum for 10 days prior to use. Cholestyramine, mevinolin, tiqueside, and CP-88488 were administered as dietary supplements in powdered chow at the indicated doses for up to 7 days. 17α-Ethinyl estradiol was administered by daily subcutaneous injection for up to 10 days. Control animals received either ground chow that contained no additions or subcutaneous injections with vehicle. Control and treatment groups contained a minimum of four animals each (typically six animals per group). At the time of sacrifice, animals were anesthetized with pentobarbitol and 1.5 g of liver tissue was removed and used as a source of hepatic LDL receptor-containing membranes. In selected experiments, at sacrifice, an additional 0.5 g of liver tissue was removed for measurement of hepatic HMG-CoA reductase activity, and plasma samples were obtained by heart puncture for determination of plasma cholesterol levels.

Bovine adrenal cortex tissues were obtained from the Prime Cuts slaughter house (Salem, CT). Tissue samples were removed within 30 min of sacrifice and stored at 4°C during transport prior to homogenization. Bovine adrenal samples were generally homogenized within 2 h of extraction.

Preparation of adrenal cortex membranes

Bovine adrenal tissues were sliced longitudinally and the medulla was gently removed. The cortex was then scraped from the musculature, placed in LDL receptor Microsomal Isolation Buffer (5 ml/g cortex tissue) and homogenized at 4°C with two 20-sec pulses of a Polytron homogenizer (setting 5). The homogenate was first centrifuged at 4°C for 10 min at 900 *g* and the resulting supernatant was centrifuged at 100,000 *g* for 1 h at 4°C. After centrifugation, the supernatant liquid was discarded and the membrane pellet was stored frozen in liquid nitrogen without resuspension for subsequent LDL receptor solubilization.

Preparation of purified fractions of bovine adrenal cortex LDL receptor

Purified preparations of the bovine adrenal cortex LDL receptor were prepared from membrane extracts by sequential DEAE-cellulose chromatography and LDL-Sepharose 4B chromatography as described by Schneider et al. (63). Briefly, bovine adrenal cortex membranes, isolated as described above, were extracted as described below using Solubilization Buffer that contained PMSF as the only protease inhibitor. The extract was applied to a 1 cm × 10 cm DEAE cellulose column equilibrated with DEAE-cellulose Equilibration Buffer. The column was washed first with 50 ml DEAE-cellulose Equilibration Buffer and then with 75 ml of DEAE-cellulose Elution Buffer. The column was eluted with an 80-ml salt gradient of from 0 mM to 200 mM NaCl in DEAE-cellulose Elu-

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tion Buffer and peak fractions were pooled. Aliquots of the pool (DEAE-cellulose fraction) were removed and stored in liquid nitrogen. A 10-ml aliquot of the DEAE-cellulose fraction was dialyzed against DEAE-cellulose Elution Buffer that lacked octylglucoside, adjusted to pH 8.0, centrifuged at 100,000 *g* for 60 min at 4°C to remove any precipitate, and applied to an LDL-Sepharose 4B column, prepared as described above, that was equilibrated with LDL-Sepharose Equilibration Buffer. The eluate from the column was recycled over the column five times and then proteins bound to the column were eluted with LDL-Sepharose Elution Buffer. Peak fractions were pooled and stored in liquid nitrogen as the LDL-Sepharose fraction.

Preparation of hepatic microsomes

For qualitative assessment of hepatic LDL receptor levels, liver tissue, isolated as described above, was rinsed with ice-cold LDL receptor Microsomal Isolation Buffer, homogenized in the same buffer (5 ml/g liver tissue) with a Polytron homogenizer (setting 5, 30 sec),[3] and centrifuged at 500 *g* for 15 min to remove cellular debris. The homogenate was first centrifuged at 10,000 *g* for 15 min at 4°C and the resulting supernatant was centrifuged at 100,000 *g* for 60 min at 4°C. After centrifugation, the supernatant liquid was discarded and the resulting microsomal pellet was frozen in liquid nitrogen without resuspension.

For measurement of HMG-CoA reductase activity, liver tissue, isolated as described above, was washed once in 4°C saline and immediately homogenized in TEDK buffer (1 ml TEDK buffer per gram liver tissue) using 15 strokes of a Dounce tissue homogenizer. The homogenate was first centrifuged at 10,000 *g* for 15 min at 4°C and the resulting supernatant was subsequently centrifuged at 100,000 *g* for 90 min at 4°C. After centrifugation, the supernatant liquid was discarded and the microsomal pellet was resuspended in 1.0 ml TEDK per gram liver and stored frozen in liquid nitrogen.

Isolation of hepatic LDL receptor-containing membranes by sucrose density gradient centrifugation

For quantitation of hepatic LDL receptor protein concentration (see Fig. 2), liver tissue, isolated as described above, was homogenized in LDL receptor Homogenization Buffer (1 ml/g liver) at 4°C with a Polytron homogenizer (setting 5, 30 sec) and the homogenate was then centrifuged at 500 *g* for 5 min at 4°C to remove cellular debris. After centrifugation, aliquots containing 6 ml of the resulting supernatant were layered onto an equal volume of 30% Sucrose Buffer and were centrifuged at 4°C in a Beckman SW40 rotor[4] for 17 h at 35000 *g*. For each 6-ml aliquot separated, the membrane fraction migrating to the homogenate/sucrose interface (approx. 1.5 ml in volume), which contained the full complement of LDL receptor-containing membranes (see Results), was removed by gentle aspiration, diluted to 3.5 ml with LDL Receptor Homogenization Buffer, and centrifuged at 200,000 *g* for 1 h at 4°C. After centrifugation, the supernatant liquid was discarded and the membrane pellet was immediately progressed, without freezing, through the extraction procedure described below.

Extraction of LDL receptor protein from membrane fractions

Microsomal pellets or membrane pellets isolated after sucrose density gradient centrifugation were resuspended in 1 ml or 400 μl of Solubilization Buffer I, respectively, with gentle vortexing. Suspensions were then drawn 3 times through a 20-gauge needle and 3 times through a 25-gauge needle. For extraction of the adrenal cortex LDL receptor, membrane suspensions were also sonicated two times at 4°C for 20 sec each using a Branson Probe Sonifier (setting 6). This step was not used for hepatic membrane suspensions as with this tissue, sonication was not necessary for achieving adequate LDL receptor solubilization. Following the above procedure, resulting suspensions were diluted with an equal volume of Solubilization Buffer II, vortexed, mixed by rotation for 10 min at 4°C, and centrifuged at 100,000 *g* at 4°C for 1 h. After centrifugation, the resulting supernatants, which contained the extracted protein and averaged 18 mg extracted protein/ml, were retained as the solubilized extract and stored frozen in liquid nitrogen until use. No detectable levels of LDL receptor remained in the pellet (see Results).

Measurement of LDL receptor concentration in membrane extracts by Western immunoblot analysis

Portions of the soluble extracts prepared as described above, that contained between 100 and 600 μg protein (maximum volume 150 μl), were adjusted to 2% SDS and 0.2 M sucrose by addition of 0.33 volumes of Sample Buffer. Samples were applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% (w/w), 0.1% SDS-containing polyacrylamide slab gel of 1.5 mm thickness. Electrophoresis was conducted at room temperature with a constant current of approximately 15 mA/gel, as described by Laemmli (66). The prestained

[3]Dounce homogenization resulted in a lower yield and less complete isolation of LDL receptor-containing membranes than that noted for Polytron homogenization.

[4]Samples processed using a fixed-angle rotor exhibited a less adequate separation of membranes into discrete fractions than that noted using a swinging-bucket rotor.

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molecular weight markers myosin (M_r 206,000), phosphorylase b (M_r 100,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), chymotrypsinogen (M_r 25,700), β-lactoglobulin (M_r 18,400), and lysozyme (M_r 14,300) were included in a separate lane to monitor the separation.

After electrophoresis, proteins migrating into the gel were electrophoretically transferred to BA85 nitrocellulose membranes at 18°C with a constant voltage of 120 V for 6–8 h in 25 mM Tris, 192 mM glycine buffer (pH 8.3) containing 20% (v/v) methanol. After electrophoretic transfer, the nitrocellulose paper was removed from the transfer apparatus and incubated with 100 ml of TBS containing 3% gelatin for a minimum of 30 min at room temperature with gentle shaking. After incubation, the nitrocellulose sheet was removed from the blocking solution, immersed, without rinsing, into 50 ml TBS containing 1% gelatin and 250 μl of anti-LDL receptor peptide antiserum (final antiserum dilution 1:200) and incubated for 2 h at room temperature with gentle shaking. The nitrocellulose sheet was then washed twice for 10 min each with TBS containing 0.05% Tween-20 and once for 10 min with TBS. The washed nitrocellulose sheet was then incubated with 50 ml of TBS containing 1% gelatin and 50 μl goat anti-rabbit IgG-horseradish conjugate (final conjugate dilution 1:1000) at room temperature for 1 h with gentle shaking. After incubation the nitrocellulose was washed as described above. During the final TBS wash, 40 mg 4-chloro-1-naphthol was dissolved in 10 ml room temperature methanol, and 50 μl cold 30% hydrogen peroxide was added to 50 ml of TBS containing 1% gelatin. After draining the final TBS wash from the nitrocellulose, the two solutions were mixed and immediately added to the nitrocellulose. The color development reaction was permitted to incubate at room temperature with gentle shaking until optimal color development was observed. The nitrocellulose sheet was then washed with running tap water for 15 min. The nitrocellulose was dried between pieces of filter paper and the intensity of color formation was quantitated by reflectance densitometry, using a Hoefer Scientific Instruments GS300 Transmittance/Reflectance Scanning Densitometer, and expressed in terms of either densitometer tracing peak height or area under the densitometer tracing curve. Variations between replicate samples averaged 7%. Interanimal variations averaged 20% (see Results).

LDL receptor visualization by biotinylated LDL ligand blotting analysis

Visualization of the LDL receptor by ligand blotting using biotin-labeled LDL was conducted essentially as described by Wade, Knight, and Sontar (50). Briefly, membrane extract aliquots containing between 10 μg and 20 μg protein were adjusted to 2% SDS and 0.2 M sucrose, and applied without heating and without addition of β-mercaptoethanol to the wells of a precast 4–20% gradient polyacrylamide minigel. Electrophoresis was conducted at room temperature with a constant current of approximately 60 mA for 2.5 h as described by Laemmli (66). The prestained molecular weight markers described above were included in a separate lane to monitor the separation. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to BA85 nitrocellulose membranes at 4°C with a constant voltage of 120 V for 18 h in 25 mM Tris, 192 mM glycine buffer (pH 8.3) containing 20% (v/v) methanol. After electrophoretic transfer, the nitrocellulose paper was removed from the transfer apparatus and incubated with Ligand Blotting Buffer I for 30 min at 37°C. After incubation, the nitrocellulose sheet was removed from the blocking solution, and incubated for 1 h in Ligand Blotting Buffer I containing 20 μg/ml biotinylated-LDL and either no further additions, 10 mM EDTA, or 100 μg/ml unlabeled LDL. After incubation, the nitrocellulose sheet was first washed four times for 1 min, 20 min, 20 min, and 1 min, respectively, with Ligand Blotting Buffer II and then incubated for 30 min at room temperature with a 1:300 dilution of streptavidin-horseradish peroxidase in Ligand Blotting Buffer I that had been adjusted to a pH of 7.4. After four washes in pH 7.4 Ligand Blotting Buffer II as described above, and an additional 1-min wash with water, color development was achieved by incubation of the nitrocellulose sheet for 10 min with 10 mM Tris Buffer (pH 7.4) containing 150 mM NaCl, 0.5% BSA, 0.4 mg/ml 4-chloro-1-naphthol and 0.03% hydrogen peroxide. After incubation, the nitrocellulose sheet was washed extensively with water and allowed to air-dry in the dark. The intensity of color formation was quantitated by reflectance densitometry as described above.

Measurement of HMG-CoA reductase activity

HMG-CoA reductase activity was measured as described by Harwood et al. (27). Microsomal aliquots containing 150 μg microsomal protein were incubated for 30 min at 37°C in a final volume of 75 μl of TEDK Buffer containing 3.4 mM $NADP^+$, 30 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 66.7 μM [^{14}C]HMG-CoA (sp act 10 cpm/pmol), 15,000–20,000 cpm [^{3}H]mevalonate (0.6–1.2 Ci/mmol) as an internal standard, and 68 mM EDTA to prevent conversion of mevalonate to phosphomevalonate during incubation. After incubation, 10 μl 6 M HCl was added to terminate the enzymatic reaction and to convert the newly formed mevalonate into mevalonolactone. The mevalonolactone was then separated from unreacted substrate by silica gel thin-layer chromatography. After development in toluene-acetone 1:1, the region of the chromatogram corresponding to R_f = 0.4–1.0 was removed, immersed in liquid scintillation fluid, and counted, using a dual-channel ^{3}H/^{14}C program. HMG-CoA reductase activity is ex-

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pressed as pmol of mevalonate formed from HMG-CoA per min of incubation at 37°C per mg of microsomal protein.

Measurement of total serum cholesterol concentration

Blood samples obtained at sacrifice by heart puncture, were allowed to clot at 4°C for 60 min. Samples were then centrifuged at 2500 g for 30 min at 4°C. Serum was removed from the cell pellet and analyzed for total cholesterol content using Abbott's A-Gent cholesterol reagent kit and an Abbott ABA-200 bichromatic analyzer. Standards containing 25 mg/dl, 50 mg/dl, and 100 mg/dl cholesterol were used to calculate serum cholesterol concentrations.

Measurement of protein concentration

Protein concentrations were determined by the method of Bradford (67) using bovine serum albumin as standard.

RESULTS

Preparation and characterization of anti-LDL receptor antiserum

Previous studies have demonstrated that polyclonal antisera prepared against synthetic peptides corresponding to the C-terminal amino acid sequence of the bovine adrenal cortex LDL receptor (64, 68, 69) are capable of recognizing the LDL receptor from several tissues of a variety of animal species (68). To obtain an antibody preparation capable of recognizing mammalian hepatic LDL receptor, a polyclonal antiserum was prepared in New Zealand White/Sandy Half Lop rabbits against a conjugate of keyhole limpet hemocyanin and a synthetic LDL receptor peptide whose 15-amino acid sequence and location in the bovine adrenal cortex LDL receptor are shown schematically in **Fig. 1.** The anti-conjugate antiserum, but not preimmune serum, exhibited, in a peptide-based ELISA, a concentration-dependent reactivity against the unconjugated LDL receptor peptide. This reactivity was lost by preincubation of antiserum with LDL receptor peptide (I. M. Varndell, Cambridge Research Biochemicals, unpublished observations).

Antiserum recognizes a single immunoreactive protein in bovine adrenal extract

Bovine adrenal cortex is known to be rich in LDL receptors (63). A membrane preparation from bovine adrenal cortex was thus used to evaluate the ability of this antiserum to recognize the LDL receptor protein. When proteins contained in membrane fractions isolated from bovine adrenal cortex were extracted using a Triton X-100-containing buffer and subjected to nondenaturing SDS polyacrylamide gel electrophoresis and Western immunoblotting analysis as outlined in **Fig. 2,** a single immunoreactive band of M_r 126 kDa, corresponding to the M_r previously noted for the bovine adrenal cortex LDL receptor (63) was observed (**Fig. 3**). Also, as previously described for the LDL receptor (51), heating with SDS or incubation with β-mercaptoethanol prior to electrophoretic separation resulted in visualization of an immunoreactive band with altered mobility (data not shown). No immunoreactivity in the 126-kDa region was noted when the incubation reaction with anti-LDL receptor peptide antiserum was omitted or when the antiserum was replaced with either preimmune serum or with peptide absorbed antiserum (data not shown). In addition, no color development was noted under conditions in which any one of the complexing reagents was omitted from the reaction sequence outlined in Fig. 2 (data not shown).

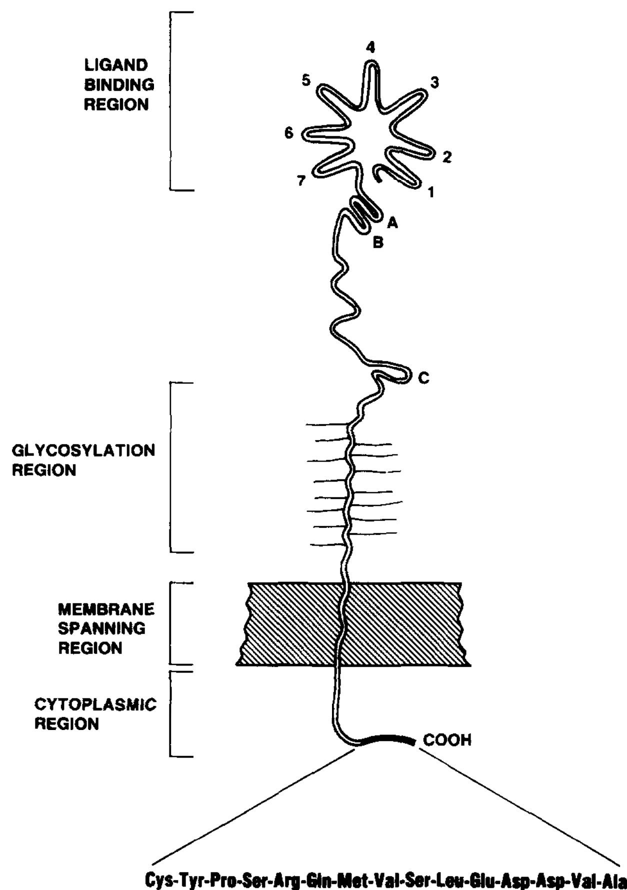

Fig. 1. Anti-LDL receptor antiserum recognition site. A polyclonal, monospecific antiserum was prepared in New Zealand White/Sandy Half Lop rabbits against a synthetic peptide-keyhole limpet hemocyanin conjugate corresponding to the 15-amino acid C-terminal sequence of the LDL receptor (Cys-Tyr-Pro-Ser-Arg-Gln-Met-Val-Ser-Leu-Glu-Asp-Asp-Val-Ala; 64). Peptide synthesis, conjugation with hemocyanin, immunization, and antibody preparation were conducted as described in Materials and Methods. Shown is a pictorial representation of the LDL receptor indicating in bold the region of the primary sequence against which the anti-peptide antiserum was prepared.

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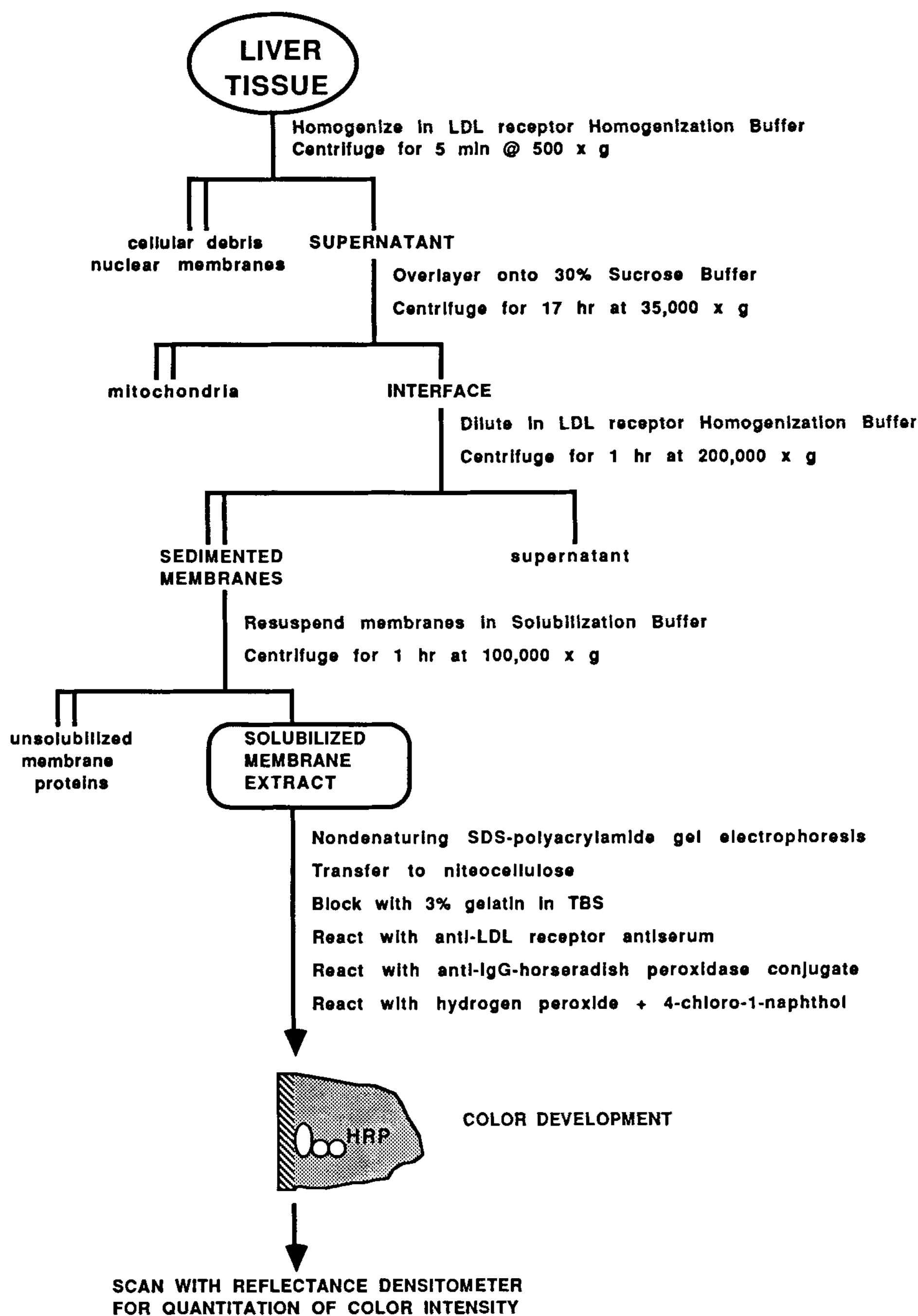

Fig. 2. Outline of the methodology for isolating and visualizing the LDL receptor.

Ligand blotting using biotinylated-LDL confirms that the antiserum recognizes the LDL receptor

To determine whether the 126-kDa immunoreactive band observed in bovine adrenal cortex membrane extracts indeed represented the LDL receptor, bovine adrenal cortex membrane extracts, purified bovine adrenal cortex LDL receptor fractions, and liver microsomal membrane extracts from estradiol-treated rats were subjected to SDS polyacrylamide gel electrophoresis and subsequent ligand blotting analysis using biotinylated LDL. As shown in **Fig. 4**, biotinylated-LDL bound to a protein of approximately 127 kDa in bovine

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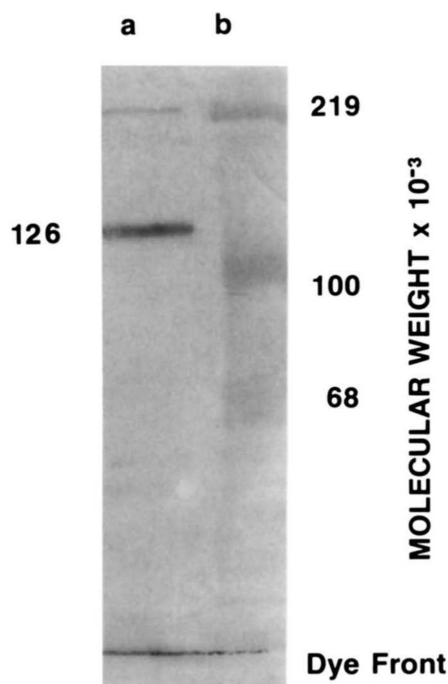

Fig. 3. Anti-LDL receptor peptide antiserum recognizes a single immunoreactive protein in bovine adrenal extracts. Bovine adrenal cortex membrane extracts were prepared as described in Materials and Methods using Solubilization Buffer I that contained PMSF as the only protease inhibitor. Aliquots of the extracts containing 100 μg protein were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel, and separated by electrophoresis for 18 h at a constant current of 10 mA. The prestained molecular weight markers were included in a separate lane to monitor separation. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to nitrocellulose and anti-LDL receptor immunoreactive proteins were visualized as described in Materials and Methods. Shown are a: immunoreactive proteins contained in 100 μg of bovine adrenal cortex membrane extract, and b: the prestained molecular weight markers indicated above.

adrenal cortex extract and purified fractions[5] and to a protein of approximately 145 kDa in hepatic extracts from estradiol-treated rats. Binding of biotinylated-LDL to proteins of both extracts was prevented by inclusion of 10 mM EDTA in the incubation reaction (Fig. 4 top) and was substantially reduced by inclusion of a 5-fold excess[6] (100 μg/ml) of unlabeled LDL in the incubation reaction (Fig. 4 bottom). These observations are consistent with both the Ca^{2+} dependence and specific binding properties of LDL/LDL receptor interactions (51, 52, 70). In addition, the apparent M_r values of the bovine adrenal LDL receptor and rat liver LDL receptor are consistent with that previously noted by immunoblotting (56) and ligand blotting (51, 52). These observations, together with the colocalization of immunoreactivity (Fig. 3) and ligand binding (Fig. 4) in the bovine adrenal cortex membrane extract indicate that the antiserum recognizes the LDL receptor.

Immunoblot analysis of hepatic microsomal extracts from estradiol-treated rats is complicated by proteolysis

When the method for visualizing the LDL receptor in bovine adrenal cortex membrane extracts by immunoblotting using the anti-LDL receptor peptide antiserum was applied to liver tissue isolated from a variety of species (rat, hamster, marmoset, cynomolgus macaque, guinea pig, rabbit), the major immunoreactive bands noted for hepatic microsomal extracts migrated with M_r values ranging between 30 kDa and 80 kDa. The intensity of the various bands differed from species to species (data not shown). This observation is exemplified in **Fig. 5** for hepatic microsomal extracts from rats treated with 17α-ethinyl estradiol at a dose of 5 mg/kg/day for 5 days. This observation differs from the results obtained for a similar preparation by ligand blotting (Fig. 4), suggesting that the appearance of the low molecular weight LDL-nonbinding immunoreactive band in the liver microsomes was the result of LDL receptor proteolysis. In this regard, similar sized cleavage products of the bovine adrenal LDL receptor have been generated in vitro by pronase digestion (64).

Addition of protease inhibitors to isolation and solubilization solutions allows recovery of intact LDL receptors from liver homogenates

To determine whether the presence of the 30-kDa immunoreactive band in hepatic microsomal extracts was the result of proteolysis during membrane isolation and solubilization, a variety of protease inhibitors, chosen in a effort to inhibit all classes of known proteases, were included in the isolation and extraction buffers.[7] Inclusion of 1 mM PMSF, 0.3 mM leupeptin, 0.3 mM TLCK, 0.3 mM TPCK, 0.1 mM iodoacetamide, 5 mM EDTA, and 5 mM EGTA throughout microsomal isolation and membrane solubilization prevented proteolysis of hamster

[5]A single band of radioactivity was also noted after ligand blotting analysis and autoradiography of the electrophoretically separated proteins contained in bovine adrenal cortex membrane extracts using ^{125}I-labeled LDL as the radiolabeled ligand (P. G. Cosgrove and H. J. Harwood, unpublished observations).

[6]That biotinylated LDL binding was not totally prevented by addition of a 5-fold excess of unlabeled LDL is consistent with observations in cultured hepatic (HepG2) cells in which a 5-fold excess of unlabeled LDL inhibited [^{14}C]sucrose-labeled LDL binding and uptake by only 80% and that 10-fold excesses were required to fully prevent nonspecific LDL binding (L. D. Pellarin and H. J. Harwood, unpublished observation).

[7]Proteinaceous protease inhibitors such as soybean trypsin inhibitor or aprotinin reduced LDL receptor recovery and/or solubilization and thus were not included in the final protease-containing buffers.

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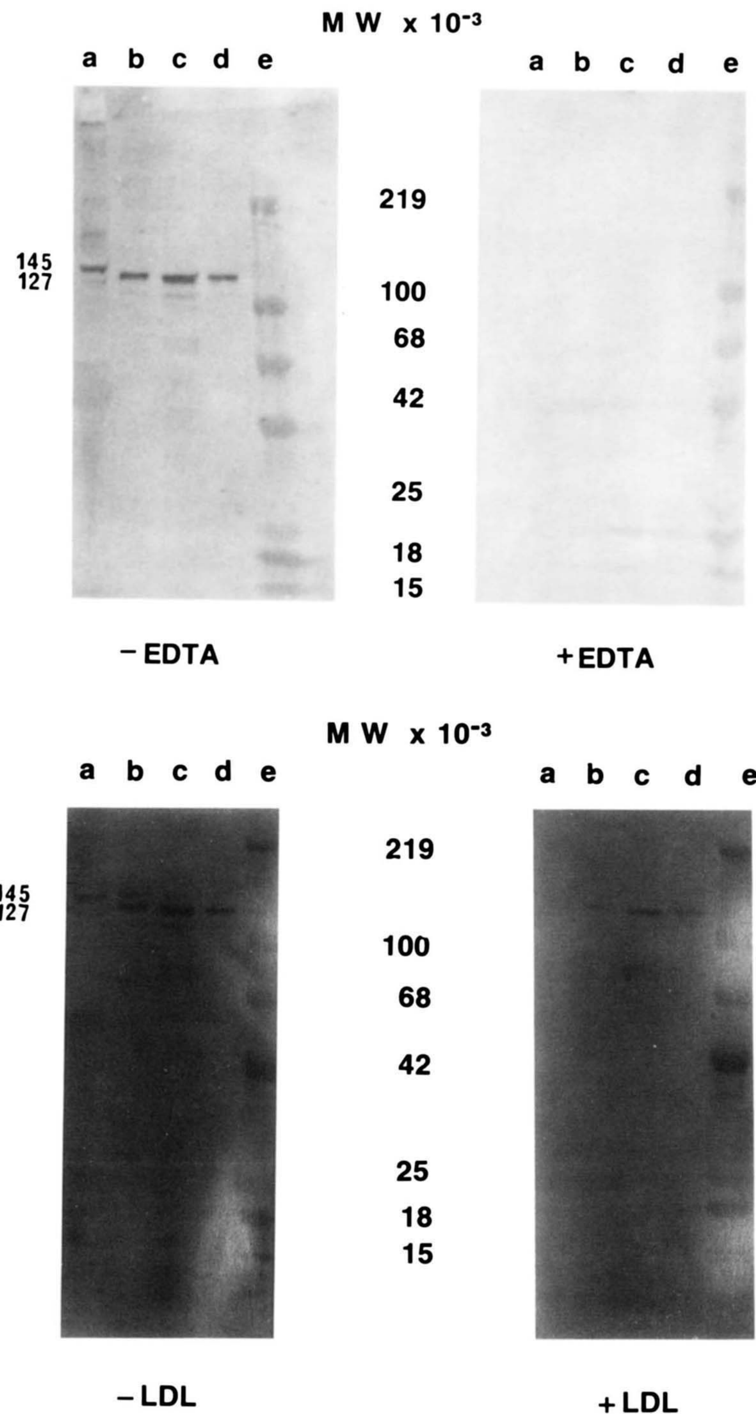

Fig. 4. Ligand blotting using biotinylated-LDL confirms that the antiserum recognizes the LDL receptor. Aliquots, 20 μl, of 17α-ethinyl estradiol-treated (5 mg/kg; 4 days) rat liver microsomal extract (2.4 μg protein, a), bovine adrenal cortex membrane extract (18 μg protein, c), bovine adrenal cortex DEAE-cellulose fraction (8 μg protein, d), and bovine adrenal cortex LDL-Sepharose fraction (2 μg protein, b) were adjusted to 2% SDS and 0.2 M sucrose and applied without heating and without addition of β-mercaptoethanol to the wells of precast, 4–20% gradient polyacrylamide minigels. The prestained molecular weight markers described in Materials and Methods were included in a separate lane (e) to monitor separation. Two identical sets of aliquots, were applied to each of two minigels. Proteins contained in the applied fractions were separated by electrophoresis for 2.5 h at a constant current of 100 mA. After electrophoresis, proteins migrating into the gels were electrophoretically transferred to nitrocellulose as described in Materials and Methods. The nitrocellulose sheet was divided into four sections, each containing the channels corresponding to one set of aliquots. Biotinylated-LDL binding proteins were visualized by ligand blotting in the presence (top right) or absence (top left) of 10 mM EDTA (minigel 1), and in the presence (bottom right) or absence (bottom left) of a 5-fold excess of unlabeled LDL (minigel 2).

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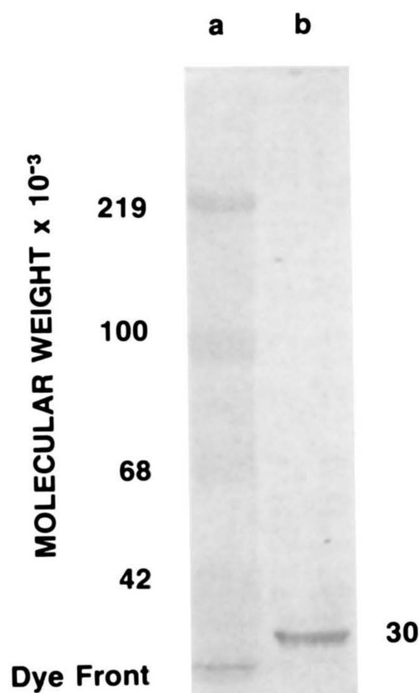

Fig. 5. LDL receptor immunoblot analysis of hepatic microsomal extracts is complicated by proteolysis. Two male Sprague-Dawley rats (400 g) that had been housed in a reversed lighting cycle room for 10 days received food and water ad libitum and were given daily subcutaneous injections of 17α-ethinyl estradiol at a dose of 5 mg/kg/day for 5 days. On the sixth day, 1 h after the final 17α-ethinyl estradiol injection, rats were anesthetized and sacrificed, and 4.5-g liver samples were obtained from each animal. Hepatic microsomes were prepared, and microsomal membrane proteins were extracted using Solubilization Buffer I that contained PMSF as the only protease inhibitor. Aliquots of the soluble extract containing 400 μg protein were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel, and separated by electrophoresis for 6 h at a constant current of 30 mA. Prestained molecular weight markers described in Materials and Methods were included in a separate lane to monitor separation. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to nitrocellulose and anti-LDL receptor immunoreactive proteins were visualized as described in Materials and Methods. Shown are a: prestained molecular weight markers, and b: immunoreactive proteins contained in 400 μg of ethinyl estradiol-treated rat liver microsomal membrane extract.

LDL receptor and resulted in the visualization of two immunoreactive bands in the hamster liver membrane extract (**Fig. 6**). The upper band, corresponding to the band visualized following ligand blotting (Fig. 4), had an M_r value of approximately 140 kDa and was not observed when the anti-LDL receptor peptide antiserum was pretreated with either the 15-amino acid LDL receptor peptide or with keyhole limpet hemocyanin[8] (**Fig. 7**). Thus the 140-kDa immunoreactive band represents the LDL receptor. As shown in Fig. 6, omission of iodoacetamide, TLCK, and TPCK from the protease inhibitor mixture resulted in loss of the immunoreactive band at 140 kDa, suggesting that a protease sensitive to either iodoacetamide, TLCK, and/or TPCK may be involved in LDL receptor proteolysis.

In contrast, the lower 110-kDa immunoreactive band was not observed either after ligand blotting (Fig. 4) or

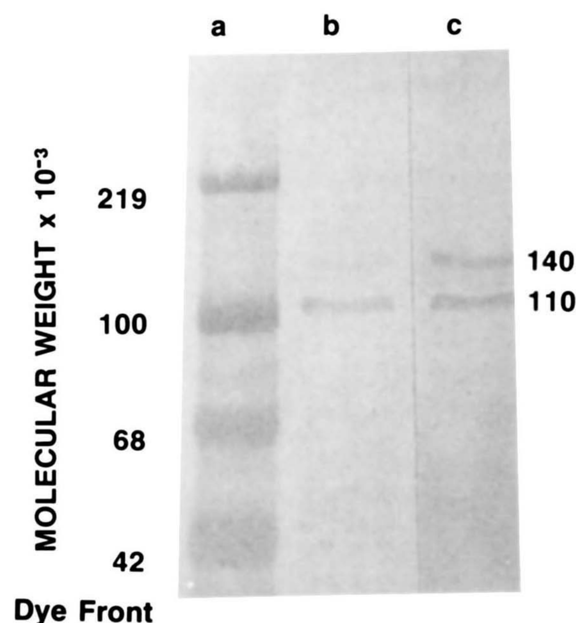

Fig. 6. Addition of protease inhibitors to isolation and solubilization solutions allows recovery of intact LDL receptors from liver homogenates. Three male golden Syrian hamsters (100-120 g) that had been housed in a reversed lighting cycle room for 1 week received water and powdered chow containing 5% cholestyramine resin ad libitum for 5 days. On the morning of the sixth day, animals were anesthetized and sacrificed, and two 1.5-g liver samples were obtained for each animal. Two hepatic tissue pools were made. One tissue pool was homogenized in LDL receptor Microsomal Isolation Buffer that was supplemented with 0.3 mM leupeptin, 5 mM EDTA, and 5 mM EGTA (lane b), whereas the second tissue pool was homogenized in LDL receptor Microsomal Isolation Buffer that was supplemented with 0.3 mM leupeptin, 5 mM EDTA, 5 mM EGTA, 0.1 mM iodoacetamide, 0.3 mM TLCK, and 0.3 mM TPCK (lane c). Microsomal membrane isolation and extraction were conducted as described in Materials and Methods using Solubilization Buffer I that contained only the above-mentioned protease inhibitors. Aliquots of soluble extracts containing 200 μg protein were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel. Prestained molecular weight markers indicated in Materials and Methods were included in a separate lane to monitor separation. After electrophoresis (6 h; 30 mA), proteins migrating into the gel were electrophoretically transferred to nitrocellulose and anti-LDL receptor immunoreactive proteins were visualized. Shown are a: prestained molecular weight markers; b: immunoreactive proteins contained in 200 μg of cholestyramine-treated hamster liver microsomal membrane extract isolated in the presence of PMSF, leupeptin, EDTA, and EGTA; and c: immunoreactive proteins contained in 200 μg of cholestyramine-treated hamster liver microsomal membrane extract isolated in the presence of PMSF, leupeptin, EDTA, EGTA, iodoacetamide, TLCK, and TPCK.

[8]The observation that immunoreactivity of the LDL receptor band could also be prevented by pretreatment of the antiserum with keyhole limpet hemocyanin suggests that the epitope against which the antibody was directed contained, in addition to the sequence of the LDL receptor peptide, portions of the sequence of the hemocyanin molecule.

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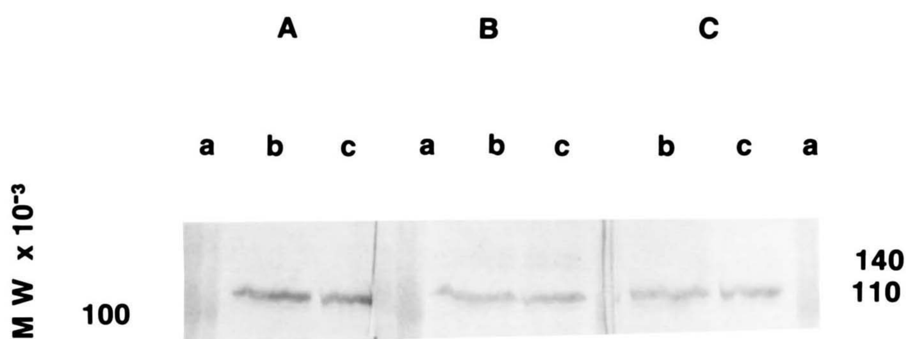

Fig. 7. Only the 140-kDa immunoreactive protein represents the LDL receptor. Six male golden Syrian hamsters (100–120 g) that had been housed in a reversed lighting cycle room for 1 week received water and powdered chow containing 5% cholestyramine resin ad libitum for 4 days. On the morning of the fifth day, animals were anesthetized and sacrificed, and 1.5-g liver samples were obtained for each animal and pooled. Liver tissues were homogenized in LDL receptor Homogenization Buffer and hepatic LDL receptor-containing membranes were isolated by sucrose density gradient centrifugation and extracted. Three sets, each containing two aliquots of the soluble extract, one of 600 μg protein (b) and one of 300 μg protein (c), were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to side by side 6-mm wells of a 7.5% SDS-containing polyacrylamide gel. The prestained molecular weight markers were included in separate lanes on either side of each set of aliquots to monitor separation and to facilitate cutting of the nitrocellulose sheet after transfer. After electrophoresis and transfer to nitrocellulose, the nitrocellulose sheet was divided into three equal parts by cutting down the middle of the lanes containing the prestained molecular weight markers. Unbound sites on the nitrocellulose sheets were then blocked in TBS containing 3% gelatin for 30 min. Nitrocellulose sheets were then incubated for 2 h with one of three preparations of rabbit anti-LDL receptor peptide antisera (1:200 dilution in TBS containing 1% gelatin) that had been incubated at room temperature for 2 h prior to dilution with either 1 mg of the 15-amino acid bovine LDL receptor C-terminal synthetic peptide per 50 μl antiserum (A), no additions (B), or 10 mg keyhole limpet hemocyanin (an amount equimolar to the peptide addition) per 50 μl antiserum (C). Nitrocellulose sheets were then washed and treated with goat-anti rabbit IgG-horseradish conjugate and subsequent color development reagents. Shown are a: prestained molecular weight markers; and b and c: immunoreactive proteins contained in 600 μg and 300 μg of cholestyramine-treated hamster liver membrane extract visualized following incubation with A: peptide-absorbed antiserum, B: unabsorbed antiserum, or C: keyhole limpet hemocyanin-absorbed antiserum.

after immunoblotting of bovine adrenal cortex membrane extracts (Fig. 3), suggesting that it does not represent the LDL receptor. Consistent with this suggestion is the observation that the 110-kDa band was also noted in hamster hepatic extracts when either preimmune serum or anti-apolipoprotein A-I antiserum was used in place of the anti-LDL receptor peptide antiserum (data not shown). In addition, preabsorption of antiserum with the 15-amino acid LDL receptor peptide or with keyhole limpet hemocyanin had no effect on the presence or intensity of the 110-kDa band (Fig. 7). The 110-kDa immunoreactive band also differed from the 140-kDa band in its sensitivity to proteolysis. In contrast to the 140-kDa LDL receptor band, omission of iodoacetamide, TLCK, and TPCK from the protease inhibitor mixture had virtually no effect on the presence or intensity of the 110-kDa immunoreactive band (Fig. 6). Taken together, these results indicate that the 140-kDa band represents the LDL receptor while the 110-kDa band represents an immunoreactive band that is unrelated to the LDL receptor.[9]

[9]In additional studies, omission of the primary antibody from the first incubation reaction resulted in loss of both the 140-, and 110-kDa immunoreactive bands. Thus, the 110-kDa immunoreactive band does not represent residual immunoglobulin contained in the extract, nor does it represent hepatic peroxidases present in the extract.

Discontinuous sucrose gradient centrifugation permits collection of all LDL receptor-containing membranes in one fraction

In order to quantitatively assess changes in LDL receptor, it was necessary to obtain a membrane fraction that contained the full compliment of cellular LDL receptors. When subjected to linear sucrose gradients, whose sucrose concentrations ranged between 0% and 50% (w/v), hepatic membranes were separated into four distinct fractions that corresponded to the expected mobility of endosomes, plasma membrane vesicles, lysosomes, and plasma membrane sheets, respectively (71), all of which contained measurable LDL receptor levels (data not shown). This observation was used to identify a step-gradient concentration that would be sufficiently dense to prevent sedimentation of all LDL-containing membranes, while still permitting other non-LDL receptor containing particles to sediment. As shown in **Fig. 8,** when liver homogenates from hamsters treated with both 5% cholestyramine and 0.05% mevinolin were overlayered onto a series of sucrose solutions containing from 0% to 45% (w/v) sucrose, and separated by density gradient centrifugation, two membrane-containing bands were obtained for all sucrose concentrations, one that sedimented (pellet) and one that migrated at the interface between the two solutions. At sucrose concentrations of 15% or less, all of the LDL receptor protein was contained in the pelleted fraction

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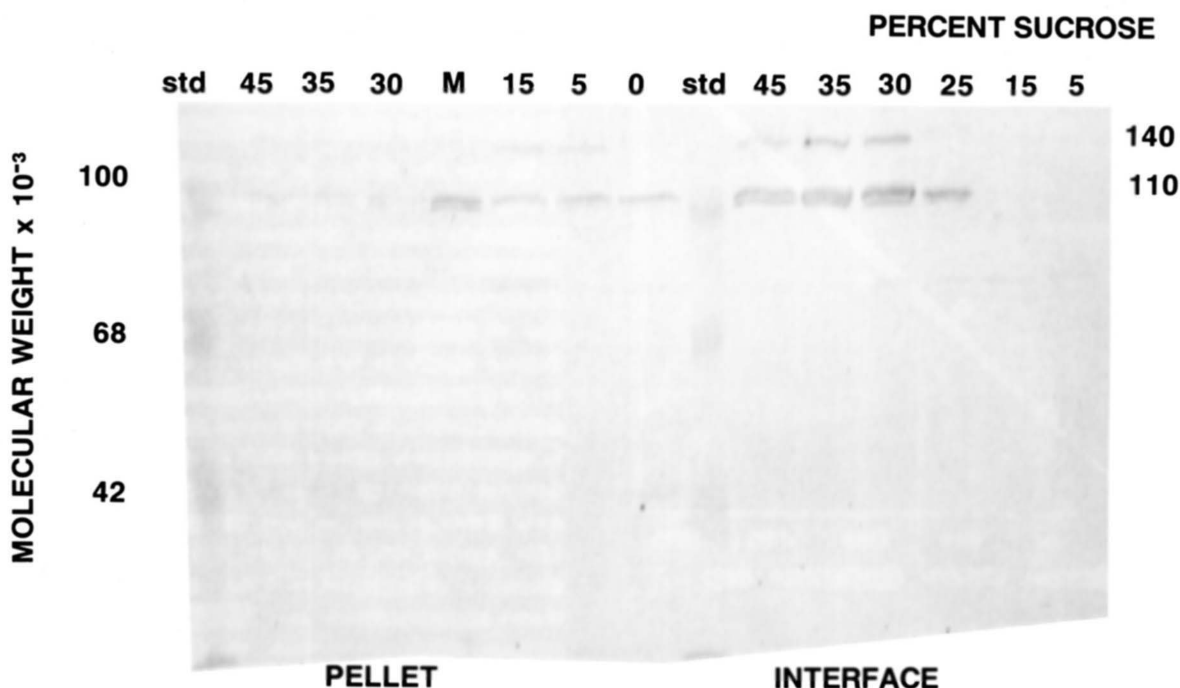

Fig. 8. Discontinuous density gradient centrifugation through 30% Sucrose Buffer permits collection of all LDL receptor-containing membranes in a single membrane fraction. Six male golden Syrian hamsters (100–120 g) that had been housed in a reversed lighting cycle room for 1 week received water and powdered chow containing both 5% cholestyramine resin and 0.05% mevinolin ad libitum for 5 days. On the morning of the sixth day, animals were anesthetized and sacrificed, and livers were removed, combined, and homogenized together in LDL receptor Homogenization Buffer (5 ml/g liver tissue). Homogenates were centrifuged at 500 *g* for 15 min at 4°C to remove cell debris. After centrifugation, aliquots of the resulting supernatant, 6 ml, were overlayered onto 6-ml volumes of each of six Sucrose Buffers containing the indicated sucrose concentrations (w/v) and centrifuged at 4°C for 20 h at 37,000 *g* in a swinging-bucket rotor (Sw40). After centrifugation, membranes migrating to the homogenate-Sucrose Buffer interface were removed by gentle aspiration, diluted to 3.5 ml with LDL receptor Homogenization Buffer, and centrifuged at 100,000 *g* for 1 h at 4°C to wash and concentrate the membranes. Membranes migrating through the Sucrose Buffer (pellets) were collected and washed by resuspension in 3.5 ml LDL receptor Homogenization Buffer and centrifugation at 100,000 *g* for 1 h at 4°C. Sedimented membrane fractions were extracted and aliquots of the resulting soluble extracts containing 400 μg protein were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel. The prestained molecular weight markers were included in a separate lane to monitor separation. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to nitrocellulose and anti-LDL receptor immunoreactive proteins were visualized. Shown are the immunoreactive proteins contained in the pellet and interface membrane fraction extracts isolated following density gradient centrifugation at the indicated sucrose concentrations (w/v).

(Fig. 8), whereas, at sucrose concentrations of 30% or greater, all of the LDL receptor protein was contained in the interface fraction (Fig. 8). Thus, a 30% sucrose solution was sufficiently dense to prevent sedimentation of all LDL receptor containing particles (Fig. 8; cf. Fig. 10). This methodology, which is outlined in Fig. 2, was thus used in all subsequent experiments.

Protein dependency of color development

Using the methodology outlined in Fig. 2, the intensity of the immunoblotting signal was a linear function of the amount of LDL receptor soluble extract applied to the gel (**Fig. 9**). Based on these observations, an application of 400 μg of LDL receptor-containing membrane extract protein was sufficient to give a reliable signal that was within the linear range of color development (Fig. 9).

Reproducibility of LDL receptor immunoblotting analysis

To examine the degree of reproducibility of the extraction, transfer, and immunoreactions leading to color development, livers from three hamsters treated with both 5% cholestyramine and 0.05% mevinolin were homogenized together, divided into six equal portions and processed as in Fig. 2. As shown in **Fig. 10,** separation of hepatic homogenates by discontinuous density gradient centrifugation over 30% Sucrose Buffer permitted isolation of the full compliment of LDL receptor-containing membranes in a single fraction. No immunoreactive proteins were detected in the pellet (Fig. 10). In addition, after extraction, virtually all of the LDL receptor immunoreactive protein was recovered in the supernatant indicating nearly complete solubilization of the LDL

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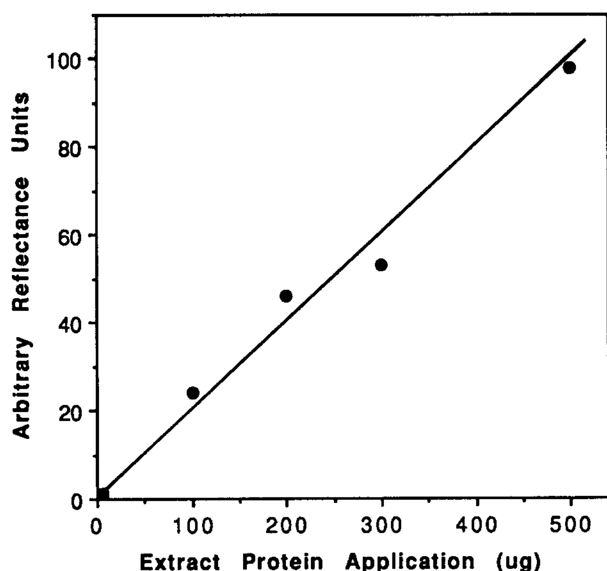

Fig. 9. Color development is dependent on the amount of membrane extract applied to the gel. Three male golden Syrian hamsters (100-120 g) that had been housed in a reversed lighting cycle room for 1 week received water and powdered chow containing both 5% cholestyramine resin and 0.05% mevinolin ad libitum for 5 days. On the morning of the sixth day, animals were anesthetized and sacrificed, and livers were removed, combined, and homogenized together in LDL receptor Homogenization Buffer (5 ml/g liver tissue). Hepatic LDL receptor-containing membranes were isolated by sucrose density gradient centrifugation and extracted. Aliquots of the resulting soluble extracts containing the indicated quantities of extracted protein were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to nitrocellulose and anti-LDL receptor immunoreactive proteins were visualized and quantitated. Shown are the arbitrary reflectance units of color intensity (peak height) as a function of amount of extracted protein applied to the gel.

receptor (data not shown). When the intensity of color development noted for the six replicates shown in Fig. 10 was subjected to quantitation based on reflectance densitometry (**Table 1**), the variability in the measurement was approximately 7%. In other experiments in which 11 hamsters were fed chow containing 3% cholestyramine for 4 days and their livers were removed and processed independently as described in Fig. 2, inter-animal variation using this methodology was approximately 20% (**Table 2**). Thus, this methodology isolates the full compliment of hepatic LDL receptors, exhibits an intra-assay variability of 7% and an inter-animal variability of approximately 20%, and hence represents a useful method for measuring total hepatic LDL receptor concentrations.

Relationship between total LDL receptor concentration, HMG-CoA reductase activity, and serum cholesterol levels after treatment with modulators of cholesterol metabolism

To test the utility of this methodology for assessing changes in LDL receptor concentration in vivo, and for evaluating whether alterations in total LDL receptor concentration, measured using this methodology, were related to physiological changes known to occur concomitant to modulations of LDL receptor transcription and hepatic uptake in vivo, hamsters were treated with a variety of pharmacologic agents known to alter LDL receptor concentrations in experimental animals in such a way as to coordinately induce HMG-CoA reductase and LDL receptor transcription and to also reduce serum cholesterol levels at least in part through increases in hepatic LDL uptake (19, 20, 22-24, 27). As shown in **Fig. 11,** treatment of hamsters for 4 days with the bile acid sequestrants cholestyramine and CP-88488, the cholesterol absorption inhibitor tiqueside, the cholesterol synthesis inhibitor mevinolin, and the combination of cholestyramine plus mevinolin, produced the predicted increases in hamster total hepatic LDL receptor concentrations that were highly correlated with the concomitant increases in HMG-CoA reductase activity (Fig. 11, top; $r = 0.81$; $P = 0.014$) and reductions in serum cholesterol (Fig. 11, bottom; $r = 0.68$; $P = 0.036$). These observations are consistent with the coordinate derepression of both LDL receptor and HMG-CoA reductase transcription that occur after these modulations of cholesterol metabolism (72) and also with a major contribution of increased receptor-mediated LDL uptake to plasma cholesterol reduction after modulation of cholesterol homeostasis induced by these agents. In addition, treatment of hamsters for 5 days with 17α-ethinyl estradiol, an estrogen analog known to increase LDL receptor activity and reduce serum cholesterol levels in experimental animals (35-38), administered by subcutaneous injection in safflower oil at the peak of the mid-dark lighting cycle at a dose of 5 mg/kg/day, also resulted in a 2.1-fold increase in total hepatic LDL receptor concentration, a finding that is consistent in magnitude to that previously noted in the rats (73), rabbits (38), and in humans (74), and a concomitant 28% reduction in serum cholesterol levels. Similarly, when 17α-ethinyl estradiol was administered for an extended duration of up to 10 days, the increase in LDL receptor concentration of up to 3.7-fold that was noted was a function of duration of dose administration (**Fig. 12**) and was inversely related to serum cholesterol concentration (Fig. 12). Taken together, these results indicate that this method is capable of measuring modulations in total hepatic LDL receptor concentration in experimental animals that are reflective of changes in

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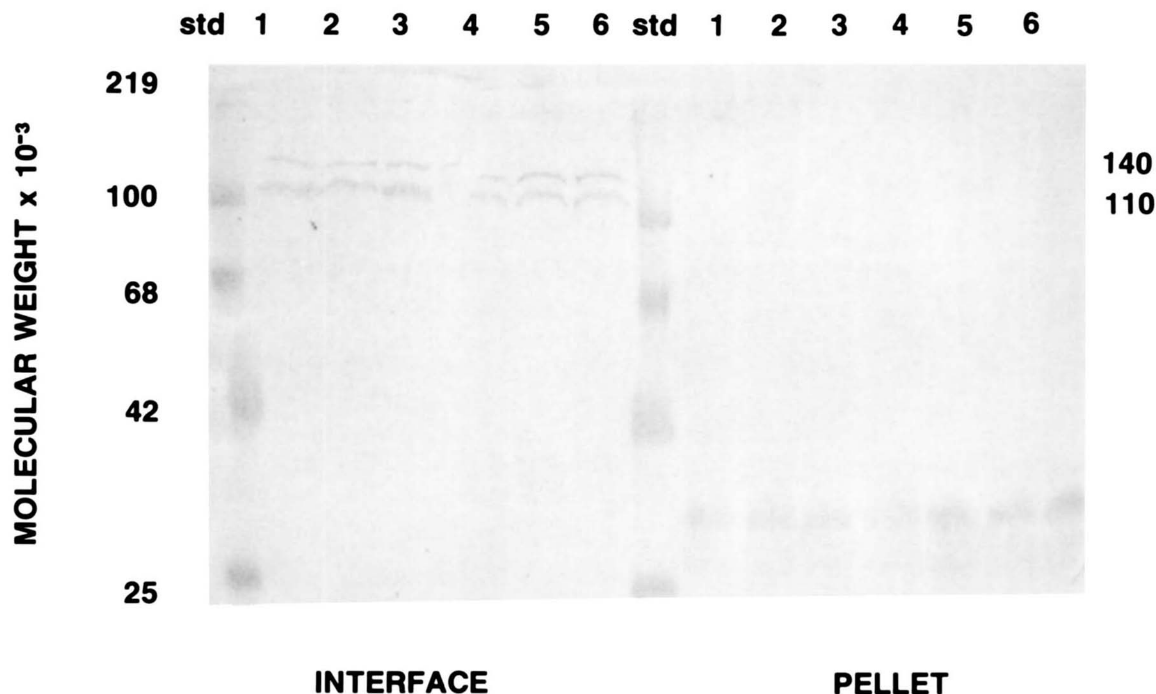

Fig. 10. Reproducibility of LDL receptor immunoblotting analysis using multiple samplings from one liver pool. Three male golden Syrian hamsters (100-120 g) that had been housed in a reversed lighting cycle room for 1 week received water and powdered chow containing both 5% cholestyramine resin and 0.05% mevinolin ad libitum for 5 days. On the morning of the sixth day, animals were anesthetized and sacrificed, and livers were removed, combined, and homogenized together in LDL receptor Homogenization Buffer (5 ml/g liver tissue). Homogenates were centrifuged at 500 *g* for 15 min at 4°C to remove cell debris. After centrifugation, six 6-ml aliquots of the resulting supernatant were overlayered onto 6-ml volumes of 30% (w/v) Sucrose Buffer and centrifuged at 4°C for 20 h at 37,000 *g* in a swinging-bucket rotor (Sw40). After centrifugation, membranes migrating to the homogenate-Sucrose Buffer interface were removed by gentle aspiration, diluted to 3.5 ml with LDL receptor Homogenization Buffer and centrifuged at 100,000 *g* for 1 h at 4°C to wash and concentrate the membranes. Membranes migrating through the Sucrose Buffer (pellets) were collected and washed by resuspension in 3.5 ml LDL receptor homogenization buffer and centrifugation at 100,000 *g* for 1 h at 4°C. Sedimented membrane fractions were extracted and aliquots of the resulting soluble extracts containing 400 μg protein were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel. The prestained molecular weight markers were included in a separate lane to monitor separation. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to nitrocellulose, and anti-LDL receptor immunoreactive proteins were visualized. Shown are the immunoreactive proteins contained in the pellet and interface membrane fraction extracts for each of the six aliquots.

cholesterol homeostasis that occur in response to diverse pharmacologic modulations to cholesterol metabolism.

DISCUSSION

In this report, we describe development of the methodology for isolating the full compliment of hepatic LDL receptors in a single membrane fraction by discontinuous sucrose density gradient centrifugation, and for quantitating the LDL receptor concentration in this fraction by a noncompetitive Western immunoblotting enzyme immunoassay procedure. For the hamster liver, this methodology, which uses an antiserum prepared in rabbits against a conjugate between keyhole limpet hemocyanin and a synthetic peptide corresponding to the 15-amino acid C-terminal sequence of the bovine LDL receptor, exhibits an intra-assay variability of approximately 7% and an inter-animal variability of approximately 20%, indicating its utility for detecting and quantitating differences in LDL receptor levels in hepatic extracts. As the antiserum used also recognizes the LDL receptor from bovine adrenal cortex and from hepatic tissues isolated from a

TABLE 1. Reproducibility of LDL receptor immunoblotting analysis using multiple samples from one hamster pool

Lane	Protein Application	Densitometer Scan Peak Height	Specific Peak Height
	μg	*mm*	*mm/mg protein*
1	444	126	284
2	540	128	237
3	600	107	178
5	600	148	247
6	600	132	220
Average			233 ± 35 SD ± 16 SE

The experimental conditions are as described in Fig. 10. The intensity of color development for each sample was quantitated by reflectance densitometry as described in Materials and Methods and is expressed in terms of peak height. Lane 4 was not included in the analysis due to a crack in the gel that precluded accurate determination of color intensity by this method.

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TABLE 2. Inter-animal variation in the measurement of hepatic LDL receptor levels

Animal Number	Serum Cholesterol	LDL Receptor	
	mg/dl	units/mg extract	units/gram liver
1	138	17.3	28.9
2	96	9.8	12.9
3	106	17.3	18.5
4	126	12.8	23.2
5	114	21.5	34.1
6	94	10.5	29.4
7	104	13.2	25.2
8	113	13.0	27.5
9	137	12.0	14.9
10	108	15.5	31.3
11	122	13.2	26.0
Mean	114	14.2	24.7
SD	14	3.3	6.4
SE	4	1.0	1.9

Eleven male golden Syrian hamsters (100–120 g) that had been housed in a reversed lighting cycle room for 1 week received water and powdered chow containing both 5% cholestyramine resin and 0.05% mevinolin ad libitum for 5 days. On the morning of the sixth day, animals were anesthetized, and sacrificed, and livers were removed and homogenized in LDL receptor Homogenization Buffer (5 ml/g liver tissue). Blood samples were also obtained at the time of sacrifice and used for determination of serum cholesterol concentration as described in Materials and Methods. For each hepatic homogenate, LDL receptor-containing membranes were isolated by sucrose density gradient centrifugation and extracted as described in Materials and Methods. Aliquots of the resulting soluble extracts containing 400 μg protein were adjusted to 2% SDS and 0.2 M sucrose and applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to nitrocellulose and anti-LDL receptor immunoreactive proteins were visualized as described in Materials and Methods. LDL receptor levels are represented in terms of arbitrary reflectance units based on the area under the curve as estimated by weighing cut outs of peak areas.

variety of species including hamster, rat, guinea pig, marmoset, and cynomolgus macaque, this methodology is applicable to determination of LDL receptor levels from a variety of experimental animals.[10]

This methodology is also applicable to measuring changes in total hepatic LDL receptor levels in response to perturbations in cholesterol homeostasis that result from pharmacologic modulations of cholesterol metabolism. For example, bile acid sequestrants such as cholestyramine (19, 20) and, CP-88488 (21–23), cholesterol absorption inhibitors such as tiqueside (27), and cholesterol synthesis inhibitors such as mevinolin (24), induce increases in LDL receptor transcription and receptor-mediated hepatic uptake of circulating LDL as a secondary consequence of their primary mechanisms of action. These increases occur in concert with increases in the expression of a number of key cholesterolgenic enzymes including HMG-CoA reductase (9, 72, 75), in an effort by the liver *1)* to replace hepatic cholesterol used as a substrate for increased bile acid synthesis in response to bile acid sequestration; *2)* to compensate for the reduction in intestinally derived cholesterol availability to the liver in response to cholesterol absorption inhibition; or *3)* to compensate for the reduction in nascent cholesterol synthesis in the liver in response to cholesterol synthesis inhibition, and play a major role in the ultimate degree of serum cholesterol lowering achieved by these agents. Our observations that increases in total hepatic LDL receptor concentration, measured using this methodology, are highly correlated with the concomitant increases in HMG-CoA reductase activity that occur in response to these pharmacologic manipulations of cholesterol metabolism, suggest that total LDL receptor concentration measured using this methodology is reflective of changes in LDL receptor transcription in experimental animals. In addition, that increases in total hepatic LDL receptor concentration in response to treatment with these agents, and also in response to treatment with 17α-ethinyl estradiol, an estrogen analog known to increase LDL receptor expression and lower serum cholesterol concentrations in experimental animals (35–38), are correlated with reductions in serum cholesterol levels, suggests that total LDL receptor concentration, measured using this methodology, is also reflective of changes in receptor-mediated LDL uptake by the liver.

It is not surprising that total hepatic LDL receptor concentration and serum cholesterol lowering are less highly correlated than are total hepatic LDL receptor concentration and HMG-CoA reductase activity in response to bile acid sequestration, cholesterol absorption inhibition, and cholesterol synthesis inhibition, since, although a primary mechanism by which serum cholesterol is lowered by such agents, increased receptor-mediated uptake of circulating LDL is not the sole mechanism through which these agents lower serum cholesterol. Sterol depletion through increased fecal excretion of bile acids (bile acid sequestrants) and cholesterol (cholesterol absorption inhibitors) and the reduction in the synthesis of cholesterol by the liver (cholesterol biosynthesis inhibitors) also plays a

[10]The presence of traces of rabbit IgG (M_r approximately 155 kDa in the absence of reduction with β-mercaptoethanol; 82) in the rabbit hepatic membrane extracts resulted in visualization of an intense immunoreactive band between 120 and 180 kDa that was present even in the absence of anti-LDL receptor peptide antiserum in the first incubation reaction. The intensity of this band precluded visualization of the rabbit LDL receptor band located in the same region (52). Preincubation of rabbit hepatic membrane extracts with Protein A-Sepharose to remove the residual IgG dramatically reduced the intensity of the IgG immunoreactive signal but did not remove it sufficiently to permit visualization of the LDL receptor band. This technical difficulty is only of consequence when estimating LDL receptor concentrations in rabbit liver as no immunoreaction was noted when hepatic membrane extracts from other species were incubated with the goat anti-rabbit IgG-horseradish peroxidase conjugate (P. G. Cosgrove and H. J. Harwood, unpublished observation). Use of alternate antisera prepared against this peptide in a species other than rabbit or use of monoclonal antibodies directed against the rabbit LDL receptor (ATCC cat# CRL 1703 or ref. 38) should render this methodology applicable to studies in rabbits.

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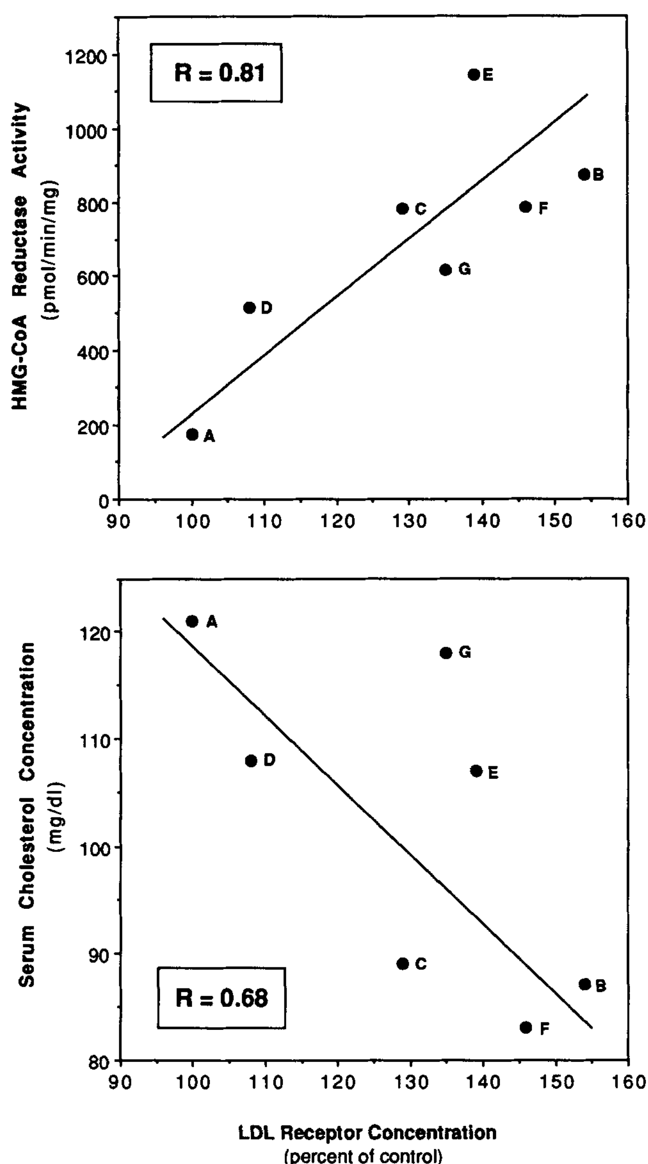

Fig. 11. Relationship between changes in total LDL receptor concentration, HMG-CoA reductase activity, and serum cholesterol levels after treatment with known modulators of cholesterol metabolism. Twenty-eight male golden Syrian hamsters (100–120 g) that had been housed in a reversed lighting cycle room for 1 week were divided into seven groups of four animals each. Animals received water and powdered chow containing either no further additions (control; A), 2% cholestyramine resin (B), 5% cholestyramine resin (C), 0.1% mevinolin (D), 5% cholestyramine resin and 0.05% mevinolin (E), 2% CP-88488 (F), or 0.2% CP-88818 (G) ad libitum for 4 days. On the morning of the fifth day, animals were anesthetized and sacrificed at the mid-dark phase of the lighting cycle, and 1.5-g liver samples were obtained for each animal for measurement of LDL receptor concentration. Liver tissues for all animals of a group were pooled and homogenized together in LDL receptor Homogenization Buffer (5 ml/g liver tissue). LDL receptor-containing membranes for each liver homogenate were isolated by sucrose density gradient centrifugation and extracted. Aliquots of the resulting soluble extracts containing 400 μg protein were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to nitrocellulose, and anti-LDL receptor immunoreactive proteins were visualized. Additional 0.5-g portions of liver tissue were obtained from each animal for measurement of hepatic HMG-CoA reductase activity. Blood samples were also obtained at the time of sacrifice and used for determination of serum cholesterol concentration. Shown are the activity of HMG-CoA reductase as a function of LDL receptor concentration (top) and serum cholesterol concentration as a function of LDL receptor concentration (bottom).

major role, to differing degrees, in the magnitude of serum cholesterol lowering by these agents. Taken together, these results indicate that changes in total hepatic LDL receptor concentration in experimental animals, as measured using this methodology, are reflective of changes in cholesterol homeostasis that occur in response to diverse pharmacologic modulations to cholesterol metabolism.

It is becoming increasingly apparent that a multitude of factors and mechanisms are involved in regulating receptor-dependent uptake of circulating LDL by the liver, and that transcriptional regulation (9, 76), post-transcriptional regulation (42, 44–47), receptor recycling regulation (77–79), and ligand affinity modulation (25, 43, 80), all play an important role in the control of cell surface LDL receptor concentration. As a result, it is unlikely that modulations in LDL receptor mRNA concentration and cell-surface LDL receptor activity will occur in parallel and accurately reflect the total concentration of LDL receptor molecules within the cell in all circumstances. It is thus important to evaluate not only modulations of LDL receptor transcription and cell surface LDL receptor activity, but also of other factors, such as total intracellular LDL receptor pool size, intracellular pool distribution, and trafficking of functional LDL receptors within the intracellular compartment and to the plasma membrane, to fully evaluate the complex regulation of LDL receptor expression.

As the methodology described in this report permits determination of total LDL receptor concentration independent of changes in mRNA concentration, cell surface to

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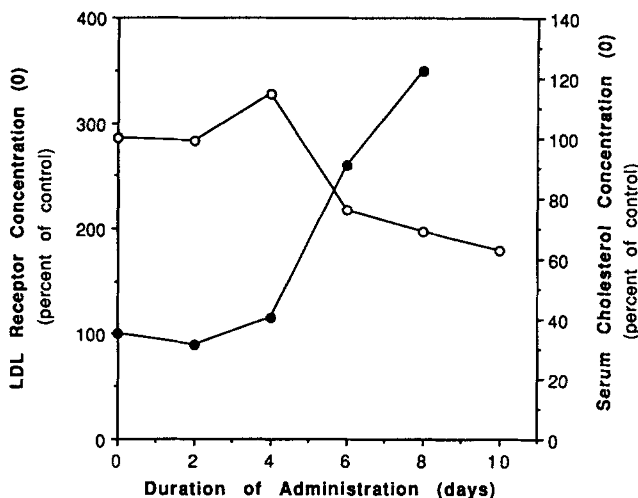

Fig. 12. Relationship between LDL receptor and serum cholesterol levels following treatment of hamsters with 17α-ethinyl estradiol. Forty male golden Syrian hamsters (100-120 g) that had been housed in a reversed lighting cycle room for 1 week were divided into ten groups of four animals each. All animals received water and powdered chow ad libitum. Animals in experimental groups were injected subcutaneously at the mid-dark phase of the lighting cycle with 17α-ethinyl estradiol in safflower oil at a dose of 5 mg/kg/day for the indicated number of days. Control groups received an identical volume of safflower oil by subcutaneous injection that lacked 17α-ethinyl estradiol. Initiation of subcutaneous injections to the various groups was staggered so that all animals reached the indicated duration of administration on the same day. On the final day of administration, 1 h after the last injection, animals were anesthetized and sacrificed, and 1.5-g liver samples were obtained for each animal. Liver tissues for all animals of a group were pooled and homogenized together in LDL receptor Homogenization Buffer (5 ml/g liver tissue). Blood samples were also obtained at the time of sacrifice and used for determination of serum cholesterol concentration. For each hepatic homogenate, LDL receptor-containing membranes were isolated by sucrose density gradient centrifugation and extracted as described in Materials and Methods. Aliquots of the resulting soluble extracts containing 400 μg protein were adjusted to 2% SDS and 0.2 M sucrose, applied without heating and without addition of β-mercaptoethanol to 6-mm wells of a 7.5% SDS-containing polyacrylamide gel. After electrophoresis, proteins migrating into the gel were electrophoretically transferred to nitrocellulose and anti-LDL receptor immunoreactive proteins transferred to nitrocellulose were visualized and quantitated by reflectance densitometry. Shown are the percentage changes in LDL receptor and serum cholesterol concentrations as a function of duration of administration of 17α-ethinyl estradiol. Control serum cholesterol concentrations averaged 118 ± 8 mg/dl.

intracellular LDL receptor ratios, and LDL binding affinity, this methodology not only circumvents difficulties associated with reliance on consistent contamination of the microsomal fraction with LDL receptor-containing membranes, but also circumvents ambiguities associated with estimation of total LDL receptor concentration based on measurements of cell surface LDL receptor activity, LDL receptor mRNA levels, or LDL binding affinities alone. Measurement of total LDL receptor concentration by this methodology should, therefore, be useful in combination with existing methods for measuring LDL receptor mRNA levels (15, 24, 35, 40), cell surface LDL binding (25, 28, 32-34, 41), LDL binding affinity (5, 25, 41, 61), and intracellular versus cell surface LDL receptor ratios (77, 78), to permit a more detailed evaluation of the molecular mechanisms responsible for in vivo modulation of LDL receptor expression.

Finally, although a variety of methods, such as ligand blotting or ELISA, exist (50-54, 59) and are also potentially useful for quantitating total LDL receptor concentration in membrane fractions after sucrose density gradient centrifugation, a Western immunoblotting procedure was chosen for these studies for the following reasons. First, immunoblotting allows for visualization of the full spectrum of immunoreactive proteins contained in a fraction and thus permits independent measurement, by reflectance densitometry, of only approximately sized LDL receptor molecules. Visualization of the LDL receptor by immunoblotting thus permits exclusion from measurement naturally occurring products of normal cellular LDL receptor degradation that may retain the C-terminal tail of the LDL receptor and thus remain antigenic. Use of an ELISA procedure for visualizing the LDL receptor using a C-terminal-recognizing antibody would not distinguish between signals produced from intact versus C-terminal-containing degraded LDL molecules and this would complicate interpretation, especially under experimental conditions that exhibit varying degrees of LDL receptor degradation (cf. ref. 79). However, for an antibody preparation whose recognition site lies sufficiently close to the LDL binding domain of the LDL receptor, such that only holoenzymes contained in the membrane extracts are measured, visualization by an ELISA technique might be an acceptable alternative to visualization by immunoblotting. Second, for an antiserum recognizing additional non-LDL receptor proteins, such as the antiserum used in these studies, it is imperative that measurement be made such that signal from extraneous immunoreactive proteins is not included in the measurement of the LDL receptor. For such an antiserum, measurement by ELISA is not a viable alternative. However, for an antibody preparation that is more highly monospecific [e.g., that of May et al. (59) or of Gherardi et al. (81)], visualization of the LDL receptors contained in extracts from sucrose density gradient-isolated membrane fractions using an ELISA procedure might represent an alternative to visualization by immunoblotting. Third, although use of a ligand blotting procedure would also allow measurement of only appropriately sized LDL receptor molecules and would similarly exclude crosstalk from extraneous LDL binding proteins, this method would only detect functional receptors and might underestimate total LDL receptor content by failing to detect those receptors in the process of recycling or translocating to the cell surface which may be in a latent, inactive form (47).

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Although not reported here, inclusion of a known concentration of purified LDL receptor in a separate lane on each immunoblot would allow conversion of LDL receptor concentrations from relative units (e.g., mm peak height) to specific units of ng LDL receptor protein per mg cellular protein which could then be used to compare, on a mass basis, amounts of LDL receptor proteins within the various intracellular pools, to compare LDL receptor protein concentration with rates of LDL receptor transcriptional activity, and also to compare "catalytic efficiency" of cell surface LDL receptors in terms of LDL binding and translocation activities per ng LDL receptor.

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