Identification of a Lipolysis-Stimulated Receptor That Is Distinct from the LDL Receptor and the LDL Receptor-Related Protein†

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ABSTRACT: This paper provides further characterization of a receptor that, in cells lacking the LDL receptor (FH fibroblasts), mediates lipoprotein binding, uptake, and degradation when incubated with oleate at concentrations not exceeding albumin binding capacity. This oleate-activated receptor is genetically distinct from the LDL receptor and is hereafter referred to as the lipolysis-stimulated receptor (LSR). Its apparent affinity was higher for triglyceride-rich lipoproteins (chylomicrons, VLDL) and for lipid emulsions supplemented with recombinant apoE, than for LDL which contains solely apoB. In contrast, VLDL isolated from a Type III hyperlipidemic patient ($apoE_{2/2}$ phenotype) failed to bind to the LSR. Five lines of evidence indicated that the LSR is distinct from the LDL receptor-related protein (LRP): (1) the LRP ligand, α_2-macroglobulin–methylamine (α_2-MG*), did not bind to the oleate-induced LDL binding site; (2) oleate had no effect on the binding of α_2-MG* to LRP; (3) the LRP-associated protein, RAP, which inhibits LRP, had no effect on the LSR; (4) binding of lipoproteins to LSR was independent of Ca^{2+}; and (5) LSR activity resolved as two proteins smaller than LRP (apparent molecular masses as determined by ligand blots: 115 and 85 kDa). That LSR provides a new candidate receptor contributing to the clearance of chylomicron remnants (CMR) is supported by the observation that LSR was inhibited by lactoferrin, a milk protein that delays CMR clearance when injected *in vivo*. Furthermore, in primary cultures of rat hepatocytes, oleate stimulated binding, uptake, and degradation of LDL with kinetic characteristics similar to that of LSR expressed in FH fibroblasts. LDL binding to LSR was also demonstrated in isolated liver membranes. Membranes from liver endocytic organelles showed marked (10–15-fold) enrichment in LSR activity when compared to total liver membranes; virtually no LSR activity was detected in liver mitochondrial membranes. We propose that LSR's primary function is to mediate the clearance of intestinally derived triglyceride-rich lipoproteins and that free fatty acids produced by the action of lipolytic enzymes on these particles are the signals activating this receptor.

Chylomicrons (CM)[1] are the lipoproteins produced by the intestine after a meal. Upon entering the circulation, CM-triglycerides (TG) are hydrolyzed by lipoprotein and hepatic lipases (Havel & Kane, 1975). This process takes place in the capillaries of muscle and adipose tissue as well as at the surface of fenestrated liver endothelium. CM lipolysis leads to the production of remnants which enter the space of Disse and are subsequently removed by hepatocytes (Sherrill & Dietschy, 1978).

The mechanism of CM remnant (CMR) removal has received considerable attention; however, a consensus on the molecular events underlying cellular uptake of these particles has not yet been reached. At least 2 and possibly 3 genetically and functionally distinct receptors are considered to be involved in the process. First, the LDL receptor, which binds CMR-apoE (Floren et al., 1981), is estimated to account for about half of their removal (Choi et al., 1991). Second, a 600-kDa membrane protein with homology to the LDL receptor, i.e., LDL receptor-related protein (LRP), was initially proposed by Herz et al. to serve as apoE-specific CMR receptor [for review, see Herz (1993)]. Indeed, LRP binds and internalizes β-VLDL enriched with recombinant apoE (Kowal et al., 1989). Also, it displays affinity for (1) chemically or enzymatically activated α_2-macroglobulin (α_2-MG*) (Strickland et al., 1990), (2) a 39-kDa receptor-associated protein (RAP) that selectively inhibits its activity (Herz et al., 1991; Williams, S. E., et al., 1992), (3) tissue-type plasminogen activator (Bu et al., 1992; Orth et al., 1992), (4) lactoferrin (Herz, 1993), (5) *Pseudomonas* exotoxin A (Kounnas et al., 1992), (6) chicken vitellogenin (Stifani et al., 1991), and (7) lipoprotein lipase (Beisiegel et al., 1991). Thus, its ability to bind apoE and lipoprotein lipase (LPL) supports the idea that LRP is a multifunctional receptor that contributes to the cellular uptake of CMR (Brown et al., 1991).

The LRP model fails, however, to explain several characteristics of CMR clearance. First, though LRP is expressed to the same extent in both hepatocytes and Kupffer cells [2 cell types which equally contribute to the removal of α_2-MG* (Moestrup et al., 1992)], CMR clearance takes place almost exclusively in hepatocytes (van Dijk et al., 1991, 1992a). Also, primary cultures of hepatocytes display decreased ability to

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[1] Abbreviations: apo, apoprotein; CM, chylomicrons; CMR, chylomicron remnants; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FFA, free fatty acids; FH, familial hypercholesterolemia; LDL, low-density lipoprotein; LPL, lipoprotein lipase; LRP, LDL receptor-related protein; LSR, lipolysis-stimulated receptor; α_2-MG, α_2-macroglobulin; α_2-MG*, methylamine-activated α_2-macroglobulin; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TG, triglycerides; VLDL, very-low-density lipoprotein.

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bind activated α_2-MG* without reducing the number of CMR and β-VLDL binding sites (van Dijk et al, 1992b). In addition, rat hepatocytes retain the ability to bind β-VLDL even after LRP is removed from the cell surface and sequestered in endocytic vesicles (van Berkel et al., personal communication). Finally, binding of β-VLDL and CMR to liver membranes was found not to require Ca^{2+}, while α_2-MG* binding was strictly Ca^{2+}-dependent (van Dijk et al., 1992a). These observations have led these investigators to postulate the existence of a specific CMR receptor biochemically distinct from LRP (van Dijk et al., 1992b).

We have recently shown that free fatty acids (FFA) activate a lipoprotein receptor that is genetically distinct from the LDL receptor, binds lipoproteins containing apo-B, and displays the highest affinity for TG-rich lipoproteins (Bihain & Yen, 1992). These observations led to the hypothesis that TG hydrolysis generates FFA that activate an otherwise silent lipoprotein receptor, here referred to as the lipolysis-stimulated receptor (LSR). To test this hypothesis, we have further characterized the biochemical properties of the LSR and searched for a possible identity with LRP. In this paper, we report that the LSR displays virtually all biochemical properties of a putative CMR receptor. LSR is distinct from LRP and the LDL receptor and appears to be linked to 2 plasma membrane proteins of apparent molecular mass 115 and 85 kDa.

EXPERIMENTAL PROCEDURES

Materials

$Na^{125}I$ was purchased from Amersham (Arlington Heights, IL). Oleic acid, bovine serum albumin (A2153) (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), human lactoferrin, insulin (bovine pancreas), dexamethasone, leupeptin, benzamidine, bacitracin, heparinase (H2519), and heparitinase (H8891) were obtained from Sigma Chemicals (St. Louis, MO). Triolein and egg phosphatidylcholine were purchased from Nuchek Prep (Elysian, MN) and Avanti Polar Lipids, Inc. (Alabaster, AL). Sodium suramin was obtained from FBA Pharmaceutical (West Haven, CT). Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin–streptomycin, glutamine, fetal bovine serum (FBS), and trypan blue were obtained from Gibco (Grand Island, NY). Collagenase A (*Clostridium histolyticum*) was purchased from Boehringer Mannheim Biochemicals (Meylan, France). Enzymatic colorimetric kits for determination of triglycerides, cholesterol, and phospholipids were purchased from Sigma Chemicals, Boehringer Mannheim Diagnostics (Indianapolis, IN), and Biochemical Diagnostics (Edgewood, NY), respectively. Polyethylene tubing, serving as catheters, was purchased from Clay Adams, Inc. (Parsippani, NJ).

Methods

Isolation and Preparation of Ligands for LSR and LRP. (*A*) *LDL.* Human LDL (1.025 < density (*d*) < 1.055 g/mL) was isolated by sequential ultracentrifugation of fresh plasma obtained from the local blood bank (Goldstein et al., 1983). All preparations were used within 2 weeks of their isolation. LDL was radioiodinated as previously described using the modified McFarlane's method (Bilheimer et al., 1972). ^{125}I-LDL was filtered (0.2-μm filter, Gelman, Ann Arbor, MI) immediately prior to use.

(*B*) *VLDL.* Human VLDL ($d < 1.006$ g/mL) was isolated by ultracentrifugation of fresh human plasma obtained from fasting normolipidemic volunteers. Type III human VLDL ($d < 1.006$ g/mL) was similarly isolated from plasma of a Type III hyperlipidemic subject (apo$E_{2/2}$ phenotype) attending the clinic of Dr. Jean Davignon (Davignon et al., 1988).

(*C*) *Chylomicrons.* Sprague-Dawley male rats (150–200 g) were lightly anesthetized with sodium pentobarbital. The abdominal cavity was opened, and catheters (PE50) were inserted in the duodenum and in the main abdominal lymph duct (Mjos et al., 1975). Through the duodenal catheter, a mixture containing 10% (w/v) glucose, 12% (w/v) olive oil, and 0.1% (w/v) egg yolk was infused at a rate of 3 mL/h. Intestinal lymph was collected and chylomicrons were isolated by centrifugation (200000*g*, 60 min, 4 °C) in a SW41 Beckman rotor.

(*D*) *Triolein/Phosphatidylcholine Emulsions.* Triolein (TO) and egg phosphatidylcholine (PC) were solubilized in chloroform and mixed to provide a final ratio of 4:1 (TO/PC, w/w) (Derksen & Small, 1989). The organic solvent was then removed by evaporation under nitrogen. Phosphate-buffered saline (PBS) containing 0.24 mM EDTA, pH 7.4, was added (10 mL per 250 mg of lipid), and the solution was sonicated (Branson sonifier, 4 × 15 min, setting 3, 50% pulse). After this, the emulsion was filtered through a 0.45-μm filter. The size of the emulsion was determined by gel filtration chromatography using Sepharose CL2B (1 cm × 100 cm) and found to correspond to that of normal human VLDL (50 × 10^6 kDa). Recombinant apoE (0.3 mg/mL water) (Vogel et al., 1985) kindly provided by Dr. R. J. Deckelbaum was added to 50-μL emulsion and incubated for 1 h at room temperature. To remove any free apoprotein, the apoE emulsion was applied to a Bio-Gel A-0.5 column (1 cm × 8 cm; Bio-Rad, Richmond, CA) equilibrated in PBS containing 0.24 mM EDTA, pH 7.4. The column was eluted with the same buffer, and fractions were collected. Emulsion not supplemented with apoE was also applied to a similar column. ApoE-containing emulsions were prepared and used immediately.

(*E*) α_2-*Macroglobulin.* Native α_2-macroglobulin (α_2-MG), α_2-MG activated by methylamine (α_2-MG*), and RAP were generously provided by Dr. D. K. Strickland. α_2-MG* preparations were iodinated using Iodobeads (Pierce, Rockford, IL), as per the manufacturer's instructions. For analysis of LRP activity, ^{125}I-α_2-MG* was incubated with FH fibroblasts at 37 °C under conditions specified in the figure legends. The cells were then washed (Goldstein et al., 1983) and recovered in 0.1 N NaOH. Degradation products were determined by TCA precipitation followed by chloroform extraction (Goldstein et al., 1983).

Human Fibroblasts. All tissue culture experiments requiring cells lacking the LDL receptor were conducted with confluent human fibroblasts isolated from a subject homozygous for familial hypercholesterolemia (GM00486A, NIGMS human genetic mutant cell repository, Camden, NJ). The cells were plated in 36-mm dishes at a density of 1.2 × 10^5 cells/dish and cultured for 4–5 days in DMEM containing 20% (v/v) FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine. Binding, uptake, and degradation of lipoproteins mediated by the oleate-activated receptor were measured in these cells following the procedure described previously (Bihain & Yen, 1992).

Primary Culture of Rat Hepatocytes. Primary cultures of rat hepatocytes were isolated by the method described by Guguen-Guillouzo and Guillouzo (1986). Briefly, livers of Sprague-Dawley male rats (250–300 g) were perfused at a rate of 30 mL/min with 500 mL of PBS buffer containing 10 mM HEPES, pH 7.65, followed by perfusion at 15 mL/min

with 300 mL of the same buffer, pH 7.65, supplemented with 0.025% collagenase and 5 mM $CaCl_2$. The liver was removed, and the Gilsson capsule opened. Cells were gently dispersed to obtain a suspension, which decanted for 20 min through a nylon membrane (60-μm pore size) to eliminate cellular debris and nonparenchymal cells. Cells were pelleted by centrifugation (50*g*, 30 s) and resuspended in PBS buffer containing 10 mM HEPES; this washing step was repeated. After the second spin, the hepatocytes were resuspended in hepatocyte culture medium: DMEM media containing 10% (v/v) FBS, 2 mM glutamine, and 100 units/mL each of penicillin and streptomycin. The cells were pelleted (50*g*, 30 s) and then resuspended in culture medium containing insulin (5 μg/mL), and dexamethasone (4 μg/mL). Cell viability, estimated by trypan blue exclusion, was always greater than 85%. Cells were then plated in 36-mm dishes at a density of 1.2×10^6 viable cells/dish and maintained in a 5% CO_2 incubator. Media were renewed after 4 and 24 h of culture. At day 2 of culture, binding, uptake, and degradation of ^{125}I-LDL in the presence or absence of 0.2 mM oleate were measured using a procedure identical to that used for FH fibroblasts (Bihain & Yen, 1992).

Rat Liver Membrane Isolation. Preparations of rat liver membranes were isolated according to the procedure of Basu et al. (1978). Briefly, livers from five male Sprague-Dawley rats (175–200 g) were perfused through the portal vein with ice-cold saline containing 2 mM EDTA, pH 7.4 (100 mL passed by syringe through 1 liver in 2 min). All subsequent isolation steps were performed at 4 °C. The livers were removed, rinsed further in saline–EDTA, and finely minced on a cold glass plate using a razor blade. The liver slices were quickly transferred to 100 mL of a solution of 0.25 M sucrose and 2 mM EDTA, pH 7.4, containing the protease inhibitors: leupeptin (10 μg/mL), benzamidine (100 μg/mL), bacitracin (800 μg/mL), and PMSF (40 μg/mL). This mixture was homogenized 5 times with a loose-fitting pestle, and once with a tight-fitting pestle, and then centrifuged at 500*g* for 10 min to pellet cell debris. The supernatant was respun at 3500*g* for 15 min to remove nuclear material. The second supernatant was homogenized 5 times with the tight-fitting pestle and then centrifuged at 120000*g* for 1 h in a Beckman SW28 rotor. The resulting membrane-containing pellet was carefully resuspended in 2 volumes of 0.1 M phosphate buffer containing 0.15 M NaCl + 2 mM EDTA, pH 8, using a 21G needle, and was then washed by repeating the latter centrifugation and resuspension steps. This membrane fraction was stored for up to 3 weeks in the dark, under N_2 at 4 °C in the presence of the proteolytic inhibitor cocktail described above.

Liver Organelle Isolation. Endosomal membranes were isolated exactly as reported in an earlier publication (Hornick et al., 1992). Purity was assessed by electron microscopy.

Mitochondrial membranes were prepared by the method of Schnaitman and Greenwalt (1968). Briefly, livers from 250–300-g Sprague-Dawley rats were excised and placed in ice-cold isolation media containing 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES buffer, and 0.5 mg/mL BSA and adjusted to pH 7.4 with KOH. Livers were homogenized with four strokes of a motorized Potter–Elvehjem homogenizer and diluted with isolation media to give a 10% homogenate solution. The homogenate was centrifuged at 560*g* for 15 min, and the pellet was discarded. The supernatant was spun at 7000*g* and the fluffy layer discarded. The pellet from this spin was resuspended and washed twice. This pellet was then assayed for total mitochondrial protein. Stock 2% digitonin solutions containing 5% BSA in isolation medium were added in equal volume to solutions containing 100 mg/mL mitochondrial protein, stirred gently for 15 min, and then diluted with 3 volumes of isolation medium. This suspension was centrifuged at 9500*g*. The resulting pellet was resuspended in an equal amount of isolation media and respun, and both supernatants were combined. The resulting "low-speed pellet" contains mainly mitochondrial inner membrane. Centrifugation of the combined supernatants at 144000*g* for 1 h yielded a "high-speed pellet" enriched in outer mitochondrial membrane based on the 30-fold enrichment of the enzyme monoamine oxidase, an established marker enzyme for outer mitochondrial membrane. Both inner and outer mitochondrial membranes were tested for LSR activity; no significant activity was found in either type.

Binding of ^{125}I-LDL to Isolated Membranes. For measurement of ^{125}I-LDL binding in the presence of oleate, membranes were diluted to a concentration of 1 mg of protein/mL with incubation buffer (0.1 M phosphate buffer containing 2 mg/mL albumin and 2 mM EDTA, pH 7.4). Immediately prior to use, this fraction was sonicated (Bioblock scientific Vibracell, 30 s, power 2.5, 25% pulse). Aliquots of membranes (10–100 μg of protein) were placed in microfuge tubes and incubated at 37 °C for the indicated times with ^{125}I-LDL in the absence or presence of oleate (final volumes ranged from 100 to 250 μL). The appropriate volume of oleate was added to this mixture from a stock solution (100 mM) in 2-propanol; the sample was then quickly and briefly agitated to disperse the oleate. Membrane-bound ^{125}I-LDL was separated from unbound LDL by layering a 80–200-μL aliquot over 200–600 μL of 5% (w/v) BSA and centrifuging at 14500*g* for 25 min at 4 °C. The supernatant was carefully removed, and the bottoms of the tubes were cut and counted to measure the amount of membrane-bound ^{125}I-LDL.

Ligand Blotting. FH fibroblasts were grown to confluency in 100-mm Petri dishes, rinsed twice in PBS, pH 7.4, and recovered either (1) by scraping or (2) by 3-min incubation at room temperature with 0.5 mg/mL trypsin [after 3-min incubation, trypsin was inactivated with 5 volumes of 10% (v/v) fetal bovine serum in DMEM]. The cells were pelleted by 5-min centrifugation (1000*g*, 5 min) and washed once with PBS. The pellets were resuspended in PBS containing 2% Triton X-100 and 20 μg/mL PMSF (35 μL/cells from one Petri dish) and sonicated (Branson sonifier, 3 × 30 s, 50% pulse, setting 1). The debris were removed by centrifugation (3000*g*, 1 min), and the supernatant was diluted with the sample buffer (30 μL of sample buffer for each 70 μL of supernatant) and applied to a polyacrylamide gradient gel (4%–12%, 1.5-mm thickness, 100 μL/lane) containing 0.1% SDS (40 V, 23 h). After separation, the proteins were transferred to nitrocellulose (Hoeffer transfer unit, 20 V, 18 h). The nitrocellulose membranes were blocked overnight at 4 °C in PBS containing 3% BSA, followed by incubation for 60 min at 37 °C in PBS, pH 7.4, containing 0.2% BSA and 20 μg/mL ^{125}I-LDL in the presence or absence of 0.5 mM oleate. The nitrocellulose membranes were washed rapidly twice in washing buffer (PBS containing 0.2% BSA) and then incubated 3 times with buffer changes every 20 min with PBS containing 0.2% BSA; the membrane was then exposed for 1 h on X-ray film (Kodak X-OMAT).

RESULTS

Our first objective was to determine LSR's ability to bind intestinally-derived lipoproteins. As shown in Figure 1, CM affinity (closed triangles) was greater than that of LDL (closed circles) and VLDL (diamonds) and was almost as high as that of triolein phosphatidylcholine emulsions supplemented

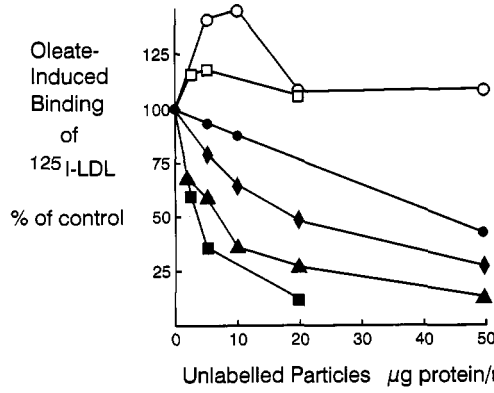

FIGURE 1: Competition of triolein/phosphatidylcholine emulsions supplemented or not with apoE, LDL, VLDL, Type III VLDL, or chylomicrons with ^{125}I-LDL for binding to LSR. FH fibroblasts were incubated for 4 h at 37 °C with 1 mM oleate, 20 μg/mL ^{125}I-LDL (specific activity 138 cpm/ng), and the indicated concentrations of unlabeled triolein/phosphatidylcholine emulsions supplemented (■) or not (□) with apoE, or LDL (●). In a separate experiment, FH fibroblasts were incubated for 1 h at 37 °C with 1 mM oleate, 20 μg/mL ^{125}I-LDL (specific activity 238 cpm/ng), and the indicated concentrations of unlabeled rat chylomicrons (▲), human normal VLDL (♦), or VLDL isolated from a Type III hyperlipidemic subject (○). After this, cell monolayers were washed, and the amounts of ^{125}I-LDL bound to the cell surface were determined as described under Experimental Procedures. Each point represents the average of duplicate determinations.

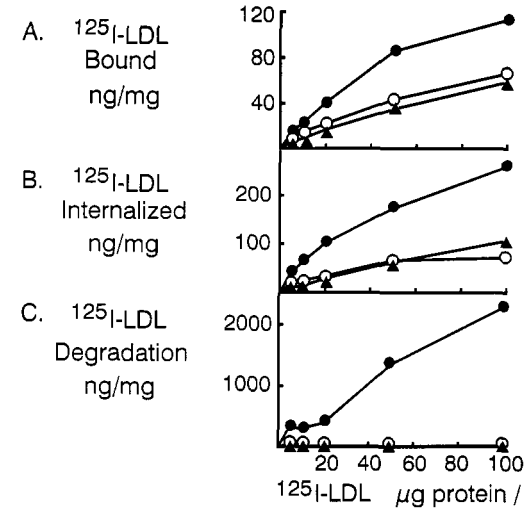

FIGURE 2: Effect of 0.2 mM oleate on ^{125}I-LDL binding, uptake, and degradation in FH fibroblasts. FH fibroblasts were incubated at 37 °C for 2 h without 0.2 mM oleate (○) or with 0.2 mM oleate in the absence (●) or presence (▲) of 1 mg/mL unlabeled LDL and increasing concentrations of ^{125}I-LDL (specific activity 114 cpm/ng). The cell monolayers were then washed, and ^{125}I-LDL binding (A), uptake (B), and degradation (C) were determined as described in the Experimental Procedures. Each point represents the average of duplicate determinations. The average cellular protein content ± standard deviation was 130 ± 2, 127 ± 5, and 137 ± 3 μg/dish for cells incubated without oleate, with oleate, and with oleate and unlabeled LDL, respectively.

with recombinant apoE (closed squares). Thus, LSR recognized lipid particles containing either solely apoB, both apoB and apoE, or solely apoE. The difference of affinity between apoE and apoB offers a plausible explanation for the relatively higher affinity of CM. In keeping with this interpretation was the lack of binding of VLDL isolated from a Type III hyperlipidemic patient with the apoE$_{2/2}$ phenotype (open circles). Therefore, LSR ligand specificity is consistent with a function as CMR receptor.

Although the concentrations of FFA existing at the sites of lipolysis have not been accurately measured, an obvious criticism of our hypothesis originated from the fact that maximal LSR recruitment occurred only with oleate concentrations exceeding albumin binding capacity (Bihain & Yen, 1992). The next issue was thus to determine whether FFA in equilibrium with albumin exerted significant stimulation of LSR. Significant increases in ^{125}I-LDL binding, uptake, and degradation were observed when FH fibroblasts were incubated with 0.2 mM oleate complexed with 2 mg/mL albumin (Figure 2). The conditions of this experiment were chosen to correspond to those recommended to efficiently deliver oleate to cultured cells (Goldstein et al., 1983). In the absence of oleate (open circles), only a limited amount of ^{125}I-LDL was bound to the cell surface and internalized. Similar values were found after incubation in the presence of oleate and excess unlabeled LDL (closed triangles). However, neither incubation in the absence of oleate nor incubation with oleate and excess unlabeled LDL led to significant degradation of the particles (Figure 2, panel C). Therefore, in the homozygous FH fibroblasts, only the oleate-stimulated pathway, but not the nonspecific binding to plasma membrane, mediated the rapid proteolytic degradation of LDL.

We next sought to identify the protein responsible for this activity. First, we postulated that LRP was responsible for LSR activity. To test for a possible identity between LRP and LSR, competition experiments were conducted. Table 1 shows that methylamine-activated-α_2-macroglobulin (α_2-MG*) at a concentration exceeding its K_d by 100-fold did not inhibit oleate-induced binding uptake and degradation of ^{125}I-LDL in FH fibroblasts, while unlabeled LDL was an efficient competitor. In addition, the binding of ^{125}I-α_2-MG* to its receptor remained unchanged by the addition of oleate, even when its concentration exceeded albumin binding capacity, or when unlabeled LDL was simultaneously added (data not shown). Thus, the oleate-induced LDL binding site and the α_2-MG* binding site are distinct; the conformation of either site remains unaffected by the occupancy of the other.

Table 1. Competition of LDL of α_2-MG* with ^{125}I-LDL for Oleate-Induced Binding, Uptake, and Degradation in FH Fibroblasts[a]

	oleate-induced (ng/dish)		
	binding	uptake	degradation
no addition	4.1	3.2	104.1
excess α_2-MG*	4.0	3.1	141.9
excess LDL	nd	nd	nd

[a] FH fibroblasts were incubated at 37 °C for 90 min with 0.5 mM oleate, 10 μg/mL ^{125}I-LDL, and either unlabeled LDL (500 μg/mL) or unlabelled α_2-MG* (100 μg/mL). Cell monolayers were washed, and the amounts of ^{125}I-LDL binding, uptake, and degradation were measured as described in the Experimental Procedures. Average values of duplicate determinations are represented as the oleate-induced binding, uptake, and degradation of ^{125}I-LDL (nd = not detectable).

Because LRP bears multiple domains, the possibility remained that it accounted for LSR activity. The findings that RAP completely blocks LRP binding of its ligands (Herz, 1993) provided the probe to further examine this hypothesis. In contrast to its pronounced inhibitory effect on ^{125}I-α_2-MG* uptake and degradation (Figure 3, panels A and B), RAP did not significantly inhibit oleate-induced binding, uptake, and degradation of ^{125}I-LDL (Figure 3, panels C and D).

In keeping with the notion that LSR was distinct from LRP, LSR activity was observed after incubations in Ca^{2+}-free media. The increase in ^{125}I-LDL uptake and degradation induced by 90-min incubation at 37 °C of FH fibroblasts with 0.1 mM oleate averaged 27 and 333 ng/mg of protein versus 20 and 339 ng/mg of protein in dishes incubated with and

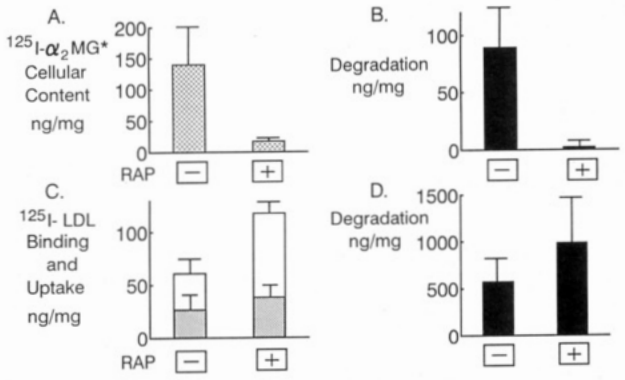

FIGURE 3: Effect of RAP on ^{125}I-α_2-MG* uptake (A) and degradation (B) and on oleate-induced ^{125}I-LDL binding, uptake (C), and degradation (D) in FH fibroblasts. One set of FH fibroblasts were incubated for 90 min at 37 °C with 1 μg/mL ^{125}I-α_2-MG* (specific activity 8177 cpm/ng) in the presence or absence of RAP (5 μg/mL) (panels A and B). A second set of cells were incubated for 90 min at 37 °C with 50 μg/mL ^{125}I-LDL (specific activity 151 cpm/ng) and 0.2 mM oleate in the presence or absence of 5 μg/mL RAP (panels C and D). Cell monolayers were washed, and the amount of ligand binding, uptake, and degradation was measured as described in the Experimental Procedures. For panel C, the ^{125}I-LDL binding to LSR is represented as a hatched bar and the amount of ^{125}I-LDL uptake is shown as an open bar. Results are mean values and standard deviations of quadruplicate determinations. Cellular protein contents were 108 ± 2 (no RAP) and 111 ± 2 μg/dish (+RAP) for panels A and B; 153 ± 8 (no RAP) and 144 ± 5 μg/dish (+RAP) for panels C and D.

without 2 mM $CaCl_2$, respectively.[2] Since LRP is a Ca^{2+}-binding protein (Herz et al., 1988; van Dijk et al., 1992a) that depends on this cation for activity, it is unlikely to account for LSR activity.

To identify the LSR protein, ligand blotting experiments were performed. Two proteins of apparent molecular masses 115 and 85 kD bound ^{125}I-LDL only when in the presence of oleate (Figure 4, lane 1 vs lane 2). These proteins are degraded after cell treatment for 3 min with 0.5 mg/mL trypsin (Figure 4, lane 3). This is consistent with their exposure on the external surface of the plasma membrane. Control experiments showed that, under the same electrophoretic conditions, LRP, detected by $^{45}Ca^{2+}$ blotting, migrated with an R_f of 0.25, i.e., far above the largest oleate-induced ^{125}I-LDL binding protein (R_f 0.55). The apparent molecular mass of the latter is very similar to that of the LDL receptor. However, cells used in these experiments lack the promoter region and first exon of the LDL receptor gene (Hobbs et al., 1987). Also, no binding activity was observed in the absence of oleate. Further, we have previously shown that binding of LDL to the LDL receptor is inhibited by oleate (Bihain et al., 1989). Finally, the ligand blot was obtained in the absence of Ca^{2+}, while binding of LDL to the LDL receptor is Ca^{2+}-dependent (Goldstein et al., 1977). Therefore, LSR activity appears to be mediated by 2 membrane proteins distinct from the LDL receptor and the LRP.

With this perspective of a new candidate for the function of the CMR receptor, we characterized the effect on LSR activity of lactoferrin—a milk protein that delays CMR clearance when injected into rats (Huettinger et al., 1988). The inhibitory effect of lactoferrin on oleate-induced binding, uptake, and degradation of ^{125}I-LDL in homozygous FH fibroblasts was parallel and dose-dependent (Figure 5, panel A). In contrast, there was no inhibitory effect on the uptake and degradation of ^{125}I-α_2-MG* (Figure 5, panel B). This

[2] We have previously shown that FFA concentrations ≥0.2 mM exert cytotoxic effects in Ca^{2+}-free media (Bihain & Yen, 1992); to prevent this, lower oleate concentrations (0.1 mM) were used in experiments using Ca^{2+}-free media.

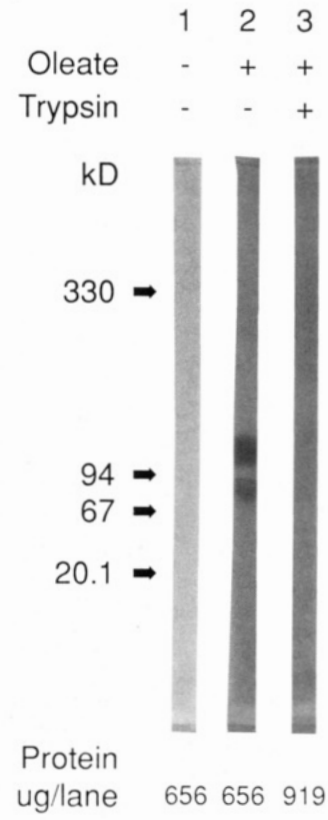

FIGURE 4: LDL binding to FH fibroblast lysates in the presence or absence of oleate after SDS–PAGE and electrotransfer to nitrocellulose membrane. FH fibroblasts were recovered by scraping (lanes 1 and 2) or by trypsinization (lane 3), solubilized in PBS containing 2% Triton and PMSF (20 μg/mL), separated on a 4%–12% gradient SDS–PAGE, and transferred to nitrocellulose. After blocking with 3% albumin in PBS, the membranes were incubated for 1 h at 37 °C with 20 μg/mL ^{125}I-LDL in the absence of oleate (lane 1) or in the presence of 0.5 mM oleate (lanes 2 and 3), washed, and then subjected to autoradiography.

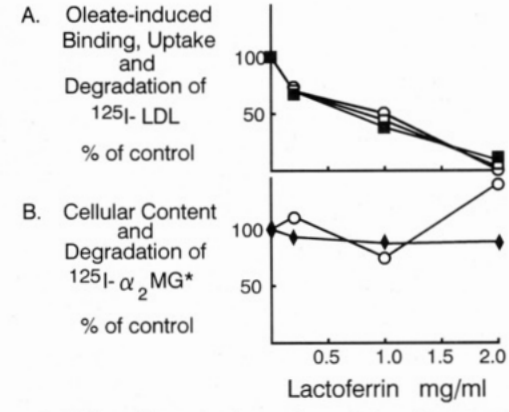

FIGURE 5: Effect of lactoferrin on oleate-induced ^{125}I-LDL binding, uptake, and degradation (A) and on ^{125}I-α_2-MG* uptake and degradation (B) in FH fibroblasts. One set of FH fibroblasts were incubated for 2 h at 37 °C with 50 μg/mL ^{125}I-LDL (specific activity 184 cpm/ng), 0.2 mM oleate, and the indicated concentrations of human lactoferrin (A). A second set of cells were incubated for 2 h at 37 °C with 1 μg/mL ^{125}I-α_2-MG* (specific activity 8177 cpm/ng) and the indicated concentrations of lactoferrin (B). After this, the cell monolayers were washed; ^{125}I-LDL binding (■), uptake (□), and degradation (○) and cell-associated (♦) and degraded (◇) ^{125}I-α_2-MG* were measured as described in the Experimental Procedures. Each point is the average of duplicate determinations. Cellular protein contents were 213 ± 15 and 223 ± 14 μg/dish for panels A and B, respectively.

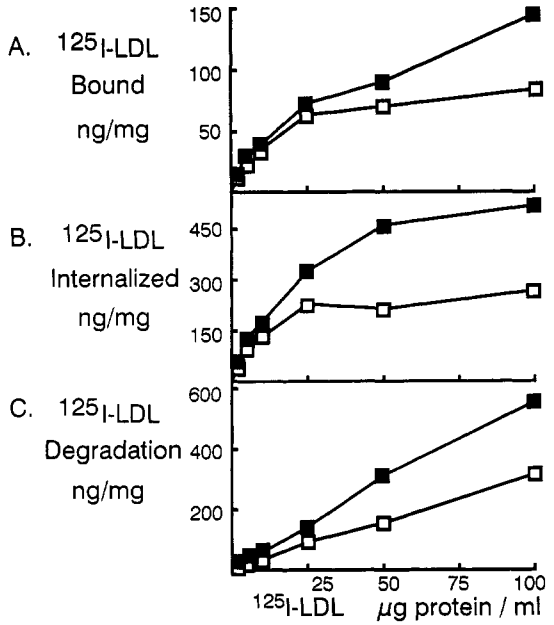

FIGURE 6: Effect of 0.2 mM oleate on ^{125}I-LDL binding, uptake, and degradation in primary culture of rat hepatocytes. Primary cultures of rat hepatocytes were isolated as described in the Experimental Procedures and plated in DMEM containing 10% FBS, insulin (5 μg/mL), and dexamethasone (4 μg/mL). Four hours after plating, the culture media were changed, and they were again replaced after 24 h. After 48 h of culture, the cells were incubated for 4 h at 37 °C with increasing concentrations of ^{125}I-LDL (specific activity 157 cpm/ng) in the presence (■) or absence (□) of 0.2 mM oleate. After this, the cells were washed, and binding (panel A), uptake (panel B), and degradation (panel C) of ^{125}I-LDL were determined as described in the Experimental Procedures. Each point represents the average of duplicate determinations. Cellular protein contents were 482 ± 27 and 448 ± 24 μg/dish for cells incubated in the absence or presence of oleate, respectively.

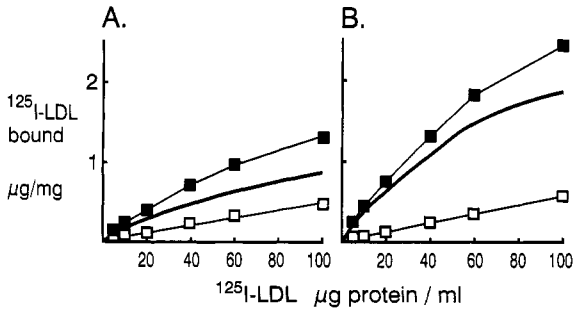

FIGURE 7: Effect of 0.2 mM oleate on the binding of ^{125}I-LDL to rat hepatocyte membranes as a function of ^{125}I-LDL concentration. Aliquots of isolated hepatocyte membrane were placed in microfuge tubes to achieve 100 μg of protein in 250 μL of 0.1 M phosphate buffer containing 2 mg/mL albumin and 2 mM EDTA, pH 7.4. The membranes were incubated at 37 °C for 4 h without (panel A) and with (panel B) 0.2 mM oleate and the indicated concentrations of ^{125}I-LDL (■). A second set of samples were incubated under similar conditions, except that unlabeled LDL were added to achieve a final concentration of 2 mg/mL (□). Membrane-bound ^{125}I-LDL were separated from unbound ^{125}I-LDL by centrifugation as described in the Experimental Procedures. After washing, the membrane pellets were cut and counted. Each point represents the average of duplicate determinations; the binding component competed for by unlabeled LDL is represented as a bold line.

latter observation is similar to those of Huettinger et al. (1992). In addition, transferrin, another member of the iron transporting protein family, but one that is unable to delay CMR clearance (Huettinger et al., 1988), had no detectable effect on LSR activity (data not shown). To determine whether LSR is also expressed in cells that directly participate in lipoprotein clearance, the effect of FFA on lipoprotein uptake by rat hepatocytes in primary culture was tested. Addition of 0.2 mM oleate to the incubation media of these cells increased binding, uptake, and degradation of ^{125}I-LDL (Figure 6). The kinetic characteristics of ^{125}I-LDL binding in the presence of oleate were similar to those observed in FH fibroblasts (Figure 2). The main difference between FH fibroblasts and rat hepatocytes was that the latter degraded significant amounts of ^{125}I-LDL in the absence of oleate; this degradation was attributed to the activity of the LDL receptor (Kovanen et al., 1979). Thus, oleate increased lipoprotein uptake and degradation in hepatocytes to levels compatible with that of an active LSR; however, the relative contribution of the LDL receptor and the LSR to this process remains undetermined.

Alternative experimental approaches were developed to address this issue. Hepatocyte membranes were prepared and tested for their ability to bind ^{125}I-LDL in conditions in which the LDL receptor is nonfunctional [Ca^{2+}-free media supplemented with 2 mM EDTA (Goldstein et al., 1977)]. In these experiments, oleate had a significant stimulatory effect on ^{125}I-LDL binding to rat liver membranes (Figure 7). However, even in the absence of oleate a limited number of LDL binding sites were detected (Figure 7, panel A). Preincubation of rat liver membranes for 15 min at 37 °C with 0.5 mg/mL trypsin, or incubation in the presence of 2 mg/mL lactoferrin or with 10 mM suramin, decreased oleate-induced binding of ^{125}I-LDL by 88%, 65%, and 85%, respectively. By contrast, the preincubation of liver membranes for 90 min at 37 °C with 10 units/mL heparinase or heparitinase, separately or in combination, had no inhibitory effect on oleate-induced ^{125}I-LDL binding. None of these treatments significantly affected the binding of ^{125}I-LDL to liver membranes in incubations without oleate. Therefore, in spite of their similar affinity for LDL, oleate-induced and oleate-independent binding sites are of a different biochemical nature. The oleate-induced binding site was mediated by a trypsin-sensitive protein distinct from proteoglycans and was inhibited by the very same compounds that reduce LSR activity in FH fibroblasts.

Taken together, these data indicate that oleate-induced binding of ^{125}I-LDL effectively represents the activity of a receptor that mediates endocytosis of lipoproteins. Because lipoproteins have been shown to bind to apparently irrelevant membrane proteins (Beisiegel et al., 1988), we sought additional evidence supporting LSR implication in lipoprotein clearance. Rat liver membranes were separated into three fractions: total liver membranes, endocytic organelles, and mitochondria. These membranes were then tested for their ability to bind ^{125}I-LDL in the presence and absence of oleate. As shown in Figure 8, LSR binding activity was increased 10–15-fold in membranes of endocytic organelles when compared to total liver membranes, but was virtually undetectable in mitochondrial membranes. These results further supported the notion that oleate-induced binding of ^{125}I-LDL represents the activity of a receptor that actively participates in the endocytosis of lipoproteins.

DISCUSSION

Data presented here indicate that oleate in equilibrium with albumin stimulates the activity of an apoB,E receptor genetically distinct from the LDL receptor. Its apparent affinity for apoE on triglyceride-rich lipid particles is greater than for LDL apoB. The affinity of VLDL from a subject with Type III hyperlipidemia was much lower than that of normal VLDL. This defective binding can result from (1)

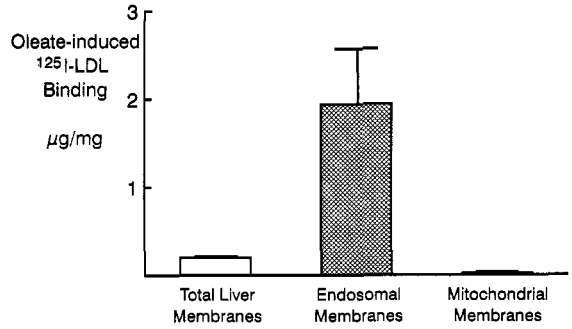

FIGURE 8: Effect of oleate on the binding of ^{125}I-LDL to rat total liver membrane, hepatocyte endosomal, or hepatocyte mitochondrial membrane fractions. Aliquots of total liver membranes, endosomal membranes, and mitochondrial membranes were added to microfuge tubes to achieve 10 μg of protein in 100 μL of 0.1 M phosphate buffer containing 2 mg/mL albumin and 2 mM EDTA, pH 7.4. After this, 20 μg/mL ^{125}I-LDL were added and the membranes were incubated for 60 min, at 37 °C, with and without 0.1 mM oleate. Membrane-bound ^{125}I-LDL were separated from unbound ^{125}I-LDL by centrifugation as described in the Experimental Procedures. After washing, the membrane pellets were cut and counted. Results are the mean ± SEM of triplicate determinations of the oleate-induced increase in ^{125}I-LDL binding.

the mutation responsible for the apoE$_{2/2}$ phenotype (Rall & Mahley, 1992), (2) the abnormal lipid composition of Type III VLDL, or (3) a combination of both. Nevertheless, the defective binding of Type III TG-rich lipoproteins to the LSR provides a new molecular model for the altered remnant metabolism that occurs in this form of hyperlipidemia. LSR therefore appears as an attractive candidate for the function of remnant receptor. In support of this hypothesis are the observations that LSR (1) is strongly inhibited by lactoferrin, a milk protein that delays the clearance of CMR; (2) is expressed not only in FH fibroblasts but also in hepatocytes, the cells that contribute to most CMR removal (van Dijk et al., 1991, 1992a); (3) is evidenced in membranes isolated from rat liver; and (4) is markedly enriched in membranes isolated from hepatocyte endocytic organelles, but not in mitochondrial membranes.

The latest candidate for the function of CMR receptor is the LRP. The experiments presented here were initially designed to determine if LRP was responsible for LSR activity. Competition studies using α_2-MG* and LDL failed, however, to demonstrate any reduction of binding induced by one ligand on the other. Considering the very large size of LRP (Herz, 1993), it remains possible that the α_2-MG* binding site and a putative LRP-mediated oleate-induced LDL binding site are located on domains sufficiently apart to prevent interference. Because LDL particles are 5–10-fold larger than LRP, it is difficult to envision that binding of LDL to LRP would fail to create any degree of steric hindrance and to decrease the binding of α_2-MG*. Nonetheless, additional evidence was sought to establish that LSR was a protein distinct from LRP. RAP was found to selectively suppress LRP function without reducing the activity of the LSR. Furthermore, in both isolated membranes and intact cells, binding of LDL to LSR was Ca^{2+}-independent. Finally, results of ligand blotting experiments using human FH fibroblasts implicates two plasma membrane proteins of apparent molecular mass 115 and 85 kDa as responsible for the LSR activity. It must be noted that since these candidate proteins were separated under nonreducing conditions, their estimated molecular masses therefore remain imprecise. However, the difference between the sizes of LRP and these candidate proteins is so large that it is unlikely to be explained by the inaccuracy inherent to this method.

Another lipoprotein receptor candidate has recently been cloned by Takahashi et al. (1992). This protein of 96 kDa exhibits striking homology to the LDL receptor, and binds VLDL and β-VLDL in transfected LDL receptor-deficient Chinese hamster ovary cells. The molecular mass of this putative VLDL receptor is similar to those of the LSR candidate proteins. Unlike LSR, the cloned VLDL receptor is reported not to be expressed in hepatocytes nor to be able to bind LDL (Takahashi et al., 1992). It should be noted however that these latter observations do not rule out the identity of LSR and the VLDL receptor since the absence of VLDL receptor expression in rabbit liver may not extend to other species. Clearly, further characterization and sequence analysis of LSR proteins are needed to determine whether the VLDL receptor is responsible for LSR activity.

Our hypothesis, therefore, is that, besides the LDL receptor and the LRP, at least one other receptor contributes to CMR removal. The issue now is to define the extent of the contribution of each of these pathways. Three sets of observations support the contribution of the LDL receptor: (1) it binds CMR-apoE and mediates cellular uptake of these particles (Floren et al., 1981); (2) it does not bind abnormal apoE-phenotypes, e.g., apoE$_{2/2}$, thereby providing a potential contribution to the pathogeny of Type III hyperlipidemia (Rall & Mahley, 1992); and (3) immunological inhibition of the LDL receptor delays CMR clearance in mice (Choi et al., 1991).

Arguments supporting LRP contribution to CMR clearance are fewer. Indeed, it is difficult to reconcile the site of LRP expression with that of CMR clearance (van Dijk et al., 1991, 1992a,b). Further analysis of the LRP hypothesis encounters significant obstacles. First, LRP binds apoE, but only when it is added to lipoproteins already containing multiple copies of this apoprotein (Kowal et al., 1989). It has been postulated that apoE is secreted to assist recapture of the remnant particles (Brown et al., 1991). Morphological evidence in support of the presence of apoE in the space of Disse has been provided (Hamilton et al., 1990). However, it has not been shown that such apoE is free of lipid particles and/or that it can associate with lipid particles entering the space of Disse. Clearly, apoE binding to LRP is of low affinity and requires cooperation of multiple apoE molecules. This is likely to increase the risk of less specific interactions with proteoglycans that are not rapidly internalized (Mulder et al., 1993; Eisenberg et al., 1992).

Finally, LPL has been shown to mediate lipoprotein binding to LRP (Beisiegel et al., 1991); this model, however, fails to provide a mechanism to explain the hyperlipidemia associated with abnormal apoE phenotype. In addition, only very small amounts of LPL (<0.2%) are displaced from the endothelium after a meal (Karpe et al., 1992). Furthermore, it can be estimated that, at the peak of postprandial lipemia, about 6 pM LPL circulates in plasma, while 2 nM CM are circulating. Thus, on a molar basis, CM exceed LPL by >300-fold.[3] This is not to say that LPL release from endothelial cells does not represent a potentially important mechanism of regulation. However, it is unlikely that LPL provides a quantitatively important ligand for the CMR receptor.

In support of LRP contribution to CMR clearance is the report that a bolus injection of α_2-MG* delays the clearance

[3] This calculation is based on the following estimated values: 500 ng of LPL/mL in post-heparin plasma (Brunzell, 1989); 0.12% of total LPL released at peak of postprandial lipemia and 154 mg/dL TG increased at peak of postprandial lipemia (Karpe et al., 1992); 1 × 10^6 molecules of TG per chylomicron particle (Mills et al., 1984).

of CMR (Hussain et al., 1991). This report is not in agreement with the finding of Herz et al. that binding of α_2-MG* to LRP occurs independently of that of β-VLDL enriched with apoE (Herz et al., 1991). Furthermore, preliminary *in vivo* studies indicate that injection of RAP to rat immediately prior to injection of either ^{125}I-α_2-MG* or ^{125}I-CMR delayed the clearance of ^{125}I-α_2-MG*, while leaving unchanged the kinetics of removal of ^{125}I-CMR (Mann et al., unpublished results).

Evidence supporting the contribution of the LSR in the removal of intestinally-derived lipoproteins has already been discussed. More information on the mechanisms through which FFA activate the LSR pathway is needed to further evaluate its potential function. Results to be reported elsewhere indicate that direct FFA interaction with the LSR protein(s) leads to reversible changes of conformation that unmask a lipoprotein binding site (Mann et al., unpublished data). Our current working hypothesis is that, at the surface of fenestrated liver endothelium, CM interact with lipolytic enzymes attached to heparan sulfate proteoglycans. The products of this reaction enter the space of Disse and reach the hepatocytes, where they serve as a signal molecule recruiting a lipoprotein receptor. The interaction of lipoproteins with heparan sulfate proteoglycans appears critical to efficiently target the signal molecule activating the LSR. However, the LSR itself is distinct from proteoglycans; indeed, it is not affected by heparitinase nor by heparinase and is precipitated in 10% TCA (Lyon & Gallagher, 1991) (Mann et al., unpublished results).

It has recently been proposed that proteoglycans mediate binding and internalization of lipoproteins either directly or through bridging of lipoprotein lipase (Williams, K. J., et al., 1992; Eisenberg et al., 1992; Ji et al., 1993; Mulder et al., 1993). Previous *in vivo* studies led to the postulate that a significant fraction of lipoproteins were bound to the endothelial surface via proteoglycans (Stalenhoef et al., 1986; Bihain et al., 1988). Furthermore, it has been suggested that Type III VLDL binding to endothelial cells is defective (Stalenhoef et al., 1986). It is therefore clear that lipoprotein interactions with proteoglycans represent a critical step in their removal process. Because heparan sulfate proteoglycans are unlikely to mediate the rapid delivery of lipoproteins to lysosomes, we do not believe that it represents the final step of their catabolism. Indeed, it has been shown that lipoprotein lipase internalized by proteoglycans is recycled to the cell surface (Saxena et al., 1990). Such a recycling process could lead to significant remodeling of lipoproteins; however, only proteolytic degradation of the apoprotein moiety represents the final step of their metabolism. Therefore, the most critical feature of the LSR pathway is its ability to lead to the rapid and massive degradation of lipoproteins (Bihain & Yen, 1992).

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