

Hepatic lipase may act as a ligand in the uptake of artificial chylomicron remnant-like particles by isolated rat hepatocytes

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Active and heat-inactivated hepatic lipase stimulated to a statistically comparable extent the uptake of chylomicron remnant-like particles by isolated rat hepatocytes by 3-fold and 2.3-fold respectively and, likewise, their binding to hepatic plasma

membranes by 5-fold and 4-fold respectively. Hepatic lipase may facilitate uptake of these particles, not only as a lipolytic enzyme, but also as a ligand anchored to extracellular glycosaminoglycans.

INTRODUCTION

Hepatic lipase (HL) (EC 3.1.1.32) is, along with lipoprotein lipase (LPL), one of the major plasma enzymes involved in lipoprotein metabolism [1]. Our group have previously demonstrated the physiological role of HL in promoting hepatic uptake of chylomicron remnants [2–4]. HL enzymic activity mainly modifies their surface phospholipids [2,5–7], which helps to expose epitopes of apolipoprotein E (apoE) [8], the ligand involved in remnant hepatic uptake (reviewed in [9]). Several types of receptor may account for the latter: the apoB,E low-density lipoprotein receptor and specific apoE receptor(s) which include the α_2 -macroglobulin/low-density-lipoprotein receptor-related protein [9–13] and/or a remnant receptor [14] that may be the lipolysis-stimulated receptor [15]. An apoE-independent pathway for remnant uptake has also been advocated [16], mediated by remnant phospholipid composition and HL remodelling of these compounds [2,7,8]. LPL was shown to be a ligand for triacylglycerol-rich lipoproteins [17–19]. In addition, cell-surface glycosaminoglycans (GAGs) that anchor LPL were closely implicated in this mechanism [11–13,19,20] in various cell types.

To explore HL involvement in remnant metabolism further, we investigated in the present study whether HL, which completes LPL action in remnant formation, could also play the role of ligand for remnant hepatic internalization. Preliminary data with apoE-containing chylomicron remnant-like particles, reported here, support this hypothesis, as heat-inactivated HL still significantly promotes both hepatocyte uptake and binding to hepatic plasma membranes of these particles.

MATERIALS AND METHODS

Materials

Collagenase (reference no. 103568) and glyceride-glycerol kit (reference no. 148270) were from Boehringer-Mannheim. [3 H]cholesteryl oleoyl ether (CET) (1.67 TBq/mmol), [14 C]cholesteryl oleate (CE) (1.85 TBq/mmol), glycerol tri[14 C]oleate and 125 I (3.7 GBq/ml) were from Amersham-France. Sephadex G-50 was from Pharmacia. Suramin was from Bayer Laboratories (Leverkusen, Germany). All other reagents were

from Sigma. Sep-Pak silicium columns were from Millipore (reference no. WAT 051900).

Animals

Male lean Zucker rats, bred in our laboratory, were used at a weight of 250–300 g. They were fed on a standard rat chow (UAR), kept at 23 ± 1 °C with a 12 h light/12 h dark cycle and killed at 09:30 h, in the fed state, under light diethyl ether anaesthesia.

Preparation of artificial remnant-like particles

The initial lipid mixture, based on 35 mg of triolein in 4.25 ml of 0.9 % NaCl plus 20 mM Tricine, pH 7.4, contained 70 % triolein, 2 % non-esterified cholesterol, 3 % CE, 25 % total phospholipids and 1.15 MBq of [3 H]CET (a non-hydrolysable CE analogue). The composition of the phospholipid moiety was: phosphatidylcholine 70.5 %, lysophosphatidylcholine 6.88 %, phosphatidylethanolamine 11 %, phosphatidylinositol 2.58 %, phosphatidylserine 2.58 % and sphingomyelin 6.54 %. This mixture was prepared and sonicated essentially as described previously [21], layered under a KBr gradient and ultracentrifuged in a Centrikon T20-70 ultracentrifuge (SW40 rotor) at 17000 g, 20 °C, for 20 min. The upper layer of grossly emulsified lipids was discarded, replaced with KBr (d 1.006) and re-centrifuged at 70000 g, 20 °C, for 1 h. GSH (50 μ g/ml) was then added to the supernatant to prevent oxidation, and the particles were stored under N_2 at 4 °C.

In the experiment involving chloroquine, the particles were labelled with 1.15 MBq [14 C]CE to measure the intralysosomal accumulation of unhydrolysed ester in the presence of the drug.

The size of the particles was determined in a Nanosizer (Coultronix-France, N-4 model).

ApoE binding to remnant-like particles

Rat apoE was isolated from plasma (pooled from 40 rats) by Sephadex G-50 and heparin-Sepharose chromatography [22]. It was further purified on an anti-(rat apoA1) immunoaffinity column and checked for purity by SDS/PAGE. It was then

Abbreviations used: HL, hepatic lipase; LPL, lipoprotein lipase; apoE, apolipoprotein E; GAGs, glycosaminoglycans; CE, cholesteryl oleoyl ester; CET, cholesteryl oleoyl ether.

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bound to the particles by incubation at 37 °C for 30 min (1 mg of apoE/7 mg of triolein) [23]. This mixture was adjusted to d 1.21 with KBr, layered under KBr (d 1.019) and ultracentrifuged in a 45.6 rotor (Kontron TFT) at 120 000 g , 10 °C, for 1 h. The upper layer, containing particles with bound apoE, was extensively dialysed overnight (4 °C) against 150 mM NaCl/100 mM EDTA, pH 7.4 (containing 0.02% sodium azide and 0.01% sodium merthiolate), then against 150 mM NaCl/20 mM Tricine, pH 7.4. Their apoE content was measured as described in [24]. The particles were routinely used within 48 h.

Experiments with isolated hepatocytes

Hepatocytes were isolated [25], and then purified on a Percoll gradient to ensure a proportion of viable cells of greater than 95% [26]. After a 2 h preincubation at 37 °C in Krebs–Henseleit medium, pH 7.45, to restore the internalization capacity of the cells [2], they were incubated for 90 min with the particles (1–2 μ g of apoE/125 μ g of triolein per 5×10^6 cells per 3 ml) under 95% CO₂/5% O₂. Effectors were added immediately before the incubation, except for chloroquine (0.1 mM) and heparinase (Sigma grade I, reference no. H 2519) (1.3 units/10⁶ cells) which were added at the start of the preincubation. The incubation procedure and the subsequent determinations of cell-associated radioactivity have been detailed previously [2].

Particle-binding experiments using hepatic plasma membranes

Membranes were prepared from a pool of five rat livers [27]. The 5'-nucleotidase activity, a plasma-membrane marker [28], in the final membrane suspension was 25.3 ± 1.0 μ mol of P_i/h per mg of protein ($n = 4$), representing a 30-fold enrichment over the initial liver homogenate. Glucose-6-phosphatase activity, an endoplasmic-reticulum membrane enzyme [27], was 2.3 ± 0.1 μ mol of P_i/h per mg ($n = 4$), which accounts for a very low contamination of the plasma membrane fraction by the endoplasmic reticulum membrane fraction. ApoE was labelled with ¹²⁵I by a modified ICI procedure [29], yielding a specific radioactivity of 101 000 d.p.m./ μ g of apoE and was bound to the particles by incubation as above.

Native chylomicron remnants for binding-competition studies were obtained from rat lymph [2] and used within 48 h of dialysis. Particle binding to plasma membranes was assayed as described in [4]. In brief, iodinated particles (0.5–10 μ g of apoE/37.5 μ g of membrane protein), native remnants and other effectors (in 50 mM NaCl/1 mM CaCl₂/0.2 mM Tris/HCl buffer, pH 7.5) were incubated in a final volume of 200 μ l for 1 h at 37 °C. Non-specific binding was measured in the presence of a 20-fold excess of unlabelled remnant-like particles. Radioactivity was measured in membrane pellets separated from medium on a mineral oil layer [dibutyl phthalate (d 1.05)/dinonyl phthalate (d 0.98); 20:11, v/v].

HL purification, heat inactivation and assay

Partially purified HL was obtained from pooled post-heparin liver perfusates of six rats [30] and assayed with a glycerol tri[1-¹⁴C]oleate emulsion [31] for its triacylglycerol hydrolase activity, which *in vitro* is 20-fold higher than that of phospholipase [32]. These preparations were devoided of LPL activity (1.5% of total lipolytic activity after addition of anti-HL antibody to the LPL assay [31]). After this partial purification, a 15-fold enrichment of HL activity was obtained over the initial liver perfusates. On SDS/PAGE of these preparations [4% (w/v) acrylamide stacking gel/12% running gel], HL showed one single band of 58 kDa by Coomassie Blue staining. Portions of the active enzyme (3.76

units/ml or 1.36 units/ml for two distinct preparations) were extemporaneously heat-inactivated (10 min at 100 °C), so that they preserved respectively a residual activity of only 3% (for binding assays) or 0.2% (for hepatocyte incubations and for remnant-like particle hydrolysis).

Treatment of remnant-like particles with active or heat-inactivated HL

Particles (53 μ g of apoE/2.33 mg of triolein) were incubated in a final volume of 1.5 ml of 0.1 M Tris/HCl buffer, pH 7.4, containing 0.5% BSA with either active HL (578 munits/tube) or the heat-inactivated HL (3 munits/tube) for 2 h, at 37 °C. They were then ultracentrifuged (1 h, 120 000 g , 10 °C) and assayed for lipid and protein content. Control particles were submitted to the same protocol, but without added lipase.

Chemical determinations

Total lipids were extracted [33]. Non-esterified and esterified cholesterol were separated on Sep-Pak silicium columns, then chemically determined [34]. Phospholipids were first separated by t.l.c. in a chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) solvent system and quantified by their P_i content [35]. Triacylglycerols were enzymically determined with a Boehringer-Mannheim kit.

Statistics

Results are means \pm S.E.M. (number of experiments or measurements). Regression curves and their linearization were obtained by using the least-square method using a computerized program (Graphpad). Statistical comparisons were by analysis of variance.

RESULTS

Characteristics of the remnant-like particles

Table 1 shows that the final lipid composition of the remnant-like particles was very similar to that reported for native remnants [7,36,37]. The chemical similarity was also true for phospholipid proportions, except for sphingomyelin and phosphatidylethanolamine (8% and 9.7% respectively) [7]. Likewise, sizes of the emulsion particles [127 ± 44 ($n = 3$) nm] were in the range of the native remnants [36].

Figure 1 shows the specific binding of the particles to hepatic plasma membranes. This binding (total minus non-specific binding which amounted to 2% of total binding) led to a K_d of 1.9 μ g/ml of medium (5.6×10^{-8} M). Particle internalization by isolated hepatocytes via receptor-mediated endocytosis was first checked with chloroquine, a lysosomal inhibitor that prevents the intralysosomal degradation of particle components. To this purpose, particles were labelled with [¹⁴C]CE, which is hydrolysed by the lysosomal esterases. As shown in Table 2, both cell-associated ¹⁴C radioactivity (CE + non-esterified cholesterol) and CE/non-esterified cholesterol ratio were doubled in the presence of chloroquine. This clearly indicated that remnant-like particles were adequately internalized by the hepatocytes and that cell-associated radioactivity might be taken as an index of their uptake. Subsequently, the non-hydrolysable analogue, i.e. [³H]CET, was used to label these particles.

Effect of native chylomicron remnants, suramin and active or inactivated HL on remnant-like particle binding to hepatic plasma membranes

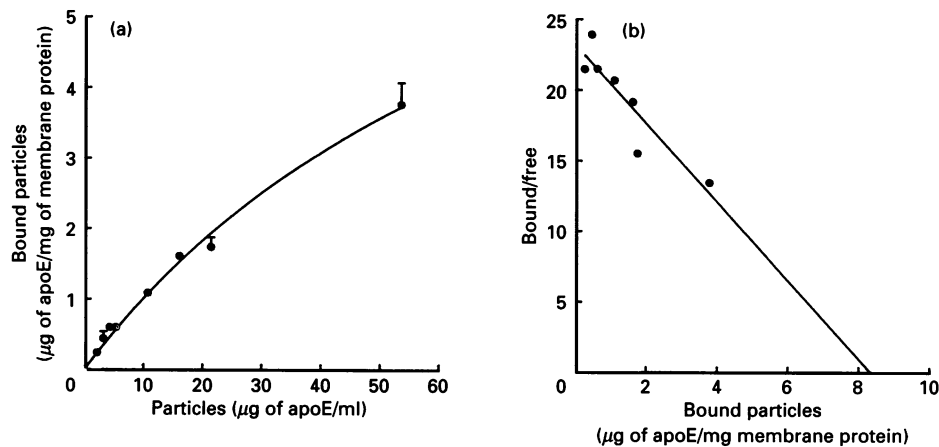
Assays (Table 3) were performed at a remnant-like particle concentration of 3.2 μ g of apoE/tube. Suramin (5 mM) inhibited

Table 1 Lipid composition of chylomicron remnant-like emulsions compared with that of native rat remnants

Emulsions were prepared by sonication of the dried lipid mixture (based on 35 mg of triolein/4.25 ml of 0.9% NaCl/Tricine, pH 7.4, and ultracentrifuged in a KBr gradient, as detailed in the Materials and methods section. TG, triacylglycerols; FC, non-esterified cholesterol; CE, cholesteryl ester; PL, phospholipids; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol, SPH, sphingomyelin. Data are means \pm S.E.M. (three emulsions).

	Lipid composition (% of total lipid mass)				Reference
	TG	FC	CE	PL	
Emulsions	74.9 \pm 1.74	2.1 \pm 0.60	3.9 \pm 0.58	19.9 \pm 0.70	
Native remnants	86.5	2.9	3.5	7.4	[2]
	75	6.5	6.5	12.9	[36]
	77.2	0.11	2.36	20.2	[37]

	Phospholipid composition (% of total phospholipids)				
	PC	LPC	PE	PS + PI	SPH
Emulsions	67.3 \pm 3.50	7.40 \pm 0.441	19.2 \pm 4.30	4.1 \pm 1.66	1.40 \pm 0.439
Native remnants	71.7	6.39	9.66	4.9	8

**Figure 1 Binding of [¹²⁵I]apoE-labelled remnant-like particles to rat hepatic plasma membranes**

Binding curve (a) and Scatchard plot for binding of [¹²⁵I]apoE emulsions (b) were determined after incubating 37.5 µg of membrane protein with 0.5–10 µg of apoE/200 µl for 1 h at 37 °C (as detailed in the Materials and methods section). K_d was 1.9 µg/ml (5.6×10^{-8} M). B_{max} was 8.3 µg of apoE/mg of membrane protein. Experimental points are means \pm S.E.M. (three determinations).

Table 2 Effect of chloroquine on hepatocyte uptake of [¹⁴C]CE-labelled remnant-like particles

Chloroquine was added to the cells during the initial 2 h preincubation as described in the Materials and methods section. CE, cholesteryl ester; FC, non-esterified cholesterol. Results are means \pm S.E.M. (four determinations). * $P < 0.05$, ** $P < 0.01$ compared with the control.

Conditions	CE (d.p.m.)/90 min per 10 ⁶ cells	CE/FC
Control	98 \pm 13	4.5 \pm 1.28
+ Chloroquine (0.1 mM)	227 \pm 25**	9.14 \pm 1.06*

particle binding by 50% ($P < 0.05$) whereas native unlabelled rat chylomicron remnants (7.4–59 µg of protein) decreased it in a dose-dependent manner by 72%. In contrast, HL addition

(100 µl, i.e. 376 munits/tube) resulted in a dramatic 5-fold increase in particle binding. Interestingly, addition of heat-inactivated HL (100 µl/tube, i.e. 13 munits) also stimulated particle binding, up to 82% of the response elicited by the active enzyme.

Effect of suramin, active and heat-inactivated HL on remnant-like particle uptake by isolated rat hepatocytes

As shown in Table 4, suramin (0.5 mM) inhibited apoE-containing remnant-like particle uptake by 50%. Addition of 100 µl (136 munits) of active HL/flask enhanced this uptake by nearly 3-fold ($P < 0.01$). Moreover, an equal volume of almost totally heat-inactivated enzyme (i.e. 0.28 munit/flask) also strongly stimulated it by 2.3-fold. Thus with heat-inactivated HL, particle uptake amounted to 72% of the uptake obtained with the active enzyme but was not statistically different from the latter.

Table 3 Effect of suramin, native chylomicron remnants and active or heat-inactivated HL on binding of [¹²⁵I]apoE-labelled remnant-like particles to rat hepatic plasma membranes

ApoE-containing remnant-like particles (3.2 µg of [¹²⁵I]apoE)/37.5 µg of membrane protein per 200 µl were incubated for 1 h at 37 °C as specified in the Materials and methods section with the different effectors at the indicated concentrations/flask. HL was inactivated for 10 min at 100 °C. Results are means ± S.E.M. (three determinations). **P* < 0.05, ***P* < 0.01 compared with controls.

Conditions	ApoE bound (µg/mg of membrane protein)
Control	1.79 ± 0.118
+ Suramin (5 mM)	0.911 ± 0.086**
+ Native remnants	
7.4 µg of protein	1.11 ± 0.287*
14.8 µg of protein	0.837 ± 0.151**
59 µg of protein	0.476 ± 0.061**
+ Active HL (376 munits)	9.06 ± 2.0**
+ Heat-inactivated HL (12.9 munits)	7.45 ± 0.211**

Table 4 Effect of suramin and active or heat-inactivated HL on rat hepatocyte uptake of apoE-containing remnant-like particles

Remnant-like particles were prepared and labelled with [³H]CET as detailed in the Materials and methods section. These particles (125 µg of triolein/5 × 10⁶ hepatocytes per 3 ml per 90 min) were incubated with suramin, active HL or heat-inactivated HL at the indicated amounts per flask. Results are means ± S.E.M. (three determinations). ***P* < 0.01 compared with controls.

Conditions	[³ H]CET uptake (nmol/60 min per 10 ⁶ cells)
Control	0.064 ± 0.006
+ Suramin (0.5 mM)	0.032 ± 0.005**
+ Active HL (136 munits)	0.199 ± 0.035**
+ Heat-inactivated HL (0.28 munit)	0.144 ± 0.01**

Effect of active and heat-inactivated HL on remnant-like particle composition

As shown in Table 5, the percentage of phospholipid was significantly decreased in particles treated with active HL (with a corresponding increment in the proportion of triolein), whereas no such change was observed in particles incubated with the heat-inactivated enzyme. Protein concentration was decreased in the active HL-treated particles, although not significantly. This may stem from a looser association between apoE and the lipid moiety after depletion of phospholipids caused by the particle hydrolysis by HL.

Table 5 Percentage composition of apoE-containing remnant-like particles incubated with active or heat-inactivated HL

Remnant-like particles (53 µg of apoE/2.33 mg of triolein) were incubated for 2 h at 37 °C in Tris/HCl medium, pH 7.4, containing 0.5% BSA, with HL, active or heat-inactivated (10 min at 100 °C). At the end of the incubation, they were ultracentrifuged as detailed in the Materials and methods section. Control particles were submitted to the same protocol in the absence of added enzyme. TO, Triolein; FC, non-esterified cholesterol; PL, phospholipid. Results are means ± S.E.M. (three determinations). ***P* < 0.01 compared with control particles.

Conditions	TO	FC	CE	PL	Protein
Control	75.4 ± 0.87	1.66 ± 0.43	4.23 ± 0.50	16.9 ± 0.28	2.15 ± 0.11
+ Active HL (578 munits)	79.3 ± 1.05	1.48 ± 0.020	5.3 ± 0.46	12.46 ± 0.74**	1.38 ± 0.59
+ Heat inactivated HL (3 munits)	75.2 ± 0.73	1.55 ± 0.104	3.43 ± 0.67	16.6 ± 0.32	2.37 ± 0.42

Table 6 Effect of HL and heparinase on rat hepatocyte uptake of apoE-devoid remnant-like particles

ApoE-devoid particles were prepared and labelled with [³H]CET as detailed in the Materials and methods section and had the following lipid composition: triolein 81%, phospholipids 15%, non-esterified cholesterol 1.7%, CE 2.1%. Hepatocytes were preincubated for 2 h with or without heparinase then incubated as in Table 4, with or without HL, at the indicated amounts per flask. Results are means ± S.E.M. (three determinations). ***P* < 0.01 compared with controls.

Conditions	[³ H]CET uptake (nmol/60 min per 10 ⁶ cells)
Control	0.040 ± 0.028
+ Active HL (231 munits)	0.160 ± 0.033**
+ Heparinase (4 units)	0.045 ± 0.084
+ Active HL (231 munits) and heparinase (4 units)	0.051 ± 0.0151

Effect of HL and heparinase on hepatocyte uptake of apoE-devoid remnant-like particles

To investigate the possibility of an interaction between HL and cell-surface GAGs in the mechanism of particle uptake, apoE-devoid remnant-like particles were used with hepatocytes preincubated for 2 h with and without heparinase, to ensure degradation of extracellular GAGs (Table 6). The uptake of these particles was 37.5% lower than that measured for apoE-containing particles, but was stimulated 4-fold by HL. Preincubation of the cells with heparinase (which itself had no effect) completely abolished HL stimulation of particle uptake.

DISCUSSION

The important observation obtained here is the nearly comparable stimulating effect of active and heat-inactivated HL on binding and cell uptake of apoE-containing remnant-like particles. Indeed the effect of heat-inactivated enzyme was only 20% lower under all the experimental conditions. In view of the phospholipase A₁-like action of HL on native chylomicron remnants, the particles used contained a complex mixture of phospholipids instead of phosphatidylcholine alone, as used in some earlier studies in rat hepatocytes, HepG2 cells [22,38] and perfused rat liver [39]. On the basis of criteria such as particle size, membrane binding and uptake by hepatocytes, these remnant-like particles display metabolic characteristics close to that of native rat remnants and human intermediary density lipoproteins [2,4,6,40]. This similarity is further documented by the inhibitory effect of suramin, a polysulphate known to decrease hepatic binding and internalization of remnants [37], and by competition by native chylomicron remnants. Likewise, lactoferrin (1 × 10⁻⁶ M) inhibited particle uptake by hepatocytes

by 50% (P. Diard, M. I. Malewiak and S. Griglio, unpublished work). Together, these data suggest a receptor-mediated internalization of these remnant-like particles, regardless of the receptor(s) involved.

The inhibitory effect of chloroquine, which blocks the receptor-mediated lysosomal degradation of lipoproteins, was not complete with either the remnant-like particles used here or native remnants [2], suggesting that their uptake could also partially proceed via a receptor-unrelated process. In this regard, phospholipid-mediated uptake [16] had been suggested. Indeed, after phospholipolysis of chylomicron remnants by HL, their uptake by isolated hepatocytes is doubled [2]. However, in the present study, although particle composition, especially the phospholipid content, is not statistically modified by heat-inactivated HL, both membrane binding and hepatocyte uptake of remnant-like particles remain 4-fold and 2.3-fold enhanced respectively. Thus variations in phospholipid content induced by active HL cannot entirely account for this increased uptake.

Interestingly, hepatocyte secretion of endogenous apoE into the incubation medium (1.2 µg/h per mg of cell protein; P. Diard, M. I. Malewiak, D. Lagrange and S. Griglio, unpublished work) is close to the amount of apoE added with the emulsions (1.04 µg/mg of cell protein) or with native remnants (3 µg/mg, recalculated from [2]). The endogenously secreted apoE may attach to the particles in the incubation medium and promote their uptake. Such a role has been suggested for newly cell-secreted apoE in studies with HepG2 cells [41]. In this regard, internalization of apoE-devoid particles was nearly 40% lower than that measured with the apoE-containing particles, which further supports the notions that (1) during the incubation, these particles may bind some cell-secreted apoE and (2) acquirement of apoE is an important prerequisite for internalization of native remnants [9,42] and lipoprotein-like particles [23,38,43]. Addition of active HL to the incubation with apoE-devoid remnant-like particles stimulated by 4-fold their hepatocyte uptake. To what extent HL facilitates apoE enrichment of such particles (or native remnants) in addition to its uncovering of remnant-bound apoE epitopes [8] requires further investigation.

Importantly, the dramatic stimulation of hepatocyte binding and uptake of particles by active HL was still observed after heat-inactivation of the enzyme. Thus the same mass, i.e. 30 µg of HL protein, either active or not, elicited nearly comparable stimulatory effects. Likewise, LPL inactivated by guanidine [19], *p*-nitrophenyl dodecylcarbamate or phenylmethanesulphonyl fluoride [11] still exerted an enhancing effect on binding of triacylglycerol-rich lipoproteins to HepG2 cells [19] or fibroblasts [11]. Together, these considerations prompt us to hypothesize that HL action on remnant-like particle uptake by rat hepatocytes may be ascribed for a large part to a non-enzymic HL protein interaction with the particles.

It has been shown in HepG2 cells, fibroblasts and monocytes/macrophages that LPL might act as a ligand for triacylglycerol-rich lipoprotein uptake, possibly by forming a bridge between these particles and their receptors and/or the cell-surface GAGs [11–13, 18–20]. Our results suggest that HL may also function through binding to such GAGs. Indeed, HL interactions with GAGs have been well established (review in [44]). In the experiment with apoE-devoid remnant-like particles, heparinase totally suppressed the stimulatory effect of HL on their uptake by hepatocytes. This indicates that the stimulatory effect of both active and heat-inactivated HL on particles, whether they contain apoE or not, requires the presence of GAGs on the hepatocyte membrane surface.

In conclusion, HL may be involved in remnant-like particle uptake by the liver through a dual action: (1) a catalytic one, by

remodelling the lipid composition and (2) by acting as a GAG-anchored ligand for remnant binding to the hepatic plasma membranes. The sequence of the molecular events involved, such as HL anchoring to the extracellular GAGs, its attachment to the particles (or native remnants) via its lipid-binding domain [45] and the subsequent interaction between these particles and their receptors triggers novel questions as to the mechanisms whereby these lipoproteins are removed from the bloodstream. In view of the atherogenic potential of remnants [46,47], these questions appear to be of clinical relevance.

We thank Dr. Martine Caron for assays of 5'-nucleotidase and glucose-6-phosphatase activities and M. Michel Joliff for expert drawing of Figure 1.

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Received 24 May 1993/1 November 1993; accepted 6 December 1993