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# Blockade of the $\alpha_2$-macroglobulin receptor/low-density-lipoprotein-receptor-related protein on rat liver parenchymal cells by the 39-kDa receptor-associated protein leaves the interaction of $\beta$-migrating very-low-density lipoprotein with the lipoprotein remnant receptor unaffected

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The nature of the liver binding site which is responsible for the initial recognition and clearance of chylomicron-remnants and $\beta$-migrating very-low-density lipoprotein ($\beta$-VLDL) is under active dispute. We have investigated the effect of the 39-kDa receptor-associated protein (RAP) on the recognition site for activated $\alpha_2$-macroglobulin and $\beta$-VLDL on rat liver parenchymal cells *in vivo* and *in vitro* in order to analyze whether both substrates are recognized and internalized by the same receptor system. Radiolabelled trypsin-activated $\alpha_2$-macroglobulin ($\alpha_2$M-T) was cleared rapidly by the liver (maximal uptake of $80.8 \pm 1.0\%$ of the injected dose). Prior injection of 5, 15, or 50 mg gluthathione-*S*-transferase-linked RAP (GST-RAP)/kg rat reduced the liver uptake to $62.2 \pm 2.3\%$, $59.3 \pm 1.1\%$, or $2.9 \pm 0.1\%$ of the injected dose, respectively. Concurrently the serum decay was strongly delayed after injection of 50 mg GST-RAP/kg rat but this did not affect the serum decay and liver uptake of $^{125}$I-$\beta$-VLDL. Binding studies with isolated liver parenchymal cells *in vitro* demonstrated that the binding of $^{125}$I-$\alpha_2$M-T was 98% inhibited by GST-RAP with an $IC_{50}$ of 0.3 μg/ml (4.2 nM), whereas the binding of $^{125}$I-$\beta$-VLDL and $^{125}$I-$\beta$-VLDL + recombinant apolipoprotein E (rec-apoE) was unaffected by GST-RAP up to 50 μg/ml (700 nM). Also, the cell association and degradation of $\alpha_2$M-T was blocked by RAP, while the association and degradation of $\beta$-VLDL and $\beta$-VLDL + rec-apoE were not influenced. The inhibitory effect of RAP on the cell association and degradation of $\alpha_2$M-T lasted for 1–2 h of incubation at 37°C. The binding of the radioiodinated RAP to isolated liver parenchymal cells was highly efficiently coupled to lysosomal degradation. Upon *in vivo* injection into rats, $^{125}$I-labeled RAP is rapidly cleared from the serum and taken up by the liver, which is also coupled to efficient degradation. Since RAP blocks binding of all known ligands to the $\alpha_2$-macroglobulin receptor/low-density lipoprotein receptor-related protein (the $\alpha_2$Mr/LRP) and at high concentrations the binding to the LDL receptor, we conclude that the initial binding and internalization of $\beta$-VLDL by rat liver parenchymal cells is not mediated by the $\alpha_2$Mr/LRP. The properties of binding of $\beta$-VLDL to rat liver parenchymal cells points to an apoE-specific recognition site for lipoprotein remnants which differs from the $\alpha_2$Mr/LRP, proteoglycans and the LDL receptor and is tentatively called the lipoprotein remnant receptor.

*Keywords:* lipoprotein remnants; low-density-lipoprotein-receptor-related protein; liver; receptor-associated protein (39 kDa).

Chylomicrons and very-low-density lipoprotein (VLDL) interact with lipoprotein lipase after entering the blood circulation. This interaction leads to hydrolysis of most of their triacylglycerols (Redgrave and Small, 1979). During this process, the apolipoprotein (apo) pattern of the formed lipoprotein remnants shows a relative increase in apoE (Windler et al., 1980). Subsequently apoE functions as a recognition marker for receptor-mediated uptake by the liver parenchymal cells (Windler et al., 1980; Van Berkel et al., 1983; Van Dijk et al., 1991). The interaction of chylomicron remnants and $\beta$-VLDL with parenchymal liver cells can be blocked by of lactoferrin (Van Dijk et al., 1991, 1992a), an $Fe^{3+}$-carrying protein with an Arg+Lys-rich sequence at positions 25–31 which resembles the binding site of apoE (amino acids 142–148) (Ziere et al., 1993).

The nature of the recognition site on liver parenchymal cells that recognizes apoE is under discussion (Van Dijk et al., 1991; Choi and Cooper, 1993; Gudmundsen et al., 1993; Jäckle et al., 1993; Nykjær et al., 1993; Ji et al., 1993; Yen et al., 1994). Choi

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*Abbreviations.* $\alpha_2$M, $\alpha_2$-macroglobulin; $\alpha_2$M-T, trypsin-activated $\alpha_2$-macroglobulin; $\alpha_2$Mr, $\alpha_2$-macroglobulin receptor; $\beta$-VLDL, $\beta$-migrating very-low-density lipoprotein, RAP, 39-kDa receptor-associated protein; LDL, low-density lipoprotein; LRP, LDL-receptor-related protein; apoE, apolipoprotein E; rec-apoE, recombinant apoE; GST, glutathione *S*-transferase.

*Enzymes.* Collagenase type IV (EC 3.4.24.3); trypsin (EC 3.4.21.4).

and Cooper (1993) found that an antibody to the low-density lipoprotein (LDL) receptor reduced the uptake of radioiodinated chylomicrons remnants by the liver to about 50%. In contrast, Kita et al. (1982) found no uptake of abnormal lipoprotein remnants in the Watanabe heritable hyper-lipidaemic rabbit, an animal with dysfunctional LDL receptors. Similarly in humans, the lack of LDL receptors does not lead to a pathological change in the metabolism of dietary fat (Rubinsztein et al., 1990).

Candidate proteins, which might function as an initial recognition site for remants, are the lipolysis-stimulated receptor, proteoglycans, and LDL-receptor-related protein (LRP). The lipolysis-stimulated receptor is a receptor that is activated by free fatty acids and has a high affinity for triacylglycerol-rich lipoproteins (Yen at al., 1994). Proteoglycans are proteins that have one or more attached glycosaminoglycan chains, with highly negatively charged sulfate and carboxylate groups. A large number of ligands are known to bind to proteoglycans, including apoE (Mahley, *1988*), apoE-enriched lipoproteins (Ji et al., 1993) and lactoferrin (Ji and Mahley, 1994; Ziere et al., 1994, 1996).

The identification by Herz et al. (1988) of a 600-kDa protein with structural similarity to the LDL receptor, called LRP, has led to the suggestion that this protein can function as lipoprotein remnant receptor (Beisiegel et al., 1989). However, LRP only recognizes lipoprotein remnants which are enriched with apoE *in vitro* (Kowal et al., 1989, 1990). The debate whether the lipoprotein remnant receptor is LRP has been intensified by the finding that the $\alpha_2$-macroglobulin receptor ($\alpha_2$Mr) and LRP are the same molecule (Strickland et al., 1990; Kristensen et al., 1990). It is generally accepted that the $\alpha_2$Mr/LRP is a multifunctional receptor as it recognizes, in addition to activated $\alpha_2$M and apoE-enriched $\beta$-VLDL, complexes between recombinant tissue-type plasminogen activator and plasminogen activator inhibitor type-1 (Kuiper et al., 1995), complexes between urokinase-type plasminogen activator and plasminogen activator inhibitor type-1 (Orth et al., 1992; Nykjær et al., 1992), *Pseudomonas* exotoxin A (Kounnas et al., 1992a), chicken vitellogenin (Stifani et al., 1991), lipoprotein lipase (Beisiegel et al., 1991), and bovine lactoferrin (Willnow et al., 1992).

A 39-kDa receptor-associated protein (RAP), as identified by Strickland and coworkers (1990) and Kristensen et al. (1990) can bind with high affinity to the LRP, thereby blocking the binding of all known ligands to the receptor (Krieger and Herz, 1994). RAP functions intracellularly as a molecular chaperone for LRP and maintains LRP in an inactive ligand-binding state (Bu et al., 1995). Recently Willnow et al. (1995) have shown that RAP-deficient mice show a significant reduction in LRP expression, resulting in an impaired clearance of methylamine activated $\alpha_2$M by the liver.

In the present studies we used RAP to study the nature of the $\beta$-VLDL recognition site on liver parenchymal cells and its relation with the $\alpha_2$Mr/LRP. The effect of the RAP on the recognition sites of activated $\alpha_2$M and $\beta$-VLDL have been compared directly *in vivo* and *in vitro* in order to analyze whether the initial recognition sites share common properties or that additional recognition systems for $\beta$-VLDL do exist on parenchymal liver cells.

## MATERIALS AND METHODS

**Chemicals.** Trypsin from bovine pancreas and soybean trypsin inhibitor were from Boehringer Mannheim. BSA (fraction V) and collagenase (clostridiopeptidase A, type IV) were from Sigma. $^{125}$I (carrier-free) in NaOH was from Amersham. Bio-Gel A-1.5m was from Bio-Rad. Dulbecco's modified Eagle's medium was from Gibco. Recombinant human apoE (rec-apoE) was a generous gift from Tikva Vogel (Bio-Technology General Ltd, Israel) and was supplied as a lyophilized powder containing 76% rec-apoE, 11.7% L-cysteine, and 12% $NaHCO_3$ (Vogel et al., 1985). All other chemicals were of analytical grade.

**Animals.** For isolation of $\beta$-VLDL, six to eight rats, with a mass of 200–220 g each, were maintained for 16 days on a cholesterol-rich chow (Hope Farms, Woerden, The Netherlands) that included 2% (by mass) cholesterol, 5% (by mass) olive oil, and 0.5% (by mass) cholic acid.

**$\beta$-VLDL, $\alpha_2$-macroglobulin and GST-RAP.** $\beta$-VLDL was obtained from cholesterol-fed rats that were starved for 20 h, after which blood was collected by puncture of the abdominal aorta. The sera were pooled and $\beta$-VLDL was isolated as described (Harkes et al., 1989). The composition of $\beta$-VLDL was $14.6 \pm 2.1$% triacylglycerols, $15.8 \pm 1.1$% phospholipids, $49.4 \pm 3.1$% esterified cholesterol, $9.9 \pm 1.0$% free cholesterol, and $10.3 \pm 0.7$% protein. Enrichment of $\beta$-VLDL with rec-apoE was performed as previously described by Kowal et al. (1990)

Human $\alpha_2$-macroglobulin ($\alpha_2$M) was isolated as described previously (Van Dijk et al., 1991) and activated by incubation with a 15-fold molar excess of trypsin for 5 min at 20°C followed by a 5-fold molar excess of soybean trypsin inhibitor over trypsin (Davidsen et al., 1985). In a subsequent step, trypsin-activated $\alpha_2$M ($\alpha_2$M-T) was separated from smaller protein complexes by gel filtration on a column (0.7×25 cm) of Bio-Gel A-1.5m eluted with phosphate-buffered saline (10 mM sodium phosphate pH 7.4 containing 150 mM NaCl and 1 mM EDTA, referred to as NaCl/$P_i$/EDTA).

A plasmid (pGEX) encoding for a fusion protein (GST-RAP) of glutathione *S*-transferase (GST) and the 39-kDa protein or receptor-associated protein (RAP), which was transformed in *Escherichia coli* (DH5$\alpha$), was a generous gift of Dr J. Herz (Dallas, Texas, USA). GST-RAP was produced as described (Herz et al., 1991). Transformed *E. coli* cells were cultured at 37°C to an $A_{600}$ of 0.4–0.5 and transcription of GST-RAP was induced with isopropyl thio-$\beta$-D-galactoside (0.01%) and growth was continued for 6 h at 37°C. Cells were harvested by centrifugation at 4°C and GST-RAP was isolated from the solubilized cells using GSH-Sepharose. The protein content of the isolated GST-RAP was determined by the method of Lowry et al. (1951) with BSA as standard and the solution subsequently concentrated to a 10 mg GST-RAP/ml using poly(ethyleneglycol) 8000. The isolated GST-RAP was for more than 95% pure as determined by analysis on 10% SDS/PAGE. Its activity was tested by determining its ability to inhibit the binding of $^{125}$I-$\alpha_2$M to freshly isolated rat parenchymal liver cells. The displacement of the binding of $^{125}$I-$\alpha_2$M (3.6 nM) by GST-RAP was 4.2 nM, which is similar to the $IC_{50}$ observed for GST-RAP kindly donated by Dr D. K. Strickland (Rockville MD, USA). The purified GST-RAP was dialyzed against NaCl/$P_i$ before administration to rats. In some experiments RAP was cleaved from the GST-RAP fusion protein by incubation with thrombin, essentially as described (Williams et al., 1992).

**Labeling of $\beta$-VLDL, $\alpha_2$M and GST-RAP.** $\beta$-VLDL was radioiodinated at pH 10 with carrier-free $^{125}$I according to a modification (Van Tol et al., 1978) of the ICl method (McFarlane, 1958). Free $^{125}$I was removed by Sephadex G-25 gel filtration with NaCl/$P_i$/EDTA as the eluent, followed by dialysis against NaCl/$P_i$/EDTA for 20 h at 4°C with repeated changes of buffer. The distribution of radioactivity in $^{125}$I-$\beta$-VLDL was $85.5 \pm 3.2$% in protein, $12.6 \pm 3.6$% in lipid, and $1.9 \pm 0.8$% unbound.

Unactivated $\alpha_2$M was radioiodinated with chloramine T as published previously (Davidsen et al., 1985). In brief, 350 µg $\alpha_2$M in 200 µl NaCl/$P_i$ pH 8.0 was mixed with 8 µl $^{125}$I in 0.1 M

NaOH followed by 214 μg chloramine T in 107 μl NaCl/$P_i$ pH 8.0. After 90 s, the reaction was stopped by the addition of 164 μg $Na_2S_2O_5$ in 82 μl NaCl/$P_i$ pH 8.0 and 120 μg KI in 60 μl NaCl/$P_i$ pH 8.0. In a subsequent step, $^{125}$I-$\alpha_2$M was directly activated with trypsin by incubation with a 15-fold molar excess of trypsin for 5 min at 20°C followed by a 5-fold molar excess of soybean trypsin inhibitor over trypsin (Davidsen et al., 1985). Isolation of $^{125}$I-$\alpha_2$M-T was similar to the isolation of $\alpha_2$M-T described above. The specific activities of $^{125}$I-$\alpha_2$M-T preparations ranged over 480–1140 dpm/ng protein. $^{125}$I-$\alpha_2$M-T was stored at −80°C for no longer than 14 days.

Recombinant GST-RAP and the RAP were radioiodinated by the Iodogen method (Fraker and Speck, 1978). Labeling of GST-RAP with $^{125}$I-tyramine-cellobiose was performed as described by Pittman et al. (1983).

**Serum decay and liver uptake.** Male Wistar rats with a mass of 225–300 g, fed with regular chow, were used in this study. Rats were anaesthetized by intraperitoneal injection of 15–20 mg sodium pentobarbital. Radiolabelled ligands were injected via the vena cava inferior. At the indicated times, blood samples of 0.3 ml were taken from the vena cava inferior and allowed to clot for 30 min. The samples were centrifuged for 2 min at 16000 *g* and radioactivity in 100-μl serum samples measured. The total amount of radioactivity in the serum was calculated as follows: serum volume (ml) = [0.0219 × body mass (g)] + 2.66 (Bijsterbosch et al., 1989).

At the indicated times, liver lobules were excised and weighed, and radioactivity was measured. At the end of the experiment, the remainder of the liver was excised and weighed. The amount of liver tissue tied off at the end of the experiment did not exceed 15% of the total liver mass. Radioactivity was corrected for the radioactivity in serum assumed to be present in the liver at the time of sampling (85 μl/g, wet mass) (Caster et al., 1955).

**Studies with freshly isolated liver parenchymal cells *in vitro*.** Liver parenchymal cells were isolated by perfusion of the liver with 0.05% collagenase by the method of Seglen (1976) modified as previously described (Casteleijn et al., 1986). The parenchymal cells obtained were resuspended in Dulbecco's modified Eagle's medium containing 2% BSA, pH 7.4. For inhibition experiments, $10^6$ parenchymal cells (>95% viable, as judged by 0.2% trypan blue exclusion) were incubated with 5 μg/ml $^{125}$I-β-VLDL or 2.6 μg/ml $^{125}$I-$\alpha_2$M-T, and the indicated amounts of competitor in Dulbecco's modified Eagle's medium supplemented with 2% BSA pH 7.4, in plastic tubes (Kartell) for 2 h at 4°C, in a circulating lab shaker at 150 rpm. For incubations at 37°C, the air in the tube was saturated with carbogen (95% $O_2$, 5% $CO_2$) every 30 min. About $10^6$ parenchymal cells (>95% viable) were incubated with 2.6 μg/ml of $^{125}$I-$\alpha_2$M-T or 5 μg/ml of $^{125}$I-β-VLDL in the presence or absence of 10 μg/ml of GST-RAP, or with $^{125}$I-GST-RAP in the presence or absence of 100 μM chloroquine, for the indicated period of time at 37°C in Dulbecco's modified Eagle's medium, pH 7.4, containing 2% BSA, with continuous shaking. After incubation, the cells were centrifuged at 50 *g* for 1 min at 4°C and washed twice with washing buffer (0.9% NaCl, 1 mM EDTA, 0.05 M Tris/HCl, 5 mM $CaCl_2$, 0.2% BSA, pH 7.4) and once with washing buffer without BSA. Cells were lysed in 1 ml 0.1 M NaOH, and subsequently radioactivity and protein content were determined. Degradation of the ligands was determined as follows: to 0.5 ml of the first supernatant, 0.2 ml 35% trichloroacetic acid was added, followed by incubation at 37°C for 30 min; the mixture was then centrifuged for 2 min at 15900 *g*. To 0.5 ml of the supernatant obtained after precipitation of the first supernatant with trichloroacetic acid, 10 μl 20% KI and 25 μl 30% $H_2O_2$ were added. After 5 min at room temperature, 0.8 ml $CHCl_3$ was added and the mixture was shaken for 15 min. After centrifugation for 2 min at 15900 *g*, the radioactivity in the aqueous phase was measured. Protein contents were determined according to Lowry et al. (1951) with BSA as internal standard.

## RESULTS

**Effect of GST-RAP on the serum decay and liver uptake of activated $^{125}$I-$\alpha_2$M-T and $^{125}$I-β-VLDL *in vivo*.** Upon injection of $\alpha_2$M-T into rats a rapid clearance is observed. The liver is mainly responsible for the uptake (Van Dijk et al., 1992b). Fig. 1A shows that $\alpha_2$M-T was cleared with a half-life of 1.3 ± 0.1 min. Maximal liver uptake was reached at 10 min after injection (80.8 ± 1.0% of the injected dose; Fig. 1B). Prior injection of 5, 15, or 50 mg of GST-RAP/kg rat prior to the injection of $^{125}$I-$\alpha_2$M-T reduced the maximal liver uptake at 10 min after injection from 80.8 ± 1.0% to 62.2 ± 2.3%, 59.3 ± 1.1%, or 2.9 ± 0.1% of the injected dose, respectively (Fig. 1B). Concurrently the serum half-life of $^{125}$I-$\alpha_2$M-T was lengthened from 1.3 ± 0.1 min to 76.2 ± 0.8 min.

Radiolabelled β-VLDL is cleared rapidly from the circulation (half-life of 0.8 ± 0.1 min) by the liver (maximal uptake of 61.5 ± 2.4% of the injected dose at 10 min after injection). Injection of 50 mg GST-RAP/kg rat prior to the injection of $^{125}$I-β-VLDL did not affect the serum clearance (half-life of 0.8 ± 0.2 min; Fig. 1C) and liver uptake (maximal uptake 60.3 ± 2.7% of the injected dose at 10 min after injection; Fig. 1D).

**Effect of GST-RAP on $^{125}$I-$\alpha_2$M-T, $^{125}$I-β-VLDL, and $^{125}$I-β-VLDL + rec-apoE binding.** Isolated rat liver parenchymal cells bind $\alpha_2$M-T with high affinity (Davidsen et al., 1985; Gliemann and Davidsen, 1986); in Fig. 2 it is shown that GST-RAP is a very effective inhibitor of $^{125}$I-$\alpha_2$M-T binding to liver parenchymal cells. An almost complete (98%) inhibition of the binding of $^{125}$I-$\alpha_2$M-T by GST-RAP with an $IC_{50}$ of 0.3 μg/ml (4.2 nM) was observed (Fig. 2A).

Isolated liver parenchymal cells bind rat β-VLDL in a saturable way by which an efficient cross-competition with chylomicron remnants is observed whereas high levels of LDL are ineffective (Van Dijk et al., 1991). GST-RAP, up to a concentration of 50 μg/ml, did not affect the binding of $^{125}$I-β-VLDL or $^{125}$I-β-VLDL + rec-apoE (Fig. 2B) whereas GST-RAP is extremely effective in blocking $^{125}$I-$\alpha_2$M-T binding.

**Effect of RAP on $\alpha_2$M-T and β-VLDL handling.** The $\alpha_2$Mr/LRP has been implicated in the cellular uptake of ligands which are initially bound to other sites on the cell surface, i.e. extracellular proteoglycans (Ji et al., 1993; Krieger and Herz, 1994). In order to test whether the degradation of $\alpha_2$M-T and β-VLDL was modified by GST-RAP, we incubated the parenchymal cells at 37°C and determined the effect of GST-RAP on the cell-association and degradation of $^{125}$I-$\alpha_2$M-T (Fig. 3A) and $^{125}$I-β-VLDL (Fig. 3B). In the absence of GST-RAP, $^{125}$I-$\alpha_2$M-T became rapidly associated to the cells, whereas after 60 min of incubation, an apparent equilibrium value was reached (Fig. 3A). The degradation of $^{125}$I-$\alpha_2$M-T followed a different time-dependency by which, after an initial lag phase of about 10 min, a constant increase in degradation products was observed (Fig. 3A). In the presence of 10 μg/ml GST-RAP, the initial cell association was almost completely blocked, but surprisingly at 120 min of incubation the effect of GST-RAP diminished and at 180 min a similar value as in the control incubation was found. GST-RAP also transiently suppressed the degradation of $^{125}$I-$\alpha_2$M-T up to

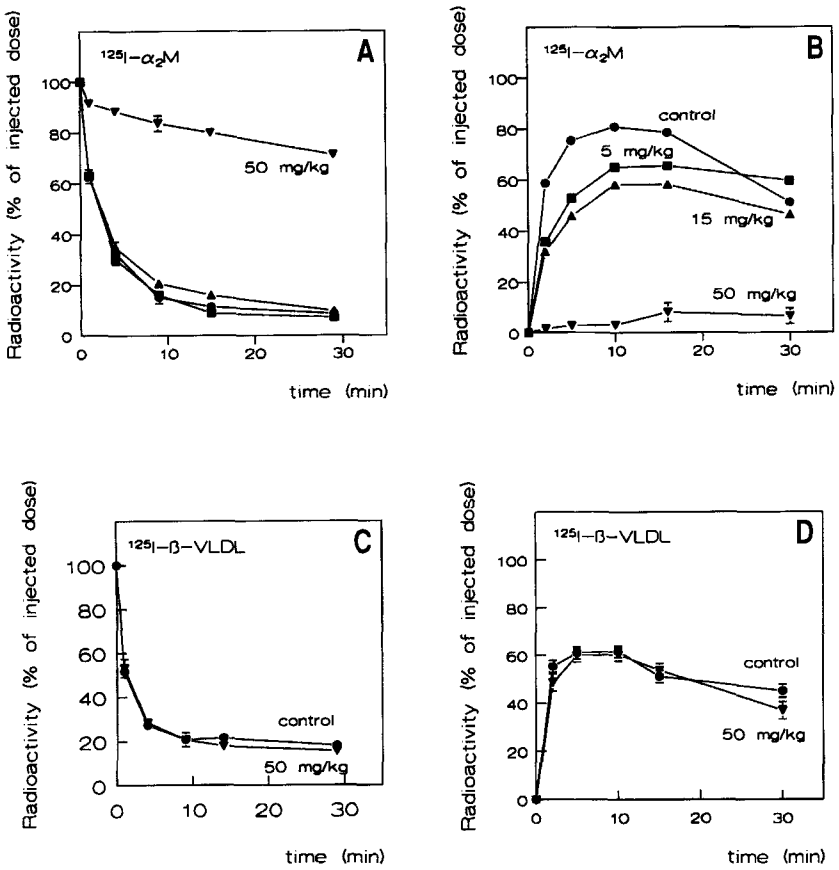


**Fig. 1. Effect of GST-RAP on the serum decay and liver uptake of $^{125}$I-$\alpha_2$M or $^{125}$I-$\beta$-VLDL in the rat *in vivo*.** Rats were injected intravenously with 0 (●), 5 (■), 15 (▲), or 50 (▼) mg GST-RAP/kg body mass 1 min prior to injection of (A, B) $^{125}$I-$\alpha_2$M-T (8 μg/kg body mass) or (C, D) $^{125}$I-$\beta$-VLDL (50 μg/kg body mass). Radioactivity in (A, C) serum and (B, D) liver were determined at the indicated times. Results are expressed as the percentage of the injected dose. When indicated, the SEM reflects data of three experiments.

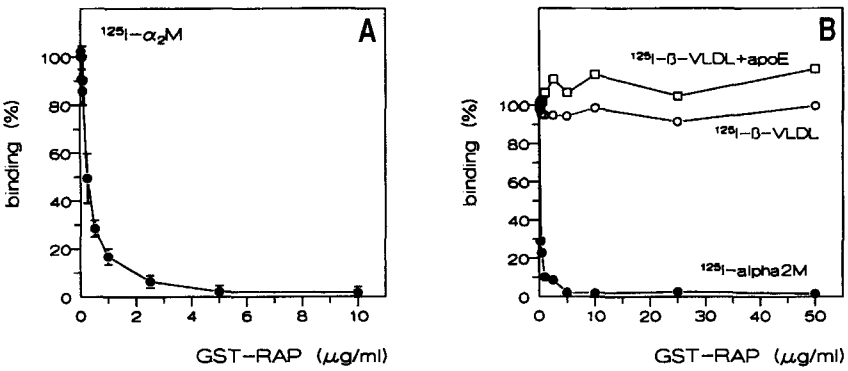


**Fig. 2. Effect of GST-RAP on $^{125}$I-$\alpha_2$M, $^{125}$I-$\beta$-VLDL and $^{125}$I-$\beta$-VLDL+rec-apoE binding to rat liver parenchymal cells.** Freshly isolated liver parenchymal cells were incubated with (●) $^{125}$I-$\alpha_2$M-T (2.6 μg/ml), (○) $^{125}$I-$\beta$-VLDL (5 μg/ml) or (□) $^{125}$I-$\beta$-VLDL + rec-apoE for 2 h at 4 °C and the indicated concentrations of GST-RAP. Binding is expressed as percentage of the binding of 2.6 μg/ml $^{125}$I-$\alpha_2$M-T or 5 μg/ml $^{125}$I-$\beta$-VLDL (+ rec-apoE) in the absence of GST-RAP.

120 min of incubation whereas between 120–180 min of incubation the increase in trichloroacetic-acid-soluble degradation products was similar to the control incubation (Fig. 3A). When $^{125}$I-$\beta$-VLDL or $^{125}$I-$\beta$-VLDL + rec-apoE were incubated with the parenchymal cells, no effect of GST-RAP on the cell association or degradation was noticed (Fig. 3B/C).

**Binding and handling of the GST-RAP by parenchymal cells.** The finding that, upon incubation of $^{125}$I-$\alpha_2$M-T with parenchymal cells at 37 °C, only a transient inhibition by GST-RAP of the cell association and degradation was noticed, suggested to us that GST-RAP might be internalized and degraded by the cells. It appears that unlabeled GST-RAP very efficiently competes for the binding of $^{125}$I-GST-RAP (Fig. 4). The glutathione *S*-transferase appears not to be responsible for this high-affinity interaction because RAP from which the GST moiety has been removed is similarly effective in the competition assay (data not shown).

Upon incubation of $^{125}$I-GST-RAP with parenchymal cells at 37 °C, an increased cell association occurred with time, which

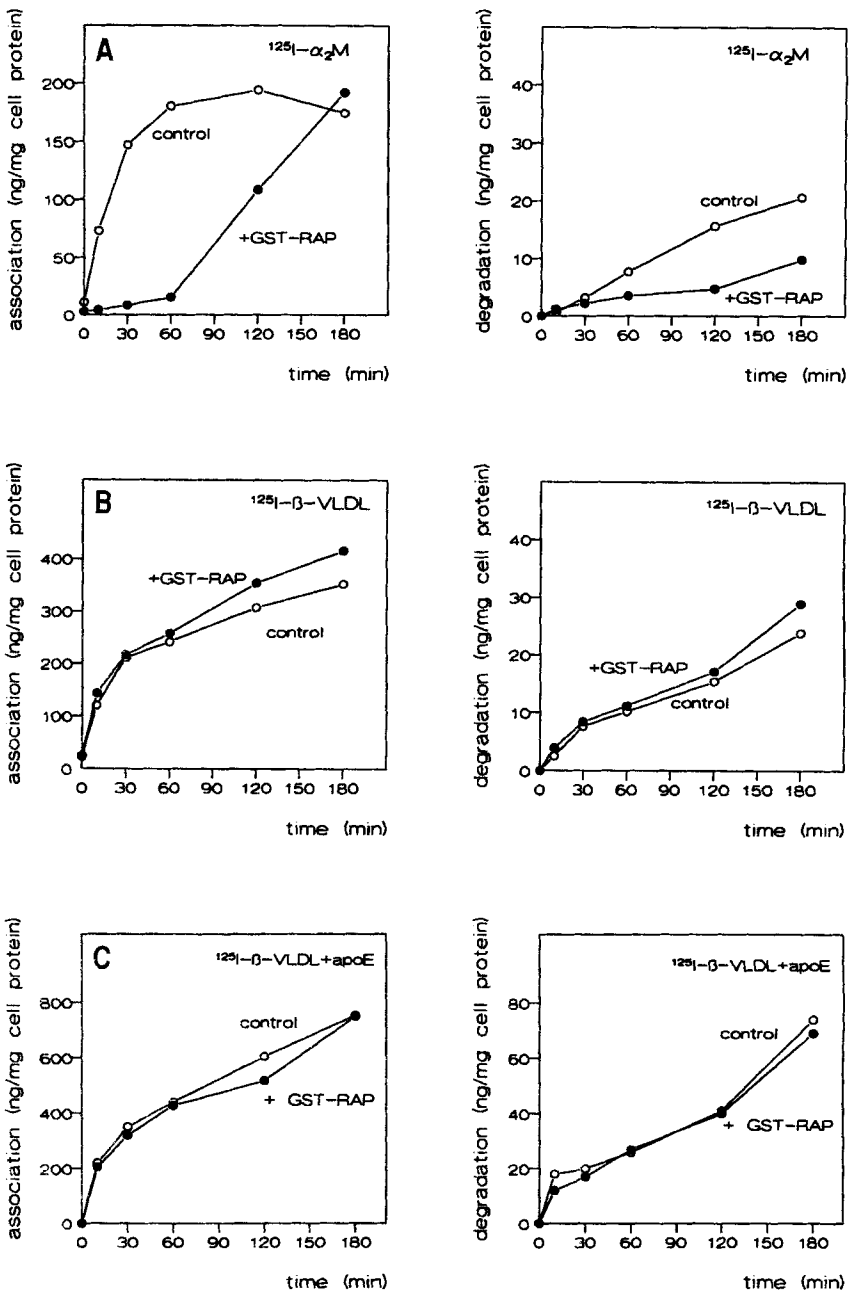


**Fig. 3. Effect of GST-RAP on the cell association and degradation of $^{125}$I-$\alpha_2$M, $^{125}$I-$\beta$-VLDL and $^{125}$I-$\beta$-VLDL + rec-apoE by liver parenchymal cells.** Freshly isolated liver parenchymal cells were incubated for the indicated period of time at 37 °C with (A) $^{125}$I-$\alpha_2$M-T (2.6 μg/ml), (B) $^{125}$I-$\beta$-VLDL (5 μg/ml) (C) or $^{125}$I-$\beta$-VLDL + rec-apoE (5 μg/ml) in the absence (○) or presence (●) of 10 μg/ml of GST-RAP. Cell association and degradation were determined and are expressed as mass of $^{125}$I-$\alpha_2$M-T or $^{125}$I-$\beta$-VLDL/mass cell protein.

reached an apparent equilibrium at 120 min of incubation (Fig. 5). Degradation of the $^{125}$I-GST RAP was observed after an initial lag phase of 10 min (Fig. 5). Chloroquine, a lysosomotropic agent which blocks lysosomal degradation, greatly inhibits the degradation of $^{125}$I-GST-RAP, suggesting that degradation occurs in the lysosomes (Fig. 5). It is clear, however, that an inhibition of the degradation is not coupled to an increased accumulation of radioactive ligand in the cells because the cell association is also lowered by the presence of chloroquine, especially at the later incubation times.

***In vivo* fate of GST-RAP.** Upon injection of $^{125}$I-GST-RAP into rats, a rapid decay from the blood circulation was noticed, coupled to a quantitative uptake in the liver (Fig. 6). Between 10–30 min after injection, the liver-associated radioactivity declined whereas the serum radioactivity remained constant. In order to test whether the decline in liver radioactivity was caused by degradation of $^{125}$I-GST-RAP, we labeled the protein with $^{125}$I-tyramine-cellobiose, a radiolabel which remains associated with cells after degradation of the labeled substrate (Pittman et al., 1983). No decline in liver radioactivity occurred (Fig. 6), indicating that the decline in radioactivity between 10–30 min after injection of $^{125}$I-GST-RAP in the liver was not caused by resecretion of intact protein, but was caused by degradation.

## DISCUSSION

The $\alpha_2$-macroglobulin receptor/low-density lipoprotein receptor-related protein (the $\alpha_2$Mr/LRP) is a member of the LDL

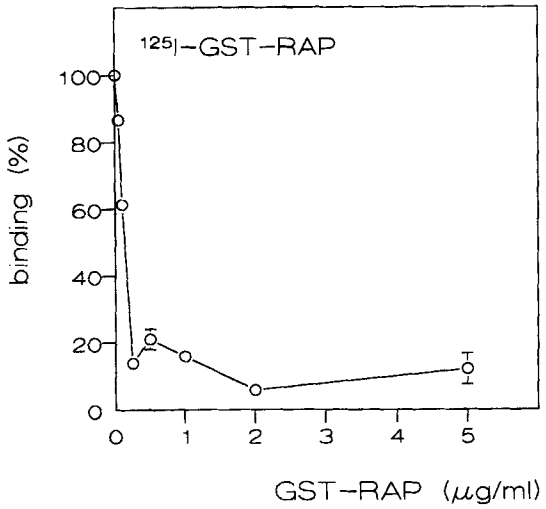


**Fig. 4. Effect of unlabeled GST-RAP on the binding of $^{125}$I-GST-RAP to liver parenchymal cells.** Freshly isolated liver parenchymal cells were incubated with 0.5 μg/ml of $^{125}$I-GST-RAP in the presence of the indicated concentrations of GST-RAP (○). Binding is expressed as percentage of the binding of $^{125}$I-GST-RAP in the absence of competitor. Results are given as means ± SD ($n$ = 3).

receptor gene family, which comprises seven known cell-surface receptors, namely (a) the LDL receptor, (b) the VLDL receptor (Takahashi et al., 1992), (c) the vitellogenin receptor (Barber et al., 1991), (d) the $\alpha_2$Mr/LRP (Herz et al., 1988), (e) an $\alpha_2$Mr/LRP-like protein from the nematode *Caenorhabditis elegans* (Yochem and Greenwald, 1993), (f) gp 330 (Raychowdhury et al., 1989), and (g) a G-protein-coupled receptor with LDL-binding motifs from the mollusc *Lymnaea stagnalis* (Tensen et al., 1994).

The $\alpha_2$Mr/LRP and the gp 330, in particular, share substrate recognition and can be considered multifunctional. Among the established substrates for the $\alpha_2$Mr/LRP, the recognition of activated $\alpha_2$M is without dispute (Krieger and Herz, 1994). In the present work we used $\alpha_2$M activated by trypsin ($\alpha_2$M-T) as a reference substrate in order to analyse the suggested role of the $\alpha_2$Mr/LRP as initial recognition site for β-VLDL and chylomicron remnants (Beisiegel et al., 1989). Furthermore we used a 39-kDa receptor-associated protein (RAP) that binds with high affinity to the $\alpha_2$Mr/LRP and blocks effectively the binding of $\alpha_2$M-T (Herz et al., 1991; Williams et al., 1992).

Prior injection of 50 mg GST-RAP/kg rat significantly reduced the liver uptake of $\alpha_2$M-T (from 80.8 ± 1.0% to 2.9 ± 0.1% of the injected dose). Concurrently the serum half-life was lengthened from 1.3 ± 0.1 min to 76.2 ± 0.8 min. The

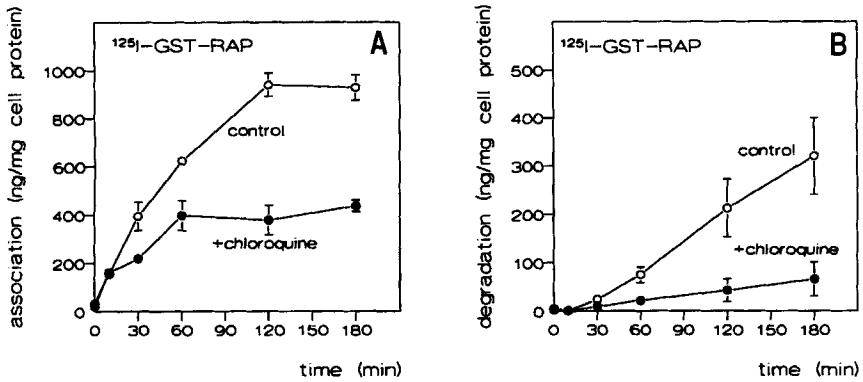


**Fig. 5. Effect of chloroquine on (A) cell association and (B) degradation of $^{125}$I-GST-RAP by liver parenchymal cells.** Freshly isolated liver parenchymal cells were incubated with 10 μg/ml of $^{125}$I-GST-RAP in the presence (●) or absence (○) of 100 μM chloroquine. Cell association and degradation were determined and are expressed as mass $^{125}$I-GST-RAP/mass cell protein. Results shown are means ± SD ($n$ = 3). When error bars are not visible, errors are within the symbol size.

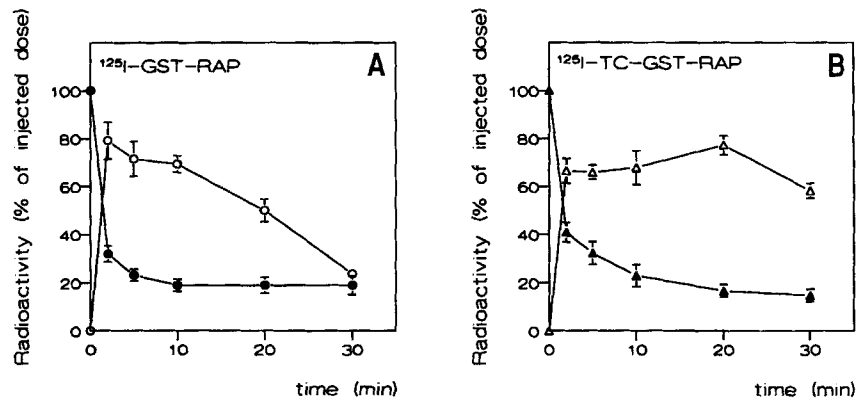


**Fig. 6. Serum decay and liver uptake of $^{125}$I- or $^{125}$I-tyramine-cellobiose-labeled GST-RAP in the rat *in vivo*.** (A) $^{125}$I-labeled GST-RAP or (B) $^{125}$I-tyramine-cellobiose-labeled GST-RAP was injected intravenously (5 μg/kg body mass) into anaesthetized rats. Radioactivity in serum (●, ▲) and liver (○, △) was determined at the indicated times. Results are expressed as the percentage of the injected dose. Results shown are means ± SD ($n$ = 3). When error bars are not visible, errors are within the symbol size.

serum decay and liver uptake of $^{125}$I-$\beta$-VLDL were not affected by prior injection with 50 mg GST-RAP/kg rat. Willnow et al. (1994) showed that adenovirus containing the RAP cDNA, upon intravenous injection, can lead to circulating levels of the RAP of 400 μg/ml. In wild-type mice no hyperlipidemia is induced, while total cholesterol is increased fourfold in LDL-receptor-negative mice at 5 days after virus injection (Willnow et al., 1994). $\alpha_2$-M clearance was reduced in these mice while no data were presented on the clearance of lipoprotein remnants. Our results are in agreement with the suggestion of Willnow et al. (1994) that the first sequestration of remnants in the liver may reside on molecules different from the $\alpha_2$Mr/LRP.

We observed with isolated rat liver parenchymal cells that GST-RAP inhibits the binding of $^{125}$I-$\alpha_2$M-T completely with an $IC_{50}$ of 4.2 nM (0.3 μg/ml), whereas the binding of $^{125}$I-$\beta$-VLDL and $^{125}$I-$\beta$-VLDL + rec-apoE to the cells was unaffected up to 700 nM (50 μg/ml) of GST-RAP. RAP blocks binding of all known ligands to the $\alpha_2$Mr/LRP (Krieger and Herz, 1994), very high concentrations of RAP (> 500 nM) also efficiently block the LDL receptor-mediated binding (Medh et al., 1994). We therefore conclude that the initial binding of $\beta$-VLDL to rat liver parenchymal cells is not mediated by the $\alpha_2$Mr/LRP or the LDL receptor. For the complex between urokinase-type plasminogen activator and plasminogen activator inhibitor type-1, it has been proposed that initial binding of this complex occurs to the urokinase-type plasminogen activator receptor, a step which precedes internalization by the $\alpha_2$Mr/LRP (Nykjær et al., 1992; Herz et al., 1992). A similar mechanism might be operative for apoE and lipoprotein lipase (Krieger and Herz, 1994). In order to test the possibility of such a mechanism for $\beta$-VLDL, we also analyzed the effect of GST-RAP at 37 °C on the cell association and degradation of $\beta$-VLDL and $\alpha_2$M-T. Both cell association and degradation of $^{125}$I-$\alpha_2$M-T are greatly blocked by the GST-RAP, whereas the cell association and degradation of $^{125}$I-$\beta$-VLDL are unaffected. From these data it appears that the internalization and degradation of $\beta$-VLDL by rat liver parenchymal cells is also not influenced by the RAP suggesting that a receptor, different from the $\alpha_2$Mr/LRP, is responsible both for recognition and intracellular handling.

A surprising finding was that the effect of the GST-RAP on the cell association and degradation of $^{125}$I-$\alpha_2$M-T lasted only for 1−2 h. Prior incubations of GST-RAP at 37 °C did not affect its capacity to inhibit the binding of $^{125}$I-$\alpha_2$M-T, indicating that the temporary inhibition is not caused by an intrinsic instability of the inhibitor at 37 °C (results not shown). It appears that GST-RAP is a high-affinity substrate for rat liver parenchymal cells and its binding is efficiently coupled to degradation. The degradation of GST-RAP is inhibited by chloroquine, suggesting that it occurs in the lysosomes. In addition to an effective inhibition of the degradation, chloroquine also inhibited the cell association especially at later incubation times. This effect was specific for GST-RAP as the association of $^{125}$I-$\alpha_2$M-T was not affected by the addition of chloroquine (data not shown). It might be possible that, in the presence of chloroquine, GST-RAP does not dissociate from the $\alpha_2$Mr/LRP, leading to intracellular entrapment of the receptor, or that chloroquine does influence the (re)-recruitment of the receptor on the cell membrane. Although the effective degradation of GST-RAP by the rat liver parenchymal cells may explain the transient inhibition, we calculated that additional (extracellular) inactivation must occur in order to explain the lack of inhibition after 1 h of incubation. Further experiments will be needed in order to analyse the receptors involved in GST-RAP binding to rat liver parenchymal cells as it was recently reported that, in addition of binding to the $\alpha_2$Mr/LRP, the RAP also binds to the LDL receptor (Mokuno et al., 1994; Medh et al., 1995) and gp 330 (Christensen et al., 1992; Kounnas et al., 1992b). It is however clear also that *in vivo* RAP is very effectively recognized and degraded by the liver. These data are consistent with results obtained by Warshawsky et al. (1993) who also showed that the liver was mainly responsible for the uptake of RAP while Iadonato et al. (1993) described rapid uptake and degradation of RAP by rat hepatoma cells.

There is no doubt that the $\alpha_2$Mr/LRP is able to interact with $\beta$-VLDL enriched in apoE *in vitro* (Beisiegel et al., 1989; Kowal et al., 1989, 1990; Lund et al., 1989). However, as shown earlier (Van Dijk et al., 1991) with isolated parenchymal liver cells, $\beta$-VLDL and chylomicron remnants do cross-compete for a high-affinity binding site (tentatively called lipoprotein remnant receptor) whereas high concentrations of LDL are ineffective. Choi and Cooper (1993) described how antibodies against the LDL receptor reduced remnant uptake in the liver by 40% while GST-RAP had little effect on the initial uptake of chylomicron remnants by the liver. However, GST-RAP does block effectively the interaction of LDL with the LDL receptor in estradiol-treated rats (Mokuno et al., 1994) while Medh et al. (1995) showed recently that high concentrations of RAP also block the interaction of VLDL and LDL with the LDL receptor of fibroblasts. The inability of the GST-RAP to affect the $\beta$-VLDL interaction with rat hepatocytes, while the interaction of $\alpha_2$M-T is very efficiently and completely blocked, thus confirms that the LDL receptor is not responsible for the primary interaction. This is also consistent with the data of Kita et al. (1982), more recently reestablished by Demacker et al. (1992) that the disappearance of chylomicrons from plasma in homozygous Watanabe heritable hyperlipidemaemic rabbits which lack functional LDL receptors, is normal. In recent studies in which mice lacking the LDL receptor were used, it was shown that the initial hepatic removal of chylomicron remnants is unaffected and therefore not mediated by the LDL receptor (Herz et al., 1995)

Our present data are thus consistent with our earlier conclusion that normal rat hepatocytes express few if any LDL receptors (Harkes and Van Berkel, 1984; Nagelkerke et al., 1986; Kleinherenbrink-Stins et al., 1990). It was recently suggested that proteoglycans may form the initial recognition site for lipoprotein remnants (Ji et al., 1993). However, treatment of rat liver parenchymal cells with heparinase and/or chondroitinase did not affect $\beta$-VLDL binding (Ziere et al., 1994, 1996). Furthermore, aminopeptidase-M-treated lactoferrin is a very efficient inhibitor of lipoprotein remnant recognition (Ziere et al., 1993) whereas it does not bind to proteoglycans (Ziere et al., 1994, 1996). In conclusion, the aforementioned properties point to an apoE-specific recognition site for lipoprotein remnants (Van Berkel et al., 1994) which differs from the $\alpha_2$Mr/LRP, proteoglycans or the LDL receptor. The further characterization of the nature of the tentative lipoprotein remnant receptor still offers an intriguing challenge for future research.

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