Synthesis and properties of the very-low-density-lipoprotein receptor and a comparison with the low-density-lipoprotein receptor

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The properties of the very-low-density lipoprotein (VLDL) receptor have been studied in Chinese hamster ovary (CHO) cells stably transfected with human VLDL-receptor cDNA and compared with those of the low-density lipoprotein (LDL) receptor expressed under the same conditions. Immunoblotting showed that the cells produced a mature VLDL receptor protein, of apparent M_r 123 000 on non-reduced and 158 000 on reduced gels, that was less extensively glycosylated than the LDL receptor. The VLDL receptor was more slowly processed than the LDL receptor, with only approx. 70 % of the precursor being converted into the mature protein. Nevertheless, the majority of the receptor in the cells was in the mature form, and most of this was present

on the cell surface. The human VLDL receptor bound rabbit very-low-density lipoprotein with β electrophoretic mobility (β VLDL), but not human LDL, and uptake through the receptor led to stimulation of oleate incorporation into cholesteryl esters. At 37 °C, the characteristics of VLDL-receptor-mediated uptake and degradation of β VLDL were essentially the same as those mediated by the LDL receptor. However, the VLDL receptor apparently did not show the increase in affinity and decrease in binding of β VLDL on cooling to 4 °C that was exhibited by the LDL receptor. Thus the overexpressed VLDL receptor in CHO cells appears to behave as a lipoprotein receptor with similar, but not identical, properties to the LDL receptor.

INTRODUCTION

The so-called 'VLDL' (very-low-density-lipoprotein) receptor, first identified in rabbit heart [1], is a member of the LDL (low-density-lipoprotein) receptor family of proteins. It is very similar in structure to the LDL receptor itself, but contains eight rather than seven repeats of a cysteine-rich element in its ligand-binding domain and cannot bind LDL with high affinity [1,2]. Unlike the LDL receptor, the VLDL receptor is not expressed in the liver, but predominantly in heart, muscle and adipose tissue [1,3]. This suggested that it could be involved in the supply of fatty acids for energy production and lipid storage. Such a proposal is supported by observations from the chicken, in which a very similar protein, also with eight repeats in its binding domain, is essential for the accumulation of VLDL-derived lipid in developing eggs [4,5]. However, other evidence casts doubt on this interpretation. Mice lacking VLDL receptors showed no abnormalities in lipoprotein profile [6], and observed changes in VLDL-receptor expression in the rat in response to starvation and thyroxine treatment were not consistent with any major role in triacylglycerol delivery to peripheral tissues [7].

Irrespective of the part that the VLDL receptor might play in triacylglycerol metabolism, there is evidence that it could also be involved in the formation of lipid-rich foam cells. It is known, for instance, that cells expressing the VLDL receptor, including THP1 cells and alveolar macrophages [2,8], accumulate lipid when exposed to apoE-rich lipoproteins. However, a detailed study of the basic properties of the VLDL receptor and a direct comparison with those of the LDL receptor has not been performed. This has become increasingly relevant with the recent proposal that expression of the VLDL receptor in the liver could be stimulated as a way of overcoming an inherited deficiency of

LDL receptors [9,10]. There are no human cell lines that express appreciable amounts of the two receptors under the same conditions, and even if there were it would be difficult to distinguish the contribution of each to the overall binding and catabolism. The properties of the LDL receptor expressed in heterologous cells are virtually the same as those observed in human fibroblasts [11], so we have chosen to study the VLDL receptor in a similar system. We have developed and used anti-peptide antibodies to examine the synthesis and maturation of the VLDL receptor and have compared the binding and uptake of lipoproteins by the two types of receptor when expressed under identical conditions in LDL-receptor-deficient Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

A 3.2 kb *EcoRI* fragment containing the whole of the coding region of the VLDL receptor [3] was inserted into the mammalian expression vector pcDNA3 under control of the simian-virus-40 promoter. The mammalian expression vector pLDLR4, containing the cDNA for the LDL receptor, was kindly provided by Dr. D. Russell (Department of Medical Genetics, Southwestern Medical Center, Dallas, TX, U.S.A.).

VLDL-receptor and LDL-receptor cDNAs were transfected by electroporation into LDL-receptor-deficient CHO cells (CHOA7 cells, kindly provided by Dr. M. Krieger, Biology Department, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.). Cells in exponential growth were trypsin-treated and resuspended at 10^7 cells/ml in Ham's F12 medium (Gibco BRL, Paisley, U.K.) without antibiotics or serum. The expression vectors were linearized with the restriction enzyme *PvuII* and 20 μ g was added to 0.8 ml of cell suspension. pSVneo (1.25 μ g)

Abbreviations used: VLDL, very-low-density lipoprotein; β VLDL, very-low-density lipoprotein with β electrophoretic mobility; LDL, low-density lipoprotein; CHO, Chinese hamster ovary.

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Figure 1 Domain diagram of the LDL and VLDL receptors

The Figure illustrates the extra cysteine-rich repeat in the ligand-binding domain and the truncated O-linked sugar domain of the VLDL receptor. The positions of the peptides used to raise antibodies (Ab) 82 and 66 are shown. Monoclonal antibody (mAb) 4B3 recognizes epitopes in the second binding repeat of the LDL receptor. The exact region of the LDL receptor recognized by the other antibody used in these studies, monoclonal antibody 10A2, is not known, but is outside the ligand-binding domain.

was added with pLDLR4. Cells were placed on ice for 10 min, pulsed once in a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA, U.S.A.) apparatus (350 V, 960 μ F) and replaced on ice for 10 min. The suspension was diluted with 7 ml of Ham's F12 containing 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% (v/v) fetal-calf serum, and left at 37 °C for 48 h before the medium was replaced with fresh medium containing 0.8 mg/ml geneticin (Gibco BRL). Stable transfectants were isolated as geneticin-resistant colonies, grown further without geneticin, and their production of receptor mRNA and protein was determined by Northern and immunoblotting. Cells expressing roughly similar amounts of each receptor in reasonable quantities were chosen for these experiments. By comparison with the expression by other cells derived from different colonies it was estimated that the cells contained approx. 12–15 copies of the receptor cDNA.

Human LDL (relative density 1.019–1.063 g/ml) and rabbit very-low-density lipoprotein with β electrophoretic mobility (β VLDL; relative density < 1.006 g/ml) were prepared and radioiodinated as described before [12]. With LDL, essentially all the radioactivity was associated with the protein component, whereas with β VLDL, 60–70% of the label was in the lipid fraction. To produce antibodies against the VLDL receptor, peptides were synthesized based on the known sequence [3]. They were coupled to bovine thyroglobulin by a cysteine residue added to the N-terminus of the peptide using 3-maleimidobenzoyl-*N*-hydroxysuccinimide ester and antibodies were raised in rabbits as described previously [13,14]. Antibody 82 was raised against a peptide comprising residues 2–17 in the first cysteine-rich repeat (Figure 1). Antibody 66 was raised to residues 708–718 next to the potential O-linked sugar region, which had only one residue in common with the same region of the LDL receptor.

For ligand and immunoblotting, cells in 90 mm dishes were washed and solubilized as described by van Driel et al. [15]. When required, the cells were pretreated with 5 μ g/ml Pronase at 4 °C as described before [16]. To examine the effects of glyco-

sidases, 20 μ l samples of extract were incubated with 1.5 units of *N*-glycanase (Genzyme, Cambridge, MA, U.S.A.), 2 m-units of *O*-glycanase (Genzyme) or 10 m-units of neuraminidase (Sigma, Poole, Dorset, U.K.) at room temperature overnight. Proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes and the receptor proteins detected with monoclonal antibodies to the LDL receptor [17], polyclonal antibodies to the VLDL receptor or 125 I-labelled β VLDL essentially as described before [17,18]. The only difference was that the antibodies were unlabelled and detected by chemiluminescence of the appropriate peroxidase-linked anti-IgG using a commercial kit (Amersham International) according to the manufacturer's instructions.

Incorporation of [35 S]methionine into immunoprecipitable LDL-receptor protein by cells in 90 cm dishes, was determined using complexes of antibody 10A2 with anti-mouse IgG as described previously [19]. For the VLDL receptor, samples of similarly prepared lysate (100 μ l) were incubated for 2 h at room temperature with 20 μ l of anti-(VLDL receptor) serum followed by 30 min at room temperature with 25 μ l of a 50% (w/v) slurry of Protein A-Sepharose (Sigma) in lysing buffer. The suspension was washed with lysing buffer, washing buffer [50 mM Tris/HCl (pH 8.0)/100 mM NaCl/1% (w/v) sodium deoxycholate/0.1% (w/v) SDS] with 1% (v/v) Triton X-100, washing buffer with 1% Triton and 0.3 M NaCl, twice with washing buffer plus 1% (v/v) Nonidet P40, and once with PBS. Proteins were solubilized and separated by SDS/PAGE as before [19].

Binding of lipoproteins to cells at 4 °C or cell-association and degradation of lipoproteins at 37 °C were assayed with cells in 24 mm Multi-wells essentially as described by Goldstein et al. [20,21]. Degradation is assayed as acid-soluble non-iodide radioactivity in the medium, which is essentially iodo-tyrosine. Thus for β VLDL, in which approx. 65% of the radioactivity was associated with lipid, degradation was calculated using a value for specific radioactivity based on the radioactivity present in the protein component alone. For cell association and binding, the specific radioactivity was based on the total radioactivity in the particles. The internalization index was calculated as the amount of lipoprotein associated with the cells at 37 °C plus that degraded at 37 °C, divided by the amount bound to the cells at 4 °C. Net incorporation of [14 C]oleate into cholesteryl esters was assayed after a 4 h incubation at 37 °C by the methods described previously [22].

RESULTS

Immunoblotting VLDL receptors

CHO cells lacking functional LDL receptors were transfected with plasmids containing either LDL-receptor cDNA or VLDL-receptor cDNA. Stable transfectants overexpressing the LDL or VLDL receptors produced single mRNA transcripts of approx. 5.5 kb and 4.2 kb respectively. Polyclonal antibodies were raised against synthetic peptides from various regions that were poorly conserved between the VLDL and LDL receptors, and were tested by immunoblotting against extracts of the transfected cells run on SDS/PAGE. Two antibodies gave a particularly strong reaction and were used for the subsequent studies. These were antibody no. 82, raised against a region of the 'extra' first cysteine-rich repeat of the VLDL receptor, and antibody no. 66, raised against a region close to the putative sugar-rich domain (Figure 1). With extracts of cells expressing the VLDL receptor, both antibodies recognized a protein of apparent M_r 123 000 on non-reduced gels (Figure 2A, lanes e and i) and apparent M_r 158 000 on reduced gels (Figure 2A, lanes l and o). This protein was absent from extracts of untransfected cells (Figure 2A, lanes

Figure 2 Immunoblotting of VLDL and LDL receptors

Cells expressing the VLDL receptor (V) or the LDL receptor (L) were lysed in buffer containing Triton X-100. Soluble proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes and the receptors detected with the appropriate antibodies as described in the Materials and methods section. Monoclonal antibodies 10A2 and 4B3 were directed against the LDL receptor, polyclonal antibodies 82 and 66 were raised against synthetic peptides from different regions of the VLDL receptor, and antibody 77 was raised against an irrelevant antigen (residues 548–560 of human hormone-sensitive lipase). (A) Immunoblots with various cells and antibodies as indicated, under reducing and non-reducing conditions. (B) Ligand blotting of the receptors with rabbit β VLDL. (C) Effect of preincubation of the cells with 5 μ g/ml Pronase for 1 h at 4 °C before extraction. Values of apparent M_r are those of protein markers run on the same gels.

f and n) or cells expressing the LDL receptor (Figure 2A, lanes g, h and m) and was not recognized by antibodies directed against the LDL receptor or an unrelated antigen (Figure 2A, lanes j and k). The minor band of apparent M_r 139000 observed on reduced gels was probably the precursor form of the protein (see below); it was not detected on non-reduced gels. Antibodies 82 and 66 did not recognize the LDL receptor, which ran with an apparent M_r of 135000 in non-reduced gels (Figure 2A, lanes c and d). The minor band of apparent M_r 115000, detected by anti-(LDL receptor) monoclonal antibody 10A2, but not by monoclonal antibody 4B3, is a breakdown product [11]. The very faint band below this (apparent M_r 122000) is the precursor form of the protein. Ligand blotting with β VLDL revealed major bands in extracts of transfected cells with mobilities similar to those of the VLDL receptor and LDL receptor detected by immunoblotting (Figure 2B).

To confirm that the transfected cells expressed the receptors on their surface, cells were treated with Pronase before immunoblotting. Treatment at 4 °C, when receptors inside the cell were trapped and inaccessible to the Pronase, greatly reduced the amount of mature VLDL-receptor protein detected (Figure 2C). No protein could be detected after treatment with Pronase at 37 °C, when trapped recycling receptors could return to the

Figure 3 Effect of glycosidases on the electrophoretic mobility of the VLDL and LDL receptors

Extracts of cells overexpressing the VLDL or LDL receptor were treated with *O*-glycanase (OG), *N*-glycanase (NG) and neuraminidase (Nse), as indicated, before immunoblotting. VLDL receptors were detected with antibody 82 and LDL receptors with monoclonal antibody 10A2. The faster band detected by antibody 10A2 is a proteolytic degradation product that is particularly evident in extracts from transfected cells overexpressing LDL receptors [11]. The Figure shows typical immunoblots, with the apparent M_r values ($\times 10^{-3}$) of the mature receptor bands obtained from three experiments shown in the diagrams below. Values from the separate experiments were virtually identical.

surface. Incubation of fibroblasts or transfected cells expressing the LDL receptor with Pronase gives a degradation product lacking the N-terminal portion of the receptor [11] (Figure 2C). The VLDL receptor also gives rise to a distinct Pronase-resistant product that is not detected by the antibody (no. 82) directed against an epitope in the N-terminal region (Figure 2C, lanes d and f).

To compare the extent of glycosylation of the VLDL and LDL receptors, cell extracts were treated with glycanases to remove N- and O-linked sugars, or neuraminidase to remove terminal sialic acid residues. Both *N*-glycanase and *O*-glycanase had a much smaller effect on the mobility of the VLDL receptor than on the mobility of the LDL receptor (Figure 3). There was also a noticeably different change with *O*-glycanase. Whereas the LDL-receptor protein ran in an indistinct broad band containing proteins with a spectrum of mobilities, the VLDL receptor gave a distinct band of only slightly faster mobility on SDS/PAGE than the untreated receptor. Moreover, neuraminidase treatment of VLDL receptors lacking N-linked sugars produced a greater effect than would be expected from the response to *O*-glycanase (Figure 3). Although the reason for this anomalous behaviour is not clear, it was not overcome by adding extra enzyme and suggests that some of the O-linked polysaccharides in the VLDL receptor could be resistant to *O*-glycanase activity. After digestion with all three enzymes the VLDL receptor ran only

Figure 4 Time course of the incorporation of [³⁵S]methionine into the precursor (○, □) and the mature (●, ■) forms of the VLDL receptor (A) or the LDL receptor (B)

Cells expressing the VLDL or LDL receptors were incubated for up to 6 h with [³⁵S]methionine (○, ●) or for 2 h with labelled methionine (pulse) followed by a chase period with 200 μM unlabelled methionine (□, ■). Cell extracts were immunoprecipitated with the appropriate antibodies and the precipitated proteins were separated by SDS/PAGE under reducing conditions. Relative amounts of radioactivity in the receptor protein bands were estimated from densitometric scans of autoradiograms of the dried gels, some of which are shown beneath the graphs. Values beside the autoradiograms refer to the apparent *M_r* ($\times 10^{-3}$) values of the VLDL receptor precursor (139) and mature (158) proteins and the LDL receptor precursor (121) and mature (168) proteins. Values were adjusted for the protein content of the cell extracts.

slightly more slowly than would be expected from the predicted amino acid sequence (*M_r* approx. 90 000).

Immunoprecipitation of VLDL receptors

To study synthesis and maturation of the VLDL receptor, cells were incubated with [³⁵S]methionine and the receptors were immunoprecipitated and separated by SDS/PAGE under reducing conditions. Antibody 82 precipitated two major radioactive bands of apparent *M_r* 158 000 and 139 000 from cells expressing the VLDL receptor (Figure 4). Antibody 66 did not precipitate

the more slowly migrating band. Neither protein was precipitated by antibody 77 raised against an irrelevant antigen. After a 2 h incubation with [³⁵S]methionine the majority of the radioactivity was present in the faster-migrating band (Figure 4). During a 4 h chase period with unlabelled methionine, the amount of radioactivity in this band declined, whereas that in the more slowly migrating band increased, indicating that the faster-migrating protein was a precursor form of the slower. The half-life of the precursor was approx. 1.4 h (Figure 4A), which is longer than that of the LDL-receptor precursor (apparent *M_r* 121 000) when stably expressed in comparable CHO cells (0.6 h, Figure 4B). Longer time-course experiments showed no evidence of accelerated breakdown of the mature VLDL receptor. Assuming little breakdown of the mature receptors during the chase period, it was estimated that only approx. 70 % of the VLDL-receptor precursor protein was eventually converted into the mature form, compared with > 90 % for the LDL receptor.

Ligand uptake

CHO cells expressing the VLDL receptor or the LDL receptor were incubated at 4 °C with ¹²⁵I-labelled human LDL or βVLDL from a cholesterol-fed rabbit. Cells expressing the VLDL receptor did not bind LDL (Figure 5a), whereas both types of cell bound βVLDL (Figure 5b). The amount of LDL or βVLDL bound was essentially unchanged if the cells were pretreated with heparinase. Untransfected CHO-A7 cells showed a small amount of βVLDL binding, which was subtracted from that of the transfected cells to give the values for binding to the transfected receptor (Figure 5c). The cells expressing the LDL receptor exhibited a much higher affinity for βVLDL and a lower maximum binding at 4 °C than those expressing the VLDL receptor.

Cells expressing both types of receptor showed much higher cell association of βVLDL at 37 °C than at 4 °C (Figure 6a). Most of this remained after trypsin treatment and so represented lipoprotein that had been internalized by the cells. They also degraded the protein component of βVLDL (Figure 6c). Again, heparinase had little effect upon βVLDL uptake or degradation. The lack of binding of LDL by cells expressing the VLDL receptor was reflected in their inability to take up or degrade LDL at 37 °C (Figures 6b and 6d). A summary of a number of experiments is given in Table 1. For cells expressing the LDL receptor, values of maximum binding and cell association of βVLDL were 20–25 % of those for LDL, and, with both ligands, the apparent affinity for the LDL receptor was markedly higher

Figure 5 Binding of LDL (a), and rabbit βVLDL (b) and (c), to untransfected cells (▲) and to cells expressing VLDL receptors (●) or LDL receptors (○)

Cells were incubated for 2 h at 4 °C with the indicated concentration of ¹²⁵I-labelled lipoprotein and extensively washed as described in the Materials and methods section. Values are corrected for non-saturable binding determined in the presence of an excess of the appropriate unlabelled lipoprotein and are the averages of duplicate incubations. Binding of βVLDL to untransfected cells was subtracted from that observed with transfected cells (b), to give the values shown in (c), with the Scatchard analysis [23] (bound/free against bound) as the inset.

Figure 6 Cell-association and degradation of VLDL and LDL at 37 °C

Untransfected cells (Δ) and cells expressing VLDL ($\bullet$) or LDL ($\circ$) receptors were incubated for 4 h at 37 °C with the indicated concentrations of 125 I-labelled LDL or rabbit β VLDL (see the Materials and methods section). They were assayed for cell-associated radioactivity and degradation of the protein component of the lipoprotein. Values are corrected for non-saturable association or degradation determined in the presence of an excess of the appropriate unlabelled lipoprotein. Cells were also incubated for 4 h at 37 °C with unlabelled lipoproteins in the presence of 0.1 mM [14 C]oleate and assayed for net oleate incorporation into cholesteryl esters. All values are the averages of duplicate incubations.

at 4 °C than at 37 °C. For the cells expressing the VLDL receptor, cell-association of β VLDL at 37 °C was approx. 65 % of that by the cells expressing the LDL receptor, with approximately the same apparent affinity. In contrast, the cells expressing the VLDL receptor bound more β VLDL at 4 °C than the cells expressing LDL receptor, and the apparent affinity was not increased at the lower temperature. The ratio of 4 °C binding to 37 °C cell association was twice as high for β VLDL in VLDL-receptor cells (0.190) as for β VLDL in LDL-receptor cells (0.092). The index of cell association plus degradation to 4 °C binding (see the Materials and methods section) for both LDL and β VLDL in the cells expressing the LDL receptor was approx. 24. That for β VLDL in cells expressing the VLDL receptor was 10.5.

DISCUSSION

CHO cells stably transfected with the cDNA for the human VLDL receptor produced a protein that ran with an apparent M_r on SDS/PAGE of 123 000 under non-reducing conditions and 158 000 under reducing conditions. This was presumed to be the VLDL receptor, since it was detected by antibodies raised against synthetic peptides from two separate regions of the receptor but not by antibodies against the LDL receptor or an irrelevant antigen. It was not produced by the untransfected cells, and it bound rabbit β VLDL on ligand blots.

Table 1 Binding and uptake of LDL and β VLDL by CHO cells expressing the VLDL and LDL receptors

Values were obtained from experiments such as those described in Figures 5 and 6. Maximum bindings and apparent K_d values at 4 °C were obtained by Scatchard analysis [23]. Maximum values for cell association and degradation at 37 °C were calculated assuming that the concentration curves followed a rectangular hyperbola. The concentrations giving half-maximum values shown in parentheses were read from the curves. Units are ng/mg of cell protein, and μ g of protein/ml for values in parentheses. Values are means $\pm$ S.E.M. for four separate experiments at 4 °C and five separate experiments at 37 °C. Each experiment used different preparations of lipoproteins.

Receptor	Ligand	Maximum (concn. for half-maximum)		
		4 °C Binding [ng/mg (μ g/ml)]	Cell association [ng/mg (μ g/ml)]	Degradation [ng/mg (μ g/ml)]
VLDL	VLDL	230 $\pm$ 24 (8.7 $\pm$ 0.2)	1209 $\pm$ 206 (8.4 $\pm$ 1.5)	1183 $\pm$ 266 (6.0 $\pm$ 1.7)
LDL	VLDL	174 $\pm$ 49 (0.75 $\pm$ 0.1)	1895 $\pm$ 396 (7.0 $\pm$ 0.8)	2473 $\pm$ 570 (6.9 $\pm$ 1.2)
LDL	LDL	865 $\pm$ 112 (6.6 $\pm$ 0.3)	7149 $\pm$ 1153 (34.4 $\pm$ 5.3)	13319 $\pm$ 1451 (25.6 $\pm$ 2.8)

Despite its similar amino acid content, the apparent M_r of the VLDL receptor on both reduced and non-reduced gels was lower than that of the LDL receptor, suggesting that it is less extensively glycosylated. This was confirmed by treating the extracted receptors with glycosidases, when removal of N-linked or O-linked polysaccharides had less effect on the mobility of the VLDL receptor than on the mobility of the LDL receptor. The mature LDL receptor contains approx. 20 O-linked polysaccharide chains [24], most of which [25] are clustered in a pattern of evenly spaced doublets or triplets along a stretch of 58 amino acid residues just outside the plasma membrane. The VLDL receptor has 14 rather than 18 potential O-linked sites in this region, but spread randomly over only 23 residues. Thus the less extensive O-linked glycosylation of the VLDL receptor and its observed resistance to O-glycanase could be related to steric hindrance between the more closely spaced sites. The VLDL receptor has naturally occurring splice variants that lack this potential O-linked sugar region [3], but it is not known if this affects its properties. Removal of the corresponding region has no effect upon the behaviour of the LDL receptor *in vitro* [25], but can give rise to a mild form of familial hypercholesterolaemia with defective receptor activity *in vivo* [26,27]. It has been estimated that the LDL receptor contains complex-type N-linked chains attached to two of the five potential sites in the protein [24,28]. The three sites in the binding region are thought to be unused. The human VLDL receptor also has two potential N-linked sites outside the binding region, one of which is in the clustered O-linked sugar region [2]. The response to N-glycanase suggests that only one of these is used. Again there is no evidence to indicate what the physiological role of this glycosylation could be, although both N-linked and O-linked polysaccharides are presumably involved in defining, maintaining or protecting the three-dimensional structure of the molecule.

When overexpressed in CHO cells the VLDL receptor was processed more slowly than the LDL receptor and not all of the precursor was converted into the mature form. Nevertheless, the majority of the receptor in the cell was present as the mature protein. Antibody 66 did not immunoprecipitate the mature protein, though it detected it apparently normally on immunoblots. This antibody was raised against a peptide immediately

adjacent to the clustered O-linked sugar site. Presumably the addition and processing of the adjacent polysaccharides subtly changed the structure of the protein in the detergent solution.

Much of the mature receptor was present on the cell surface, since treatment with Pronase at 4 °C, when only receptors on the surface were degraded, greatly reduced the amount of mature protein detected. Although Pronase is a general protease, some of the receptor was degraded to a distinct product that must have been protected from further digestion. As with the LDL receptor [11], which also gives rise to a distinct product on treatment with Pronase, this band was not detected by antibodies directed against the N-terminal region, and presumably represents a product with some or all of this region removed.

The results presented here confirm that the human VLDL receptor, when overexpressed in cultured cells, binds rabbit β VLDL but not human LDL. At 37 °C, cell association and degradation of β VLDL mediated by the VLDL and LDL receptors showed similar properties. However, there were marked differences when the cells were cooled. Whereas the LDL receptor showed a greatly increased apparent affinity for β VLDL at 4 °C, the VLDL receptor showed no such change. Furthermore, the ratio of 4 °C binding to 37 °C cell association of β VLDL was twice as high for cells expressing the VLDL receptor as for cells expressing the LDL receptor. It is known that the binding of LDL to the LDL receptor in fibroblasts is decreased and the affinity increased when they are cooled to 4 °C [29]. This does not occur with certain mutant receptors and is thought to be related to an aggregation or change in the configuration of the receptors in the membrane during cooling that modifies their binding properties [11,17,29,30]. The VLDL receptor appears to resemble the mutant LDL receptors in being unable to undergo this change in configuration.

As far as can be ascertained from these experiments, the VLDL receptor shows a complete inability to bind LDL, rather than binding with a greatly reduced affinity. It is known that only the fifth cysteine-rich repeat at the N-terminal region of the LDL receptor is essential for the binding of β VLDL [31]. Efficient binding of LDL requires, in addition, the two repeats on either side of repeat 5, together with the adjacent repeat A in the growth factor homology region [31,32]. Although the predicted sequence for the VLDL receptor shows differences from the LDL receptor in each of these repeats, the cluster of electronegative residues thought to be important for binding [33] is always conserved [3]. Thus it is likely that it is the presence of the extra repeat in the VLDL receptor that prevents LDL binding, possibly by disrupting the special arrangement of the repeated modules. Manipulation of the various repeated sequences by mutagenesis *in vitro* should help to resolve this issue.

Since the VLDL receptor is expressed in extra-hepatic tissues, it has been proposed that the therapeutic transfer and expression of the receptor in the livers of homozygous familial hypercholesterolaemic subjects could help to correct their hyperlipidaemia without triggering the immune response that occurs with the LDL receptor itself [9,10]. Two recent studies have used adenovirus-mediated gene transfer to deliver VLDL receptors to the livers of LDL-receptor-deficient mice [9,10]. Both showed that injection of the transgene produced a marked and prolonged decrease in the hypercholesterolaemia of animals fed a high-cholesterol diet, but without any great increase in the turnover of VLDL. This raised the possibility that the receptor could only act as an anchor on the surface of the cell to facilitate lipolysis of the VLDL particles. However, the experiments presented here show that the VLDL receptor can function as more than merely an anchoring receptor. If it is assumed that the amount of β VLDL bound by the LDL receptor at 37 °C is double that

observed at 4 °C, the ratio of internalization plus degradation to binding at 37 °C was essentially the same for the cells expressing the VLDL receptor (10.5) as for those expressing the LDL receptor (12.5). Thus the VLDL receptor can act in the same way as the LDL receptor to internalize intact particles and deposit cholesterol for cholesteryl ester formation. If it is assumed that the VLDL receptor behaves *in vivo* as it does in transfected heterologous cells, our results support the proposition that a VLDL receptor transgene would increase the clearance of apoE-rich intermediate-density lipoproteins [10], and so reduce the production of LDL. It remains to be established if the cholesterol deposited in the liver by this process is available for bile acid synthesis and secretion in the bile.

The projected use of the VLDL receptor in gene transfer therapy makes it increasingly important that the properties of the receptor are fully evaluated. The data presented here provide detailed information on its synthesis and ability to mediate ligand uptake. However, the range of ligands that it can bind, both natural and pharmacological, remains to be determined. Once this is known it should be possible to elucidate the true physiological role of the VLDL receptor and to assess its potential as a therapeutic agent.

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