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The role of O-linked sugars in determining the very low density lipoprotein receptor stability or release from the cell

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Received 12 February 1999; received in revised form 1 April 1999

Abstract The very low density lipoprotein receptor is a member of the low density lipoprotein receptor supergene family for which two isoforms have been reported, one lacking and the other containing an O-linked sugar domain. In order to gain insight into their functionality, transient and stable transformants separately overexpressing previously cloned bovine variants were analyzed. We report evidence that the variant lacking the O-linked sugar domain presented a rapid cleavage from the cell and that a large amino-terminal very low density lipoprotein receptor fragment was released into the culture medium. As only minor proteolysis was involved in the other very low density lipoprotein receptor variant, the clustered O-linked sugar domain may be responsible for blocking the access to the protease-sensitive site(s). To test this hypothesis, a mutant Chinese hamster ovary cell line, ldlD, with a reversible defect in the protein O-glycosylation, was used. The instability of the O-linked sugar-deficient very low density lipoprotein receptor on the cell surface was comparable to that induced by the proteolysis of the variant lacking the O-linked sugar domain. Moreover, our data suggest that the O-linked sugar domain may also protect the very low density lipoprotein receptor against unspecific proteolysis. Taken together, these results indicate that the presence of the O-linked sugar domain may be required for the stable expression of the very low density lipoprotein receptor on the cell surface and its absence may be required for release of the receptor to the extracellular space. The exclusive expression of the variant lacking the O-linked sugar domain in the bovine aortic endothelium opens new perspectives in the physiological significance of the very low density lipoprotein receptor.
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Key words: ldlD cell; Cleavage; Variant; Trypsin treatment; Glycosylation

1. Introduction

The very low density lipoprotein (VLDL) receptor is a membrane glycoprotein present in two isoforms whose physiological function has not yet been established. It is a member of the low density lipoprotein (LDL) receptor supergene family and is structurally related to the LDL receptor. Five functional domains have been defined, corresponding to different exons in the gene: (i) a ligand-binding domain composed of multiple cysteine-rich repeats, (ii) an epidermal growth factor precursor homologous domain, (iii) a highly O-glycosylated domain, (iv) a short transmembrane domain and (v) a cytoplasmic domain with a coated pit targeting signal. The O-linked sugar domain is a serine/threonine-rich domain close to the cell membrane that corresponds to exon 16 in the gene and can be differentially spliced in humans, rats, rabbits and bovines [1–5], but not in mice [6]. The VLDL receptor is modified by asparagine-linked (N-linked) glycosylation and most of the mucin-type serine/threonine-linked (O-linked) oligosaccharides are bonded to the O-linked sugar domain. This clustered O-linked sugar domain is analogous to that in the LDL receptor [7] and decay accelerating factor [8]. The VLDL receptor is a multiligand receptor. The mammalian receptor shows affinity for several ligands, including receptor-associated protein (RAP) [9–11], apoE-containing lipoproteins [12–17], lipoprotein lipase [18], serine proteinase/serpin complexes [18–20], lipoprotein Lp(A) [21] and the minor group of human rhinoviruses [22]. No differences in the ligand specificity or intracellular processing have been found between the two VLDL receptor variants.

The predominant sites of VLDL receptor expression in all mammals have been found in extrahepatic organs like heart, skeletal muscle and adipose tissue [12,23–26]. The relative expression levels of the two VLDL receptor variants in different tissues has not been analyzed in detail. However, recent studies show that both variants are expressed in certain human, rabbit and bovine tissues [24,4,5], whereas the variant lacking exon 16 is a major component in the white matter of human brain [27], in some rabbit non-muscle tissues [4] and in the bovine kidney, liver and mammary gland [5]. Cell-specific expression of the variant lacking the O-linked sugar domain has been demonstrated for epithelial cells from breast carcinomas [28], for endothelial cells from bovine aortas [5] and for the growing oocyte in the chicken homologue of the VLDL receptor [29]. Expression of VLDL receptor variants is cell- and tissue-specific and, thus, their role or regulation could be different

We have recently cloned the bovine VLDL receptor variants, the one lacking and the other containing the O-linked sugar domain [5]. We studied the expression of these variants in three different cell lines, one of them with stable overexpression, and using a mutant Chinese hamster ovary (CHO) cell line, ldlD, with a reversible defect in the addition of galactose (Gal) and *N*-acetylgalactosamine (GalNAc) to oligosaccharide chains of glycoproteins [30]. All the results indicate that the presence or absence of clustered O-linked sugar

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Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; RAP, receptor-associated protein; CHO, Chinese hamster ovary; Gal, galactose; GalNAc, *N*-acetylgalactosamine

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PII: S0014-5793(99)00494-9

chains may be crucial to VLDL receptor stability or release to the extracellular space.

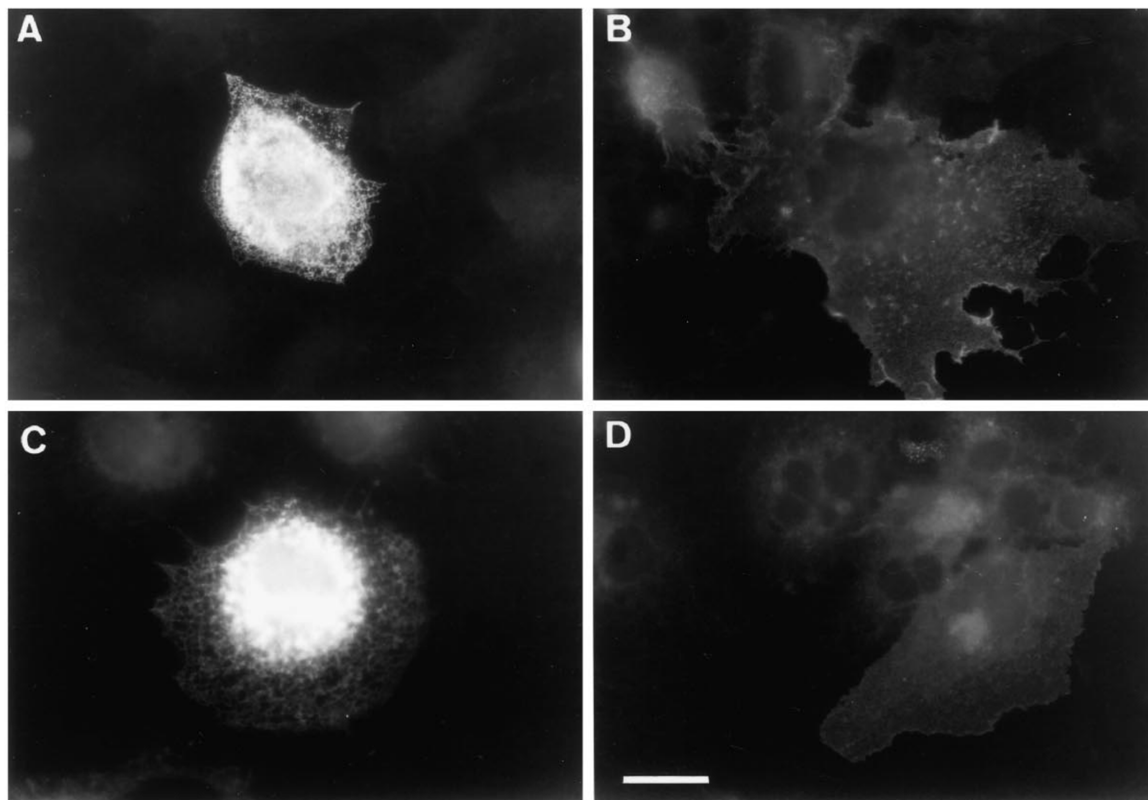
2. Materials and methods

2.1. Materials

FITC-conjugated and peroxidase-conjugated swine anti-rabbit were obtained from Dako (Santa Barbara, CA, USA). The antibody against the amino-terminal 160 amino acids of the human VLDL receptor was as previously described [31]. Rabbit polyclonal antibodies against the 20 amino acids carboxyl-terminal domain of the human, rabbit and mouse VLDL receptors were as described [32]. RAP and polyclonal antibodies against the recombinant RAP protein were prepared as described elsewhere [33]. UDP-Gal/UDP-GalNAc 4-epimerase-deficient mutant (ldlD) CHO cells were described previously [30]. Cell culture reagents were from Whittaker (Walkersville, MD, USA). All other chemicals were of analytical grade.

2.2. Cell culture and transfection experiments

Bovine VLDL receptor cDNA variants ([5], GenBank accession



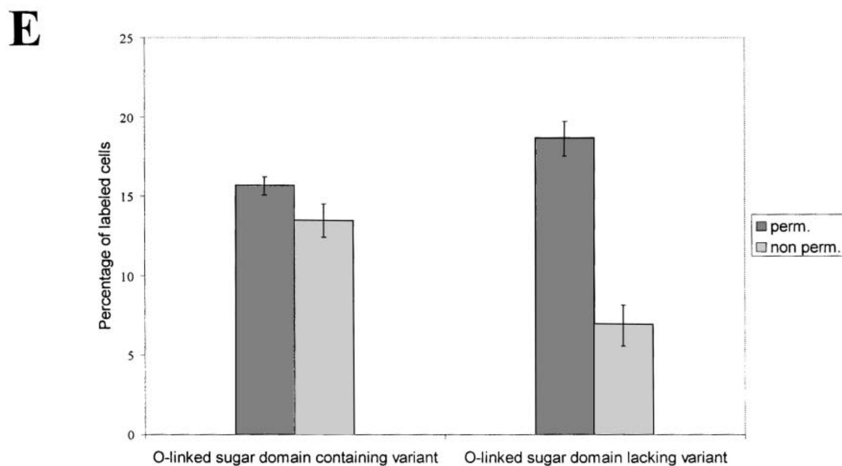


Fig. 1. Immunofluorescence studies of VLDL receptors on transfected COS1 cells. COS1 cells were transfected with a vector encoding the bovine VLDL receptor variant either containing (A, B) or lacking (C, D) the O-linked sugar domain and processed 48 h later. To detect the intracellular pattern of the VLDL receptor (A, C), cells were processed as described in Section 2. The cell surface pattern of expression was detected on fixed cells (B, D) using the same primary and secondary antibodies. Film exposure times: 1 min for A, B and C, 2 min for D. Bar: 30 μm. VLDL receptor immunodetection in permeabilized and non-permeabilized COS1 cells transfected with bovine VLDL receptor cDNAs (E). Percentages of labelled cells refer to the number of cells overexpressing the VLDL receptor out of the total number of cells counted. The mean of three independent experiments is shown. Differences between the triplicates were less than 4%.

numbers are AF016537 and AF034420) were separately subcloned into the pCR3.1 vector (Invitrogen, Groningen, The Netherlands).

COS1 cells, used for transfection experiments, were cultured in Dulbecco's modified Eagle's medium (DMEM), with 4.5 g/l glucose and 10% fetal bovine serum. Transient transfection experiments were performed as described [34].

MDCK cells were cultured in DMEM, with 1.0 g/l glucose and 10% fetal bovine serum. Plasmids encoding the bovine VLDL receptor variants were separately introduced to MDCK cells using lipofectamine reagent (Life Technologies, Gaithersburg, MD, USA) as recommended by the manufacturer. Stable transformants were selected in 600 μg/ml active G418 (Life Technologies). G418-resistant clones were isolated using cloning rings and screened for VLDL receptor expression by immunofluorescent staining and Western blot. 14 positive clones were found. The colonies transfected with the bovine VLDL receptor variant lacking or containing the O-linked sugar domain are designated in this study as #m or #p, respectively.

Wild-type CHO cells (CHO-K1) and ldlD cells were grown in Ham's F-12 medium with 5% fetal calf serum, 100 U/ml penicillin, 10 μg/ml streptomycin and 2 mM glutamine. 20 μM Gal was added to ldlD cells culture medium to allow synthesis of glycoproteins with normal N-linked oligosaccharides [35].

2.3. Western and ligand blotting analyses

Media were collected from cells after 2 days without changing the medium. Intact cells were removed from the medium by centrifugation at 1000 rpm in an Omnifuge 2.0 RS Heraeus centrifuge for 5 min at 4°C.

Cell extracts were prepared from cells grown to confluence in 60 mm dishes. Cells were scraped into a solution that contained 50 mM Tris (pH 7.4), 2 mM EDTA, 150 mM NaCl, 1% Igepal Ca-630, 0.4% sodium deoxycholate, 0.4% SDS and 1 mM PMSF. The extracts were passed through a syringe provided with a 0.9 mm×40 mm needle and used for electrophoresis and Western blot.

To study cell surface expression of the VLDL receptor, cells were pre-treated with trypsin-EDTA solution (Whittaker) for different times at 37°C and then, cell extracts were obtained.

Samples were run on 7.5% SDS-polyacrylamide gels under reducing conditions. VLDL receptor was immuno-detected with antibodies against the N-terminal domain or against the C-terminal domain. The immunoreaction was visualized by a chemiluminescence system (Amersham). For RAP ligand blotting, the samples were run on 7.5% SDS-PAGE under non-reducing conditions. The nitrocellulose membranes were incubated with 25 nM RAP in 10 mM Tris (pH 7.4) containing 3% not-fat milk, 0.02% Tween-20 and 5 mM $CaCl_2$, overnight at 4°C. Filters were then incubated with a rabbit anti-RAP IgG.

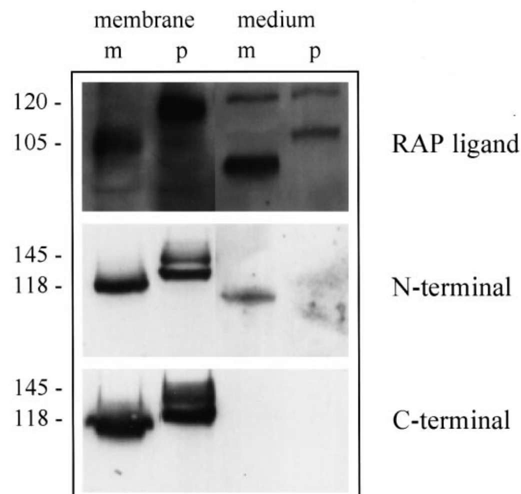
2.4. Immunofluorescence microscopy

For immunofluorescence studies, cells were grown on coverslips and, after washing, fixed with 3% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 containing 60 mM sucrose. To detect cell surface VLDL receptor, cells were incubated at 37°C for 45 min with antibodies against the N-terminal domain of the human VLDL receptor. To detect the intracellular pattern, cells were previously permeabilized with 0.1% Triton X-100. Both cell preparations were then processed for immunofluorescence as described [34] with FITC-conjugated swine anti-rabbit immunoglobulins to detect the VLDL receptor. The different percentages of labelled cells were obtained by counting more than 500 cells in each of the three independent experiments.

3. Results

In order to gain insight into the functionality of previously described bovine VLDL receptor variants [5], we transfected COS1 cells with expression vectors separately containing these variant forms. The intracellular pattern in permeabilized cells was the same for both variants (Fig. 1A and C), suggesting that they were equally processed. Although the cell surface VLDL receptor showed a similar punctate pattern in both variants (Fig. 1B and D), cells transfected with the bovine VLDL receptor lacking the O-linked sugar domain showed a lower fluorescent intensity (compare Fig. 1B, exposure time 1 min, with Fig. 1D, exposure time 2 min). The efficiency of transfection with both variants was the same as they presented similar percentage values when intracellular expression of transfected cells was considered (Fig. 1E). In addition, the percentage of cells overexpressing the receptor lacking the O-linked sugar domain on the cell surface was less than half that of the variant containing the O-linked sugar domain (Fig. 1E). As the antibodies against the N-terminal domain recognize

A



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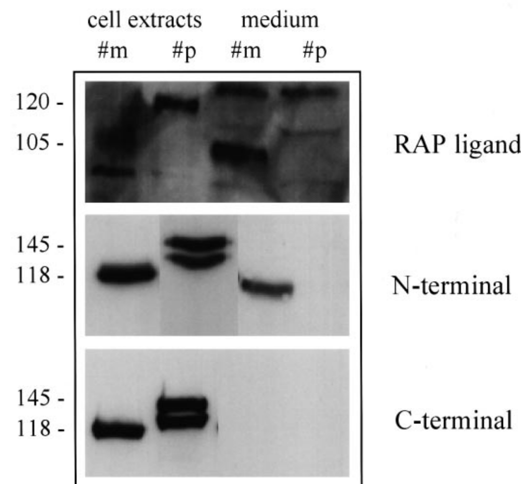

Fig. 2. Blot analysis of VLDL receptor in cell extracts and media from transfected COS1 cells and stable MDCK cell lines. A: Membranes and media of COS1 cells transiently overexpressing the bovine VLDL receptor variant either lacking (m) or containing (p) the O-linked sugar domain. B: Cell extracts and media of two MDCK clones stably overexpressing the bovine VLDL receptor form either lacking (#m) or containing (#p) the O-linked sugar domain. Samples were size-fractioned on a 7.5% SDS-polyacrylamide gel under either non-reducing conditions (RAP ligand blotting: upper panels) or reducing conditions (Western blotting: middle and lower panels), electrophoretically transferred to a nitrocellulose filter and incubated with 25 nM RAP followed by anti-RAP IgG or N-terminal antibodies against the VLDL receptor or C-terminal antibodies, as indicated. 30 μg of protein from cell extracts and 50 μl of medium were loaded to each lane.

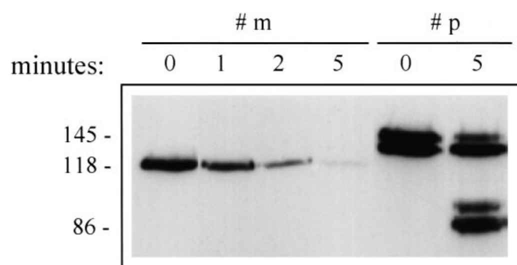

Fig. 3. The effect of trypsin-EDTA treatment on VLDL receptor variants. Two MDCK clones stably overexpressing the bovine VLDL receptor form either lacking (#m) or containing (#p) the O-linked sugar domain were used. Cells were incubated with trypsin-EDTA for increasing times at 37°C. Homogenates were obtained, size-fractioned by SDS-PAGE under reducing conditions and subjected to Western blotting with antibodies against the C-terminal domain of the VLDL receptor. 30 μg of protein was loaded to each lane.

both variants, the variant lacking the O-linked sugar domain may present an anomalous cell surface expression.

Membrane extracts and media from COS1 cells transiently and separately overexpressing both variants were obtained and subjected to RAP ligand blotting assays and Western blotting using the antibodies against the N-terminal domain of the human VLDL receptor and against the C-terminal domain of the protein. As shown in Fig. 2A (upper panel), RAP bound to 105 kDa and 120 kDa proteins from the membrane extracts that corresponded, respectively, to bovine VLDL receptor variants lacking and containing the O-linked sugar domain. In Western blotting assays (Fig. 2A, middle and lower panel), a 118 kDa band which corresponded to the bovine VLDL receptor lacking the O-linked sugar domain was immuno-detected. This band could correspond to both the precursor and mature form of the receptor because of the small difference in molecular weight between them. The 145 kDa and 127 kDa proteins could be, respectively, the mature and the precursor form for the bovine VLDL receptor containing the O-linked sugar domain. RAP also bound to a 94 kDa protein in medium from cells overexpressing the variant lacking the O-linked sugar domain, which was also immuno-detected by the antibodies against the N-terminal domain (108 kDa under reducing conditions), but not with the C-terminal antibodies (Fig. 2A). This VLDL receptor product was 11 kDa shorter than the receptor from the cell extracts and it was hardly detectable in medium from cells overexpressing the variant containing the O-linked sugar domain (107 kDa band, only in RAP ligand blotting assays). The other band of 124 kDa that appeared in media (Fig. 2A, upper panel) was non-specific as it was also detected in control filters incubated with only anti-RAP IgG, but not with N-terminal antibodies.

To extent these analyses to a different cell line, stable MDCK transformants separately overexpressing one of the variants were selected and isolated. In all positive clones analyzed, the same bands described above for the cell extracts were detected by RAP ligand blotting assays and Western blotting using the antibodies against the N-terminal domain and against the C-terminal domain of the protein (Fig. 2B). The 88 kDa band which was detected in some cell extracts by RAP blotting assays (Fig. 2B, upper panel) was non-specific as it also appeared in control filters incubated with only anti-RAP IgG, but not in Western blotting assays. These results suggest that the VLDL receptor lacking the O-linked sugar domain is proteolized and that the cutting site should be closed to the membrane-spanning domain of the protein. Thus, two products were given in the variant lacking the O-linked sugar domain, a 94 kDa product (108 kDa under reducing conditions), which was released into the medium, and a 11 kDa product, which contained the transmembrane and the cytoplasmic domain of the bovine VLDL receptor. The 107 kDa band could be a consequence of a similar, but much less extensive, proteolysis/release of the receptor containing the O-linked sugar domain.

To study the sensitivity to extracellular proteolysis of the bovine VLDL receptor on the cell surface, two different stable MDCK clones were treated with trypsin-EDTA for increasing times at 37°C and their homogenate extracts were analyzed by Western blotting (Fig. 3). Trypsin unspecifically cuts the extracellular domain of the bovine VLDL receptor at more than 70 sites [5]. After trypsin-EDTA treatment, the amount of VLDL receptor lacking the O-linked sugar domain decreased markedly in cell extracts. In contrast, the effect of this proteolysis on the VLDL receptor containing the O-linked sugar domain was much lower. Although some surface receptors were proteolyzed, the O-linked sugar domain still conferred some protection, as 92 kDa and 86 kDa forms of the receptor were found. These proteins were produced by the proteolytic removal of a fragment from the N-terminus of the mature receptor, as they were detected by the antibodies against the C-terminal domain of the protein (Fig. 3), but not against the N-terminal domain (data not shown).

To analyse the function of the O-linked sugar domain on the VLDL receptor, we compared VLDL receptor expression in wild-type CHO cells and in a mutant CHO cell line, a

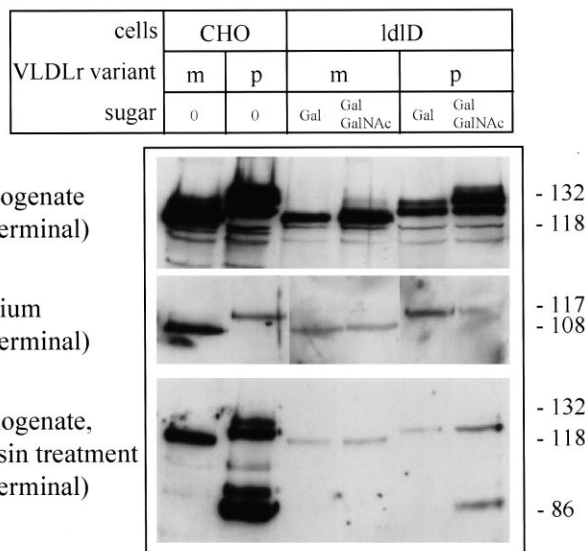

Fig. 4. VLDL receptor studies on ldlD cells. Wild-type CHO or ldlD cells were transfected with the VLDL receptor variant either lacking (m) or containing (p) the O-linked sugar domain. Cells were grown in a medium that contained the following: no additions (0), Gal at 20 μM or both Gal and GalNAc (at 200 μM). At 48 h post-transfection, homogenates and media were obtained and trypsin-EDTA treatment was performed. Proteins were separated by SDS-PAGE and subjected to Western blotting with the N-terminal antibodies against the VLDL receptor or the C-terminal antibodies, as indicated. 30 μg of homogenate protein and 50 μl of medium were loaded to each lane.

UDP-Gal/UDP-GalNAc 4-epimerase-deficient mutant (ldlD) [30]. ldlD cells have a defect in protein O-glycosylation that can be rapidly reverted by addition of GalNAc to Gal-supplemented culture medium [35].

To examine the effects of preventing O-glycosylation on VLDL receptors, wild-type CHO and ldlD cells were transfected with expression vectors encoding bovine VLDL receptor variants lacking or containing the O-linked sugar domain. Homogenate extracts and media were obtained and analyzed by Western blotting using the antibodies against the N-terminal domain of the protein. High levels of receptor were expressed in both ldlD and wild-type CHO cells (Fig. 4, upper panel). No band corresponding to the VLDL receptor was seen in blots from untransfected ldlD or wild-type CHO cells (data not shown). The electrophoretic mobilities observed for the VLDL receptor in CHO cells were similar to those observed in MDCK clones. The small differences may be due to changes in the sugars added to the receptor, as described previously for the human and CHO LDL receptors [36]. A precursor/mature protein of 118 kDa corresponded to the VLDL receptor variant lacking the O-linked sugar domain. The variant containing the O-linked sugar domain was synthesized as a 123 kDa precursor and a 132 kDa mature protein. In the absence of added GalNAc, ldlD cells normally synthesized the 118 kDa and 123 kDa precursors but failed to process them to the mature form (Fig. 4, upper panel). The electrophoretic mobility of the O-linked sugar-deficient VLDL receptors (O^d VLDL receptors) only decreased in the variant containing the O-linked sugar domain (Fig. 4, upper panel).

A VLDL receptor degradation product was mainly present in media from those ldlD and wild-type CHO cells which had VLDL receptors lacking the O-linked sugar domain (108 kDa band: Fig. 4, middle panel) or O^d VLDL receptors (117 kDa band: Fig. 4, middle panel). This VLDL receptor degradation product was only detected with the antibodies against the N-terminal domain of the protein but not against the C-terminal domain (data not shown). Although much less extensive, some proteolysis/release of receptors with normal O-linked chains also occurred (Fig. 4, middle panel). Therefore, the VLDL receptor only showed protection against proteolysis in ldlD cells transfected with the bovine variant containing the O-linked sugar domain and grown in the presence of Gal and GalNAc. These data indicate that the clustered O-linked sugars are required for the stable expression of VLDL receptor in ldlD cells.

The role of O-glycosylation of VLDL receptor in ldlD or wild-type CHO cells in the absence or presence of exogenously added sugars was analyzed by sensitivity to extracellular digestion with trypsin-EDTA (Fig. 4, lower panel). Homogenate extracts were examined by Western blotting using the antibodies against the C-terminal domain of the protein. The effect of trypsin digestion on VLDL receptor variants was similar to that described for MDCK clones in Fig. 3. After trypsin-EDTA treatment, the amount of VLDL receptors lacking the O-linked sugar domain or O^d receptors markedly decreased. Only in cells transfected with the VLDL receptor variant containing the O-linked sugar domain, and with the O-glycosylation defect fully reversed, was the effect of trypsin proteolysis lower. Although some surface receptors were proteolyzed in these cells, the O-linked sugar domain still protected them, as partially degraded receptors were found (92 kDa and 86 kDa bands: Fig. 4, lower panel). These partially degraded receptors were a consequence of the proteolytic removal of an N-terminal fragment from the mature receptor, as they were only immuno-detected with the antibodies against the C-terminal domain of the protein, but not against the N-terminal domain (data not shown).

4. Discussion

Previous studies [37] have shown that deletion of the clustered O-linked sugar domain of the human LDL receptor does not affect the normal stability or function of these receptors. The O-linked sugars required for the maintenance of LDL receptor stability are the sugars dispersed on the receptor itself [38]. The VLDL receptor variants have three dispersed potential N-linked glycosylation sites and clustered O-linked chains in the variant containing the O-linked sugar domain. Previous studies of O-glycosylation of many other glycoproteins have shown that O-linked sugars play important roles in determining the receptor stability and function [30,38–41]. Here, we demonstrate that the presence of the clustered O-linked sugar domain may be required for the stablility of the VLDL receptor on the cell surface. The physiological implications of the presence of a VLDL receptor variant lacking the O-linked sugar domain require further study.

We showed that the receptor lacking the O-linked sugar domain presented a rapid cleavage/release from the cell. This proteolysis occurred on the extracellular part of the receptor, near the membrane-spanning domain. It is still unknown how this mechanism takes place. As only minor proteolysis was involved in the VLDL receptor containing the O-linked sugar domain, we assumed that access to the protease-sensitive site(s) was prevented by the clustered O-linked sugar domain. To test this hypothesis, a mutant ldlD CHO cell line, with a reversible defect in protein O-glycosylation, was used. No variants in O-glycosylation have been described for cell surface membrane glycoproteins in the ldlD system. Thus, the presence of two naturally occurring VLDL receptor variants, one lacking and the other containing an O-linked sugar domain, provides an ideal system to determine the role of the clustered O-linked sugars. The instability of the O-linked sugar-deficient VLDL receptors on the cell surface was comparable to the proteolysis of the variant lacking the O-linked sugar domain.

The O-linked sugar domain not only prevents cleavage/release of a large VLDL receptor N-terminal fragment but may also have protected the receptor against unspecific proteolysis. After trypsin treatment, an intermediate degradation product (92–86 kDa) was only recognized in cells overexpressing the VLDL receptor containing the O-linked sugar domain. Its electrophoretical mobility suggests that proteolysis may occur in the multiple cysteine-rich N-terminal region of the receptor. A degraded form of the CHO LDL receptor has also been described [36,42] after the removal of an apparently unglycosylated portion of the receptor's cysteine-rich N-terminus.

The critical role of the O-glycosylation in establishing the normal surface glycoprotein stability and function has already been described [30,38–41]. Our findings are consistent with those observed for various O-glycosylated cell surface membrane glycoproteins analyzed to date. In four cases, inhibition of O-glycosylation markedly reduced the amount of these proteins on the cell surface. In three of these cases, the LDL receptor [30,38], decay accelerating factor (DAF) [40]

and the major envelope glycoprotein of the Epstein-Barr virus [39], rapid proteolytic cleavage after reaching the cell surface resulted in the release of a large fragment of the extracellular domains of these proteins. In the case of the interleukin-2 receptor [39], intracellular missorting prevented the O-linked deficient protein from reaching the cell surface. A marked reduction in cell surface expression of the attachment glycoprotein of human respiratory syncytial virus [41] and the human glycophorin A [43] has been found in cells in which both the N- and O-linked glycosylation is blocked. In contrast, inhibition of O-glycosylation did not affect either the biosynthesis and secretion of β-amyloid precursor protein [44] or the sorting, expression and processing of the precursor for transforming growth factor-α [45]. The LDL receptor and DAF contain an O-linked sugar domain [46,47] at a position analogous to the VLDL receptor, between the amino-terminal domain composed of multiple cysteine-rich repeats and the cell membrane. Naturally occurring variants lacking the O-linked sugar domain have not been reported for the LDL receptor and DAF.

The presence in the culture medium of a soluble VLDL receptor fragment has also been reported for the human VLDL receptor in other cell-types [4,48]. The role of the large amino-terminal VLDL receptor fragment released to the medium has not yet been established. However, it has recently been shown that a homologous VLDL receptor fragment released from HeLa cells binds to the minor group of human rhinoviruses (HRV) and inhibits HRV infection [48]. There is also evidence that a soluble form of the α-chain of the LDL receptor-related protein is present in normal human plasma [49] and that a 28 kDa N-terminal fragment of the LDL receptor is active against vesicular stomatitis virus infection [50]. Although no soluble form of VLDL receptor has yet been found in body fluids or the extracellular matrix, the exclusive expression of a VLDL receptor variant lacking the O-linked sugar domain in bovine aortic endothelial cells [5] and, therefore, its potential cleavage/release into the lumen of the aorta, open new possibilities on the physiological significance of these receptors.

Acknowledgements: We are grateful to David García and Ana Marcos for his expert technical assistance and to Robin Rycroft for his expert editorial help. We also thank Dr Monty Krieger for his helpful comments and for providing the ldlD cell line. This study was funded by the Fondo de Investigaciones Sanitarias from Ministerio de Sanidad (Grant 96/2099), the Generalitat de Catalunya, the Swedish Medical Research Council and the Comisión Interministerial de Ciencia y Tecnología (SAF98-0135). J.M. was partially supported by the CIRIT of the Generalitat de Catalunya.

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