Bovine aortic endothelial cells express a variant of the very low density lipoprotein receptor that lacks the O-linked sugar domain[1]

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Abstract **The very low density lipoprotein (VLDL) receptor is a member of the low density lipoprotein supergene family of receptors in which differential splicing of mRNA has been reported. We present several lines of evidence showing that bovine aortic endothelial cells exclusively express a VLDL receptor isoform that lacks the O-linked sugar domain *i*) Western and receptor-associated protein (RAP) ligand blotting gave a single band of about 99 kDa in membrane extracts of bovine aortic endothelial cells (BAEC). *ii*) Screening of the BAEC cDNA library with the previously characterized human VLDL receptor cDNA as a probe gave several C-terminal-positive clones; all lacked the 84 nucleotides corresponding to exon 16. Polymerase chain reaction (PCR) confirmed that VLDL receptor cDNA encoding exon 16 was absent from the library. *iii*) Reverse transcription (RT)-PCR analysis of the BAEC mRNA using a pair of oligonucleotide primers that flank the deletion gave only one band of 136 nt. *iv*) Semiquantitative RT-PCR analysis showed that only the non-O-glycosylated variant was expressed in BAEC. Cell-binding studies with antibodies against the N-terminal domain showed that the BAEC VLDL receptor is present at the plasma membrane, suggesting that the non-glycosylated variant could be functional. In addition, RT-PCR performed in bovine tissues showed that the variant containing the O-linked sugar domain is preferentially expressed in heart, brain, and skeletal muscle, whereas the non-O-glycosylated spliced variant is found in all tissues analyzed. Taken together these results suggest that the differential splicing of the VLDL receptor is cell- and tissue-specific and that the functions of the receptor could depend on the cell type.—**Magrané, J., M. Reina, R. Pagan, A. Luna, R. P. Casaroli-Marano, B. Angelin, M. Gåfvels, and S. Vilaró. **Bovine aortic endothelial cells express a variant of the very low density lipoprotein receptor that lacks the O-linked sugar domain. *J. Lipid Res.* 1998.** 39: **2172–2181.**

Supplementary key words sequence • differential splicing • mRNA • isoforms • RT-PCR

The very low density lipoprotein (VLDL) receptor is a member of the low density lipoprotein receptor supergene family (1, 2 for review). The VLDL receptor is structurally related to the LDL receptor and, like the recently identified apoE receptor 2 (3), it consists of five functional domains: *i*) an amino-terminal ligand-binding domain composed of multiple cysteine-rich repeats; *ii*) an epidermal growth factor (EGF) precursor homologous domain; *iii*) an O-linked sugar domain; *iv*) a transmembrane domain; and *v*) a cytoplasmic domain with a coated-pit targeting signal (4–13). In contrast to the LDL receptor, which is highly expressed in the liver and steroidogenically active organs, the highest levels of VLDL receptor expression have been found in extrahepatic organs like heart, skeletal muscle, adipose tissue, kidney, and brain (4–6, 8, 10, 11).

The mammalian VLDL receptor has been shown to bind and mediate the endocytosis of several ligands such as apoE-containing lipoproteins in vitro (4, 14–17), lipoprotein Lp[a] (18), complexes of uPA:PAI-1 (19, 20), lipoprotein lipase (19), and the 39 kDa receptor-associated protein (RAP) (19, 21, 22). However, the physiological ligand(s) have not yet been identified with certainty. In vivo studies on VLDL receptor knockout mice showed a lack of effect on lipoprotein levels and only a slight deficiency in the relative amount of adipose tissue in receptor-deficient animals versus intact controls, which argues against a major physiological role of VLDL receptors in lipid metabolism in vivo (23). VLDL receptors transiently

Abbreviations: BAEC, bovine aortic endothelial cells; EGF, epidermal growth factor; LDL, low density lipoproteins; LPL, lipoprotein lipase; RAP, receptor associated protein; VLDL, very low density lipoprotein; RT-PCR, reverse transcription-polymerase chain reaction; nt, nucleotides.

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overexpressed in livers of LDL receptor-knockout mice fed a high cholesterol diet could, however, reverse the hypercholesterolemia (24, 25), indicating that the VLDL receptor could perform the function of a lipoprotein receptor, albeit in an organ where it is not normally expressed. The oocyte vitellogenin receptor, the chicken homolog of the VLDL receptor, plays a critical role in the transport of vitellogenin and VLDL into growing oocytes (12). In fact, a single point mutation in the gene leading to a $Cys^{682} \rightarrow Ser$ substitution renders affected hens susceptible to sterility, hyperlipidemia, and premature atherosclerosis (26). This indicates that, at least in avian species, the VLDL receptor is clearly involved in lipoprotein metabolism.

VLDL receptor pre-mRNA of both mammalian and avian species may be subjected to specific post-transcriptional differential splicing of the clustered O-linked sugar domain, which consists of approximately 30 amino acid residues enriched in serines and threonines (7, 10–12). The VLDL receptor, together with the apolipoprotein E receptor 2 (27), are the only members of the LDL receptor supergene family in which differential splicing of pre-mRNA has been demonstrated. However, up to now, cell-specific expression of the spliced variant protein has only been demonstrated for the growing oocyte (12, 28) and for epithelial cells from breast carcinomas (29).

Recently, the VLDL receptor has been shown to be expressed in the vascular endothelium of large vessels and capillaries both in vivo and in vitro (19, 30, 31). These findings, together with the binding capacity of the VLDL receptor in vitro reported above, suggest that it could be involved both in lipid metabolism and in fibrinolysis. In order to gain insight into the cell-specific expression of the VLDL receptor we have characterized its structure in bovine aortic endothelial cells (BAEC). By several approaches we found that the VLDL receptor expressed in this cell type lacks the O-linked sugar domain. In addition, our results indicate that the VLDL receptor variant lacking the O-linked sugar domain is present at the plasma membrane of BAEC and has RAP binding activity. RT-PCR analysis on bovine tissues showed that the two spliced variants of the VLDL receptor are differentially expressed. Taken together, these results indicate that the VLDL receptor spliced variant lacking the O-linked sugar domain has cell- and tissue-specific expression, suggesting that the role of the two isoforms could be different.

MATERIALS AND METHODS

Materials

Antibodies against von Willebrand factor, FITC-conjugated and peroxidase-conjugated swine anti-rabbit were obtained from Dako Corp. (Santa Barbara, CA). The antibody against the 160 amino acids N-terminal domain of the human VLDL receptor were as previously described (30). The antibody against the carboxy-terminal (C-terminal) domain was a gift from Dr. J. Gliemann (University of Aarhus, Aarhus, Denmark). Receptor-associated protein (RAP) and polyclonal antibodies against the recombinant RAP protein were prepared as described elsewhere (19). ldla-7 cells were a gift from Dr. M. Krieger (Massachusetts Institute of Technology, Cambridge, MA). V-7 cells, which overexpress the human VLDL receptor, were obtained as described (17). PCR and RT-PCR reagents were from Perkin-Elmer (Roche Molecular Systems, Inc., Branchburg, New Jersey, USA). Oligonucleotides were from MWG-Biotech (Ebersberg, Germany). ECL kit, Sequenase 2.0 kit, ^{32}P- and ^{35}S-labeled nucleotides and nylon membranes (Hybond™-N) were from Amersham Corp. (Bucks, UK). Cell culture reagents were from Whittaker (Walkersville, MD). All other chemicals were of analytical grade.

Cell culture and transfection experiments

Bovine aortic endothelial cells (BAEC) were isolated from fresh bovine thoracic aortae obtained in the local slaughterhouse (Mercabarna, Barcelona) as described (32). BAEC were cultured in medium 199 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 10 μg/ml streptomycin, 2 mm glutamine, 0.5–1 ng/ml basic fibroblast growth factor, and 50–100 μg/ml heparin. Medium was changed every 48 h, and cells were used at passages 2–8. ldla-7 and V7 cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 10 μg/ml streptomycin, 2 mm glutamine, and G-418 (400 μg/ml). COS1 cells, used for transfection experiments, were cultured in Dulbecco's modified Eagle's medium, with 4.5 g/l glucose and 10% fetal bovine serum. Transfection experiments were performed as described (33) with the vector pRCMV containing the full-length human VLDL receptor cDNA (5).

Western and ligand blotting analyses

Cell extracts were prepared from cells grown to confluence in 150-mm dishes. Cells were scraped into a solution that contained 250 mm sucrose, 2 mm EGTA, 20 mm HEPES, 1 mm PMSF, 0.5 U/ml aprotinin, and 5 μg/ml leupeptin. The extracts were passed through a syringe provided with a 0.9 × 40 needle, and centrifuged 20 min at 28,000 *g*. To obtain the membrane preparation, the lysate was centrifuged 5 min at 750 *g* and the supernatant was centrifuged 60 min at 31,000 *g*. Pellets were used for electrophoresis and Western blot. All centrifugations were carried out in a Sorvall centrifuge with rotor SS-34. Samples were run on 7.5% SDS-polyacrylamide gels under non-reducing conditions. VLDL receptor was immunodetected with antibodies against 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 membranes were incubated with 25 nm RAP in TBS containing 3% non-fat milk, 0.05% Tween 20, and 5 mm $CaCl_2$, overnight at 4°C. Filters were then incubated with a rabbit anti-RAP IgG.

Immunofluorescence microscopy

For immunofluorescence, BAEC or transfected COS cells were grown on coverslips. To detect cell-surface VLDL receptor and bound RAP, cells were incubated at 4°C for 30 min with antibodies to the N-terminal domain of the human VLDL receptor or 25 nm of recombinant RAP. After washing, cells were fixed with 3% paraformaldehyde in 0.1 m phosphate buffer, pH 7.4, containing 60 mm sucrose, and processed for immunofluorescence as described (33) with FITC-conjugated swine anti-rabbit immunoglobulins to detect VLDL receptor or rabbit-anti RAP and FITC-conjugated swine anti-rabbit immunoglobulins to detect cell-surface RAP. To detect von Willebrand factor, BAEC cells were fixed as above and permeabilized with 0.1% Triton X-100. Immunofluorescence was performed with rabbit antibodies against human von Willebrand factor and FITC-conjugated swine anti-rabbit immunoglobulins. Coverslips were viewed and images were recorded (33) in a Leica TCS 4D (Leica Lasertechnick GmbH,

Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope.

Cloning and characterization of bovine VLDL receptor complementary DNA

A bovine endothelial cell cDNA library cloned into Lambda Zap II vector (Stratagene, La Jolla, CA, cat. no 936705) was screened for potential cDNAs homologous to the human VLDL receptor. Phages (2×10^6) inoculated into XL 1 Blue MRF′ were seeded in top agarose onto LB-agar plates. The filters were hybridized with a random-primed ^{32}P-labeled human cDNA probe encompassing nt 392–892 (5) corresponding to the ligand-binding domain. Positives were confirmed by reprobing the same filters with a probe positioned 3′ to the first probe. Five double positives were found. cDNA inserts were recovered by *Eco*RI digestion of phage DNA and agarose gel electrophoresis, and subcloned into the pGEM 3Z f (−) plasmid vector. The inserts were sequenced using the primer walking strategy. Sequence data were assembled using the DNASTAR software (DNASTAR Inc., Madison, WI) and analyzed by the Genetics Computer Group (GCG) (Wisconsin, WI) package at the Spanish node of EMBNet.

RNA isolation and reverse transcriptase (RT)-PCR analysis

RNA isolation from BAEC or frozen bovine tissues was performed according to standard procedures. For PCR amplification of bovine VLDL receptor mRNA, 1 μg of total RNA was reverse-transcribed using random primer hexamers. The following oligonucleotides were used for PCR primers: WBV-1, 5′-ATGGA GGATGTGAATACC-3′; WBV-17, 5′-GCTCTGGTCACATTGATC 3′. PCR amplifications of cDNA were performed with the two pairs of primers, WBV-1/WBV-17 (4 min at 97°C, 35 cycles of 30 sec at 94°C, 30 sec at 50°C, and 30 sec at 72°C) and the GeneAmp RNA PCR and PCR kit (Perkin-Elmer) on a Perkin-Elmer thermal cycler model 2400. GAPDH specific primers (GAPDH-5′, 5′-TGATGACATCAAGAAGCTGGTGAAG-3′; GAPDH-3′, 5′-TCTT GGAGGCCATGTGGGCCAT-3′) were used as internal controls. PCR parameters for GAPDH-5′/GAPDH-3′ primers were 7 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. For semiquantitative analysis of bovine VLDL receptor mRNA, PCR amplification of bovine samples for a different number of cycles in the presence of ^{32}P-dCTP was performed using WBV-1 and WBV-17 primers. DNA variant fragments were separated by polyacrylamide gel electrophoresis, and the relative amounts of the two different receptor variants were assessed by using the Fuji Bio-Image analyzer system Bas 2000 (Fuji Photo Film). For Southern blotting analysis two probes were used. One, named C-terminal probe, expands from nucleotide 1924 to 2543 of the reported full-length sequence (AF016537), and does not contain the O-glycosylation sugar domain. The other, named exon 16 probe, corresponds to the O-glycosylation sugar domain sequence, and expands from nucleotide 1 to 84 of the reported sequence (AF03442).

RESULTS

Western, ligand blotting, and immunofluorescence analyses of the VLDL receptor on BAEC

Recent reports (30, 31) indicate that the VLDL receptor is present and could be functionally important on the vascular endothelium. In order to identify the VLDL receptor in bovine endothelial cells, membrane extracts from cultured BAEC were isolated and subjected to immunoblotting analysis with antibodies against the N-terminal domain (**Fig. 1A**) of the human VLDL receptor. Membranes from V7 cells, which constitutively overexpress the human VLDL receptor, and membranes from bovine heart were used as a control. The sizes of the immunoreactive proteins detected with the anti-VLDL receptor were different in the various samples tested. Under non-reducing conditions the VLDL receptor appeared as a major band of 124 kDa and a minor band of 91 kDa, which could represent the precursor form of the VLDL receptor in V7 cell membrane extracts. Two bands of 140 kDa and

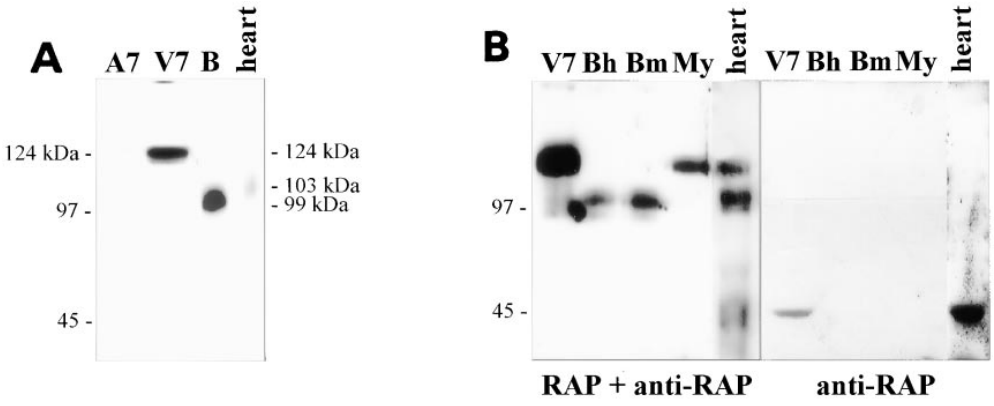


Fig. 1. Immunoblot analysis (A), and RAP ligand blot analysis (B) of the VLDL receptor in cell membrane extracts from V7 cells, BAEC, and bovine heart. A: Cell membrane extracts of ldl-A7 (A7) or with V7 cells (V7), BAEC (B) and of bovine heart were size-fractionated on a 7.5% SDS-polyacrylamide gel under non-reducing conditions and subjected to Western blotting with N-terminal antibodies against the human VLDL receptor. Twenty-five μg of membrane protein was used from A7, V7 and BAEC, and 75 μg from bovine heart. B: For RAP ligand blotting, cell extracts were subjected to 7.5% SDS-PAGE under non-reducing conditions, transferred to nitrocellulose, and incubated with 25 nm RAP. RAP was detected using an anti-RAP IgG. Control filters were incubated with anti-RAP. Sample exposures were for 5 min; control exposures were for 10 min. Twenty-five μg of V7 cell membrane extracts (lane 1), homogenates (Bh) and cell membranes (Bm) of BAEC (lanes 2 and 3), and 75 μg of human myocardium (My) and of bovine heart were used.

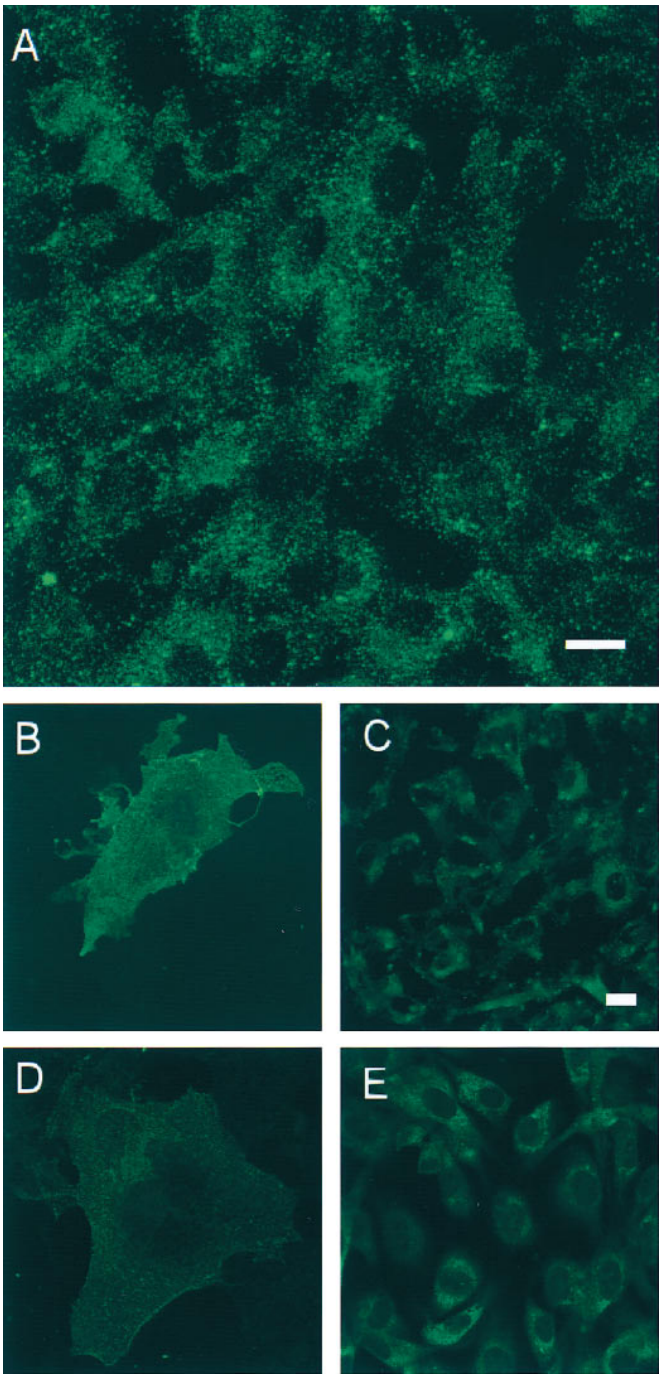


Fig. 2. Immunofluorescence studies of VLDL receptor on BAEC and transfected COS1 cells. BAEC (A, C, and E) were obtained and cultured as indicated in Materials and Methods. COS1 cells (B and D) were transfected with a vector containing the full-length human VLDL receptor and processed 48 h afterwards. To detect extracellular VLDL receptor, BAEC (A) and transfected COS1 cells (B) were incubated with antibodies against the N-terminal domain of the human VLDL receptor and then fixed and processed for immunofluorescence with FITC-conjugated swine anti-rabbit immunoglobulins. To detect cell-surface bound RAP, BAEC (C) and transfected COS1 cells (D) were incubated with 25 nm RAP, fixed, and processed for immunofluorescence with rabbit anti-RAP antibodies and FITC-conjugated swine anti-rabbit immunoglobulins. To detect von Willebrand factor (E), fixed and permeabilized BAEC were processed following conventional immunofluorescence methods with anti-human von Willebrand factor and FITC-conjugated swine anti-rabbit immunoglobulins. Images show a single optical section (xy: 0,31 μm) taken by the confocal microscope from the top plasma membrane (A, B, C, and D) and from the middle (E) of the cells. Bar: 10 μm.

116 kDa have been described under reducing conditions (17). Unexpectedly, in BAEC membranes all anti-VLDL receptor antibodies identified a single band with an apparent electrophoretic mobility of about 99 kDa in non-reducing acrylamide gels (Fig. 1A) and of 110 kDa in reducing conditions (not shown). Two bands with an apparent electrophoretic mobility of 124 kDa and 103 kDa were also identified in membrane extracts from bovine heart in non-reducing conditions. To confirm that the 99 kDa protein detected in membrane extracts from BAEC corresponded to the VLDL receptor, homogenates and membranes from BAEC and human and bovine myocardium were used for RAP ligand blotting assays. As shown in Fig. 1B, a 99 kDa protein of BAEC bound RAP. In bovine heart membranes, RAP bound two major bands in the range of 124 and 100 kDa. In human myocardium, RAP bound only to a 124 kDa protein. In control filters incubated with anti-RAP antibodies, only endogenous RAP was detected, and no binding to the bands identified with the anti-VLDL receptor or in ligand blotting assays was observed (Fig. 1B), indicating that the RAP antibodies used did not recognize the VLDL receptor in these conditions.

To determine whether the VLDL receptor in BAEC could be efficiently secreted and present in the cell membrane, binding of the N-terminal antibodies and RAP was performed on cells. Cell-surface bound proteins were detected by immunofluorescence and confocal microscopy. Transfected COS1 cells were used as a control to test the efficiency with which the N-terminal antibodies and RAP detect cell-surface VLDL receptor. Both anti-VLDL receptor and RAP were able to bind to the BAEC surface (**Fig. 2A** and **C**) and to the cell-surface of COS1 cells transfected with the full-length human VLDL receptor (Fig. 2B and D). Control experiments in BAEC and transfected COS1 cells, not incubated with RAP but incubated with anti-RAP antibodies, did not stain the cell surface**.** In addition, non-transfected COS1 cells did not stain when incubated with anti-VLDL or RAP (not shown). Immunofluorescence with anti-von Willebrand factor indicated that BAEC cultured in vitro maintained their differentiation state (Fig. 2E). Anti-VLDL receptor staining showed that the receptor was distributed as small dots all over the cell surface, with higher intensity in central areas of the cells (Fig. 2A). Parallel experiments performed with antibodies against the C-terminal domain gave similar results (not shown). Bound RAP showed a similar immunofluorescence distribution. These results indicate that VLDL receptor in BAEC is sorted to the plasma membrane.

Isolation of cDNA for bovine VLDL receptor and analysis of domain structure in different species

In order to characterize the shorter VLDL receptor variant expressed in BAEC, we screened a BAEC cDNA library using a DNA probe corresponding to bp 392–892 of the

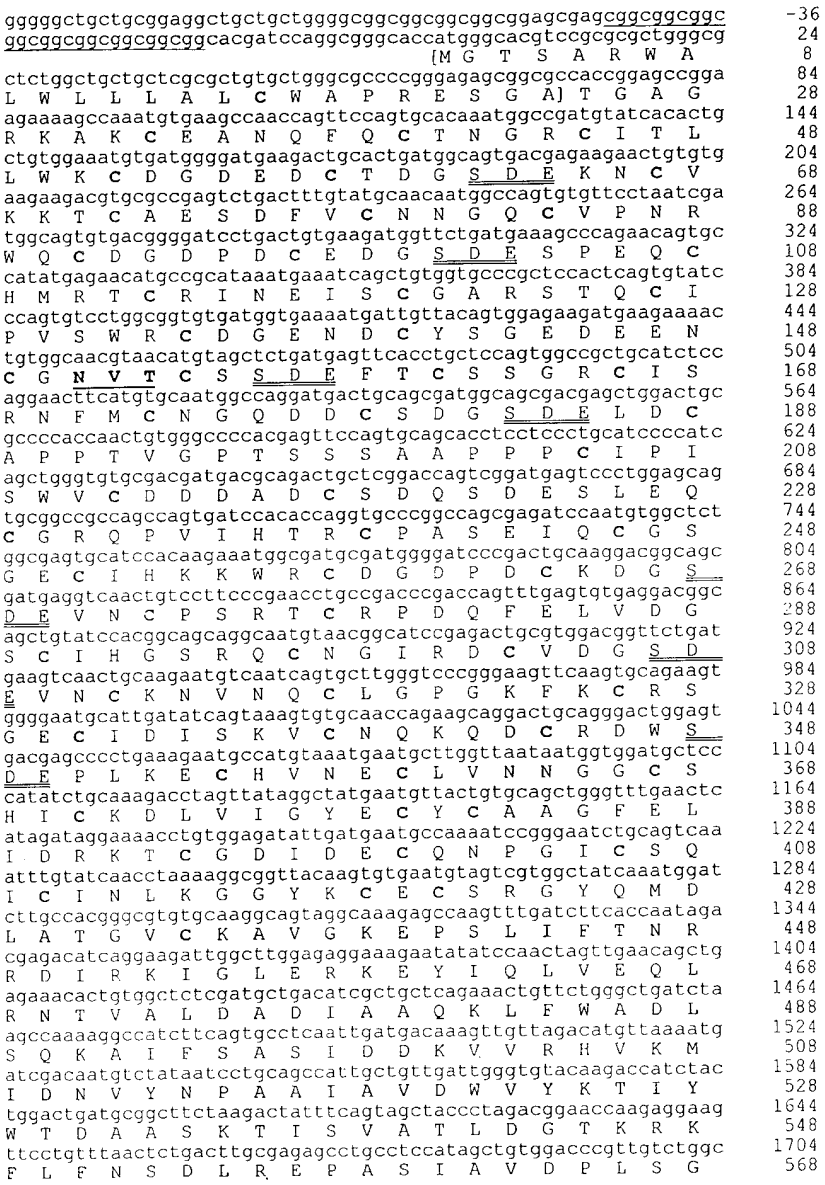


Fig. 3.

human VLDL receptor (5). A cDNA covering the whole polypeptide region was isolated and sequenced. The 3113 bp cDNA encompassed an open reading frame of 845 amino acid residues (**Fig. 3**). The proposed leader sequence spans residues 1–24, as shown by Simonsen et al. (21) leaving by deduction 821 residues for the mature peptide. By alignment analysis of the VLDL receptor structure described for other species, it was found that the bovine cDNA lacked the stretch of residues corresponding to the O-linked sugar domain of exon 16 of the gene (7). Analysis of the five different subdomains of the VLDL receptor indicated 92–100% similarity between the bovine and the other mammalian species, whereas the similarity to VLDL receptor analogs of other species was lower (**Fig. 4**).

Analysis of VLDL receptor mRNA

Northern blot of bovine total RNA extracted from heart, skeletal muscle, and adipose tissue showed a single band of 3.4 kb (data not shown). To assess which RNA variant of the VLDL receptor is predominant in BAEC and in heart, we performed RT-PCR using as primers WBV-1 and WBV-17 (**Fig. 5**), which surround the O-linked sugar domain. In RNA from heart, two bands of 220 and 136 bp were amplified (Fig. 5A, right panel). When comparing RT-PCR-amplified RNA obtained from BAEC, as well as total DNA extracted from the BAEC cDNA library used for cloning the VLDL receptor with the isolated full length cDNA, only one band of 136 bp was detected. Southern blotting of the RT-PCR-amplified products with probes containing O-glycosylated region sequences or not further demonstrated that primers WBV-1 and WBV-17 did not amplify exon 16 from BAEC RNA (Fig. 5A, left panel). To study the tissue distribution of the bovine VLDL receptor variants, RT-PCR analysis was performed on RNA from bovine tissue using the same primers, WBV-1 and WBV-17.The results indicate that the O-linked sugar

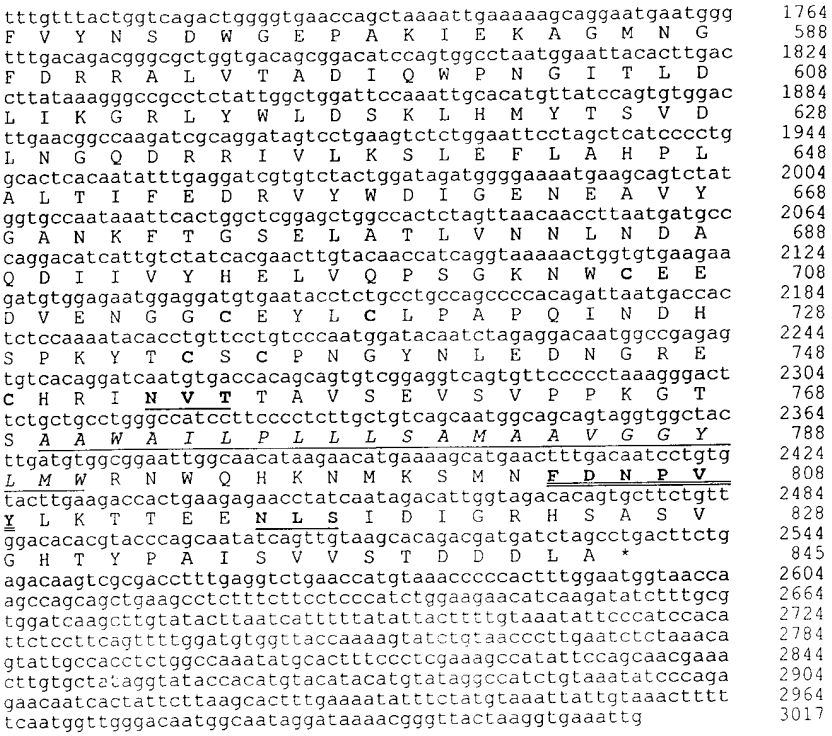


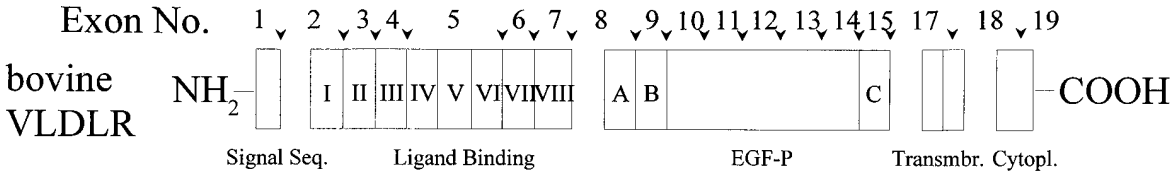
Fig. 3. Complementary DNA and deduced amino acid sequence of the VLDL receptor variant found in BAEC. Numbering of nucleotides starts from the A of Met. The triplet repeat in the 5′-untranslated region is underlined. Cleaved signal sequence is boxed. Cysteine residues are in bold. Three potential N-linked glycosylation sites are in bold and underlined. Different ligand binding motifs within the ligand binding domain are indicated by double underline. The transmembrane domain is in italics and underlined. The FDNPVY sequence targeting the receptor to coated pits is in bold and double underlined. The stop codon is indicated by an asterisk. The GenBank accession number of the complete cDNA sequence is AF016537.

variant was predominantly expressed by brain, heart, and skeletal muscle. This variant was not detected in kidney, liver, or mammary gland. However, these tissues mostly expressed the spliced variant, which could also be detected in adipose tissue, heart and skeletal muscle by using the probe encoding the C-terminal portion of the VLDL receptor that did not contain the O-linked sugar domain. These results suggest that the two isoforms are differentially expressed by bovine tissues. The Southern blotting showed that the C-terminal probe had more affinity to hybridize with the RT-PCR product corresponding to the short variant than with that corresponding to the long, which could be a consequence of the continuous homology between the C-terminal probe and the PCR product. However, longer exposure showed a weak band of 220 bp detected with the C-terminal probe, especially in heart and skeletal muscle. Sequencing of both the 220 and 136 bp PCR products revealed that they correspond to VLDL receptor RNA variants possessing and lacking the O-linked sugar domain, respectively (Fig. 5B). The number of serine and threonine residues of the bovine O-linked sugar domain was nine. Although serines and threonines in the O-linked sugar domain of other mammalian species number ten to twelve (Fig. 5C), the lack of three such residues in this region does not substantially reduce the apparent molecular weight of the bovine VLDL receptor versus the human as determined by Western analysis (Fig. 1).

To semiquantitatively assess the amount of the two VLDL receptor variants, we PCR-amplified BAEC and bovine heart cDNA for a different number of cycles in the presence of ^{32}P-dCTP and using the pair of oligonucleotide primers that flanks the exon 16. In heart, both variants were detected; the amount of the long variant was two times the amount of the short variant. In contrast, in RNA of BAEC, the unspliced variant containing the O-glycosylation domain corresponding to exon 16 of the gene was almost undetectable (not shown).

DISCUSSION

Although the physiological function of the VLDL receptor is not well understood, recent studies have identified the VLDL receptor on vascular endothelial cells (30, 31), suggesting that it could play a critical role in the vascular endothelium by regulating lipoprotein catabolism and fibrinolysis (19, 20). Here we report the cloning and sequencing of the bovine VLDL receptor and show that BAEC express exclusively a short variant of the VLDL receptor that lacks the entire O-linked sugar domain. In addition, the present study demonstrates that the two VLDL receptor variants are differentially expressed by bovine tissues.



	Signal Sequence	Ligand Binding	EGF Prec. Homol.	Transmembrane	Cytoplasmic
bovine vs. human	92,3	92,4	97,2	100	98,1
bovine vs. rabbit	92	93	96,9	100	98,1
bovine vs. rat	92,6	91,5	97,7	100	98,1
bovine vs. mouse	92,6	91,2	97,5	100	98,1
bovine vs. chicken	63,6	84,1	88,8	71,4	94,3
bovine vs. Xenopus	54,5	77,6	82,4	85	86,8

Fig. 4. Exon organization, protein domains, and amino acid conservation of bovine VLDL receptor lacking the O-linked sugar domain. Model representing the domains in the VLDL receptor variant found in BAEC. Only four domains are present in the mature protein (signal sequence is cleaved). Note that no O-glycosylated domain is located towards the C terminus of the bovine protein, because of the lack of exon 16. The presence of introns as deduced from the human gene sequence (7) is indicated by arrowheads. The percentage of amino acid similarity among bovine VLDL receptor and VLDL receptors from other species in a given domain calculated by the clustal method (a multiple sequence alignment algorithm available within the DNASTAR software) using PAM290 residue weight table is indicated.

Several lines of evidences indicate that BAEC express a VLDL receptor variant that lacks O-linked sugars: *i*) Western and RAP ligand blotting gave a single band of 99 kDa in membrane extracts of BAEC; *ii*) screening of the BAEC cDNA library with the previously characterized human VLDL receptor cDNA as a probe gave three C-terminal positive clones (all the clones lacked the 84 nucleotides corresponding to exon 16 and PCR analysis confirmed that VLDL receptor cDNA encoding exon 16 was absent from the library); *iii*) RT-PCR analysis of the BAEC RNA gave only one band of 136 nt using a pair of oligonucleotide primers that flanks the deletion; and *iv*) semiquantitative RT-PCR analysis showed that only the variant lacking the O-linked sugar domain was amplified in BAEC.

The VLDL receptor, together with the apolipoprotein E receptor 2, are the only members of the LDL receptor supergene family that have been shown to have two variants (7, 10, 11, 27–29). RT-PCR studies (7, 10) performed in some mammalian cell lines (TPH-1 monocytic leukemia cells, normal and FH fibroblasts, smooth muscle cells, and HepG2 cells) and in human brain (34) have shown that the two isoforms are expressed in different proportions. However, in HepG2 cells and human kidney, the mRNA variant devoid of the O-glycosylation domain was predominant (10, 34). Biochemical studies have shown that bovine mammary gland- and human mammary gland-derived cell lines only present a low molecular weight (105 kDa) form of the VLDL receptor (20, 21), which represents the short variant (29). In laying hens, the VLDL receptor expressed by the oocytes lacks the O-linked sugar domain (12, 26), but somatic tissue cells express the non-spliced isoform of the VLDL receptor (28). The present RT-PCR analysis performed in bovine tissues indicated that heart, brain, and skeletal muscle express both variants in similar proportions, while kidney, mammary gland, and adipose tissue predominately expresses the non-O-glycosylated variant. Therefore, together with the results presented above, the emerging picture suggests that expression of VLDL receptor isoforms is cell- and tissue-specific and that the role or regulation of the two VLDL receptor isoforms could be different. Further studies are necessary to establish whether the different cell types present in these tissues could only express one VLDL receptor variant.

The O-linked sugar domain shows high homology between mammalian species (81.4% of bovine vs. human), suggesting that it may have an important influence on some properties of the VLDL receptor. In the present study we found that the short variant of the VLDL receptor was present at the plasma membrane of endothelial cells, indicating that the lack of the O-linked sugar domain did not impair its intracellular processing. In addition, ligand blotting studies indicate that it binds with high affinity to RAP, and preliminary studies (J. Magrané, unpublished observations) also indicate that it binds to βVLDL. Moreover, studies by Schneider and his colleagues (22, 35–38) on the chicken homolog, lacking the O-glycosylation domain, showed a broad ligand specificity similar to mammalian VLDL receptors. Binding to apoB (35), apoE (36), vitellogenin (37), RAP and lactoferrin

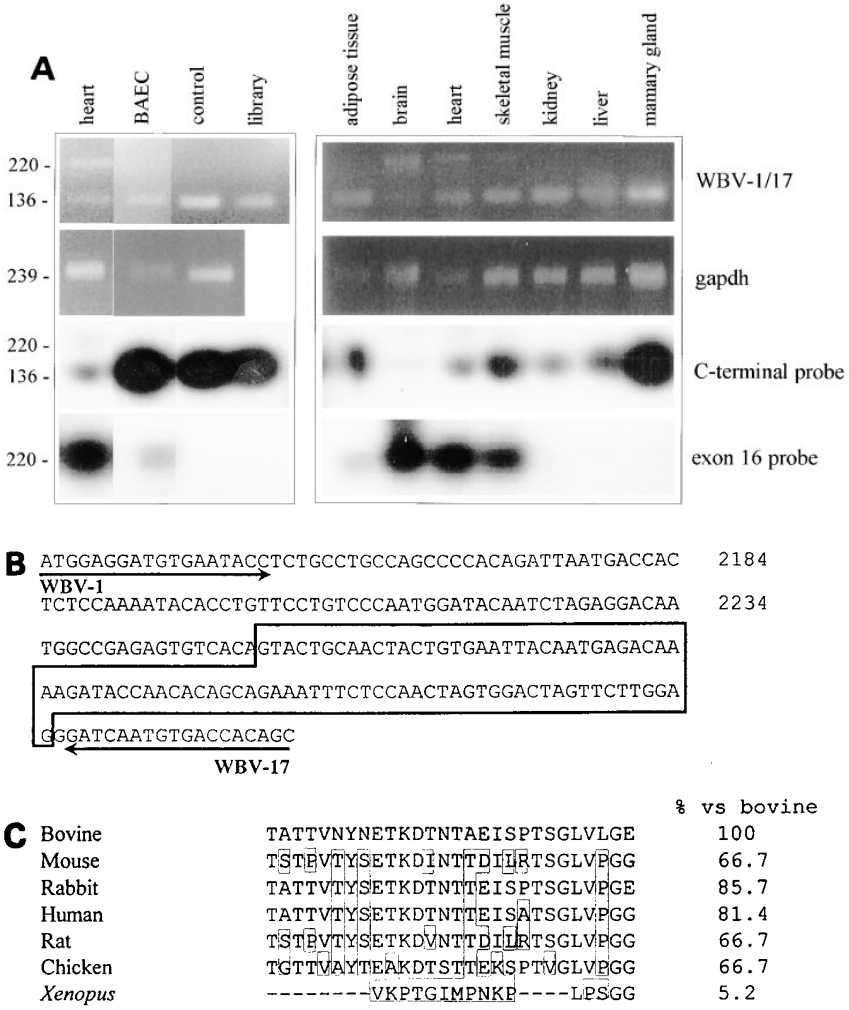


Fig. 5. Bovine VLDL receptor O-linked sugar domain. A: RT-PCR analysis performed using exon 16 flanking primers (WBV-1 and WBV-17) and GAPDH. DNA bands from the WBV-1/17 RT-PCR agarose gel were transferred to nitrocellulose and hybridized with probes corresponding to the C-terminal part of the VLDL receptor cDNA or to exon 16. Right panel, samples analyzed were: a positive clone from the BAEC cDNA library clones (control), total DNA extracted from the BAEC cDNA library (library), and cDNA obtained by reverse transcription of total RNA from BAEC and bovine heart. Left panel, seven different bovine tissues were analyzed. Sequencing of the 220 bp band from bovine heart was found to correspond to VLDL receptor mRNA variant possessing the O-linked sugar domain. All 136 and 220 bp bands were sequenced and found to correspond to VLDLR. B: bovine VLDL receptor exon 16 sequence is boxed. The position of WBV-1 and WBV-17 primers is indicated by arrows. C: alignment of the residues of the O-glycosylation domains of bovine VLDL receptor and others species. The GenBank accession number of the bovine exon 16 sequence is AF034420.

(22,) and α_2-macroglobulin (38) was shown. Recently, Martensen et al. (29) demonstrated that the mammalian VLDL receptor variants have the same in vitro binding affinity to RAP and serine proteinase/serpin complexes. Thus, no differences in ligand specificity or intracellular processing have been found between the two receptor isoforms to date. However, a careful comparative study of these variants with regard to their ligand capacity, intracellular processing, and cell regulation is required.

The LDL receptor contains an O-linked sugar domain at a position analogous to the VLDL receptor. This domain is 29 residues shorter in the VLDL receptor than the corresponding region of the LDL receptor (4–11). The sequence identity of VLDL and LDL receptors is the lowest in the O-linked domain (i.e., 19% of identity between the human VLDL and LDL receptors, 7, 9). The precise function of the O-linked sugar domain of the LDL receptor is not known. When the clustered O-linked sugar domain of the LDL receptor is removed by site-directed mutagenesis, no apparent defect in its ligand binding capacity or stability was observed (39). However, O-linked carbohydrate chains on the LDL receptor appear to protect the receptor from cleavage by proteolytic enzymes (40). Whether this is the case for the VLDL receptor remains to be studied, but given the low level of homology between the VLDL and LDL receptors as regards the O-linked sugar domain, the function of this domain may be different.

The highest expression of VLDL receptor mRNA in mammals is found in tissues that have a continuous endothelium such as heart, skeletal muscle, and adipose tissue (31). Immunofluorescence studies have indicated that in VLDL receptor-expressing tissues, endothelial cells present higher levels of VLDL receptor than the neighboring parenchymal cells (31). The presence of the VLDL receptor on the endothelium of both capillaries and larger vessels (30, 31) and its expression in rabbit atherosclerotic lesions (41) is suggestive of a critical function in the uptake and trans-endothelial transport of lipoproteins and/or other ligands. VLDL receptor of human endothelial cells mediates the endocytosis of u-PA:PAI-1 complexes (19), suggesting that it could participate in fibrinolysis and angiogenesis. Lipoprotein lipase (LPL) promotes the uptake of lipoproteins through the VLDL receptor (14, 19) as it enhances binding and uptake of triacylglyceride-rich lipoproteins to other members of the LDL receptor family. Given that the site of the LPL action is on the luminal surface of the endothelial cells, VLDL receptor may provide a specific mechanism for endothelial lipoprotein catabolism. The finding of the endothelial-specific expression of the spliced variant of the VLDL receptor provides a framework to investigate its role in vascular metabolism. BAEC is therefore a suitable model for further studies to address the physiological function as well as the molecular mechanisms for differential splicing and functional properties of VLDL receptor variants lacking the O-linked sugar domain.

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