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Expression and Characterization of a Very Low Density Lipoprotein Receptor Variant Lacking the *O*-Linked Sugar Region Generated by Alternative Splicing[1]

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The very low density lipoprotein receptor (VLDLR) gene contains an exon encoding a region of clustered serine and threonine residues immediately outside the membrane-spanning sequence, and this region has been proposed to be the site of clustered *O*-linked carbohydrate chains. Two forms of VLDLR transcripts, with and without the *O*-linked sugar region, are generated through alternative splicing. Reverse transcription polymerase chain reaction with RNAs from various rabbit tissues revealed that the VLDLR transcript with the *O*-linked sugar region (type-1 VLDLR) is the major transcript in heart and muscle, while the VLDLR transcript without the *O*-linked sugar region (type-2 VLDLR) predominates in non-muscle tissues, including cerebrum, cerebellum, kidney, spleen, adrenal gland, testis, ovary, and uterus. Hamster fibroblasts expressing type-2 VLDLR bound with relatively low affinity to β-migrating very low density lipoprotein compared with type-1 VLDLR-transfected cells. In contrast, the internalization, dissociation, and degradation of the ligand were not significantly impaired in either type of VLDLR-transfected cell. The receptor proteins in type-2 VLDLR-transfected cells underwent rapid degradation and accumulated in the culture medium, while those in type-1 VLDLR-transfected cells were stable and resistant to proteolytic cleavage. Analysis of the *O*-linked sugars of both types of transfected cells suggested that the *O*-linked sugar region is the major site for *O*-glycosylation.

Key words: lipoprotein, *O*-linked sugar, splicing variant, VLDL receptor.

Very low density lipoprotein (VLDL) receptor (VLDLR) (*1, 2*) and recently identified apolipoprotein E (ApoE) receptor 2 (apoER2) (*3, 4*) bind with high affinity to apoE-containing lipoproteins, including apoE-rich β-migrating VLDL (β-VLDL), and closely resemble low density lipoprotein (LDL) receptor (LDLR) in structure (*5*). The three lipoprotein receptors consist of five functional domains: (i) a ligand binding domain composed of multiple cysteine-rich repeats; (ii) an epidermal growth factor (EGF) precursor homology domain which functions in the acid-dependent dissociation of the ligands from LDLR (*6*); (iii) a serine- and threonine-rich domain immediately outside the membrane-spanning sequence, which has been proposed to be the site of clustered *O*-linked carbohydrate chains (*1-4*); (iv) a transmembrane domain; and (v) a cytoplasmic domain with a coated pit targeting signal (*7*). The chromosome locations and tissue distributions of VLDLR, apoER2 and LDLR are completely different, even though they all recognize apoE (LDLR binds both apoE- and apo B-100-containing lipoproteins), and are closely similar in their protein and gene structures (*2, 4*).

In mammals, VLDLR mRNA is most highly expressed in heart, skeletal muscle, brain, and adipose tissue, with only a trace amount in the liver (*1, 8-12*). Based on the expression of VLDLR in tissues responsible for active fatty acid utilization and the role in chicken (see below), mammalian VLDLR is postulated to play a role in the uptake of triacylglycerols into fat and muscle cells (*13*). Frykman *et al.* have produced mice lacking immunodetectable VLDLR, and revealed that the knockout mice exhibited modest decreases in body weight, body mass index, and adipose

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Abbreviations: apo, apolipoprotein; apoE, apolipoprotein E; apoER2, apolipoprotein E receptor 2; EGF, epidermal growth factor; LDLR, low density lipoprotein receptor; RT-PCR, reverse transcription polymerase chain reaction; VLDL, very low density lipoprotein; VLDLR, very low density lipoprotein receptor; β-VLDL, β-migrating very low density lipoprotein.

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tissue mass, while their plasma cholesterol levels, triacylglycerol levels, and lipoprotein profiles were not altered (*14*). In contrast to the apparently normal plasma lipoprotein metabolism in VLDLR knockout mice, adenovirus-mediated gene transfer of VLDLR into LDLR knockout mice resulted in the overexpression of VLDLR in the liver. This hepatic overexpression markedly reduced the plasma levels of cholesterol as well as apoE- and apoB-100-containing lipoproteins (*15, 16*), suggesting that VLDLR recognizes apoE *in vivo*.

In chicken, VLDLR is expressed almost exclusively in oocytes (*17*) and mediates their uptake of yolk precursors, VLDL and vitellogenin (*18*). This receptor-mediated process is critical in non-mammalian vertebrate oogenesis. Mutations in the chicken VLDLR cause female sterilization through impaired ovulation. This mutant phenotype is caused by an amino acid substitution in the EGF precursor homology domain of the chicken VLDLR gene located on the Z chromosome (*18*). In *Xenopus laevis*, VLDLR is also abundant in oocytes and binds vitellogenin with high affinity (*19*), suggesting that it also plays a key role in oogenesis in the Amphibia.

We have previously shown that, in human monocytic leukemia THP-1 cells, a variant VLDLR lacking the *O*-linked sugar region is co-expressed with a receptor with five functional domains (*2*). Since the *O*-linked sugar region of human VLDLR is encoded by a single exon, alternative splicing of VLDLR pre-mRNA possibly leads to two different types of VLDLR transcript (*2*). The amino acids in the *O*-linked sugar region of VLDLR are highly conserved various species, while those in the LDL receptor are poorly conserved (*1, 2, 8-12, 20*). Although deletion of the *O*-linked sugar region does not impair the function of LDLR in hamster fibroblasts (*21*), the high degree of amino acid conservation suggests the functional importance of the *O*-linked sugar region in VLDLR.

To address the function of the *O*-linked sugar region of VLDLR, we expressed the two variant human VLDLRs in hamster cells and analyzed their properties. In this paper, we describe the effects of the deletion of the *O*-linked sugar region of VLDLR expressed in hamster cells.

EXPERIMENTAL PROCEDURES

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was isolated from adult rabbit tissues and human THP-1 cells by lysis in guanidinium isothiocyanate and purified by CsCl gradient ultracentrifugation (*22*). Single-stranded cDNA was synthesized from 10 μg of total RNA using SuperScript™ reverse transcriptase (Life Technologies) and random primers. One-tenth of the cDNA was subjected to PCR (*23*) with a sense primer (oligo 2035: 5′-GCCACTCTAGTCAACAACCT-3′) and an antisense primer (oligo 2390R: 5′-GAAGTCCCTTTTGGGGGAAC-3′) that span the *O*-linked sugar regions of human and rabbit VLDLR mRNAs (*2*). The cDNA was amplified with 250 nM each primer and 0.75 unit of Taq DNA polymerase (Takara Shuzo, Kyoto) in 50 μl of the buffer recommended by the supplier. The reaction mixture was heated to 94°C for 3 min, followed by 33 cycles of reannealing at 62°C for 1 min, elongation at 72°C for 1 min, and denaturation at 94°C for 1 min. The PCR products were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. To confirm the identity of the amplified cDNAs, the PCR products were directly sequenced using an Applied Biosystems automated DNA sequencer (model 373A).

Isolation of Transfected Cell Lines—Human VLDLR expression plasmids containing (phVLR2) or lacking (phVLR1) the *O*-linked sugar region (*2*) were separately introduced into *ldlA*-7 cells, a mutant Chinese hamster ovary cell line lacking the functional LDL receptors (*24*). Each plasmid (9.5 μg) was co-transfected with 0.5 μg of pSV2-Neo by the transfection protocol described by Chen and Okayama (*25*). On day 4 after transfection, the cells were placed in selective medium, which contained 700 μg/ml G418 (Life Technologies). On day 14, colonies of G418-resistant cells were pooled and assayed for VLDLR expression using fluorescently labeled lipoproteins at 37°C: fluorescently labeled β-VLDL was prepared by incorporating 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, as described previously (*1*). The transfected cells were grown in a monolayer culture in medium A (Ham's F-12 containing 20 mM HEPES, pH 7.4, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine) supplemented with 5% fetal bovine serum and 200 μg/ml G418 at 37°C under a humidified atmosphere of 5% CO_2 and 95% air.

Standard Format for Cell Culture—All cells were used for experiments according to a standard format. Cells from stock flasks were dissociated with trypsin/EDTA. On day 0, $4\text{-}6\times10^4$ cells were seeded into each Petri dish (60×15 mm) in 3 ml of medium A supplemented with either 5% fetal bovine serum or 4% newborn calf lipoprotein-deficient serum plus 1% fetal bovine serum. On day 2, the cells were refed with medium of the identical composition. For lipoprotein receptor assays, on day 3 the cells were switched to minimal Eagle's medium supplemented with 5% lipoprotein-deficient serum and used for experiments on day 4. For studies on the biosynthesis of radiolabeled VLDLR, on day 4 the cells were refed medium A supplemented with 5% lipoprotein-deficient serum and used for experiments on day 5.

Monoclonal Antibodies—Monoclonal antibody IgG 6B6-2b, which recognizes the extracellular regions of human and rabbit VLDLRs, was generated by multiple immunization of BALB/c female mice with a synthetic peptide, SLEQC-GRQPVIHTK (human VLDLR amino acids 198-211) (*2*). Similarly, a monoclonal antibody (IgG C4) to the C-terminal-cytoplasmic regions of human and rabbit VLDLRs was generated by immunization with the synthetic peptide, GHTYPAISVVSTDDDLA (amino acids 830-841) (*2*). These peptides were coupled to keyhole limpet hemocyanin and used to immunize mice. Mice showing the highest anti-VLDLR immunoreactivity titer were used to create fusions with myeloma cells using standard protocols (*26*). Clones that were positive on enzyme-linked immunosorbent assaying were examined by immunoblotting of extracts from VLDLR-transfected cells. Positive hybridomas were cloned twice by limiting dilution and injected into mice to produce ascites fluid. IgGs were purified by affinity chromatography on HiTrap protein A columns (Pharmacia).

Lipoprotein Preparation and Lipoprotein Binding Assays—Newborn calf lipoprotein-deficient serum ($d>1.21$ g/ml) was prepared by ultracentrifugation (*27*). Rabbit

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β-VLDL ($d < 1.006$ g/ml) was prepared from the plasma of 1% cholesterol-fed animals (*28*). ^{125}I-labeled β-VLDL was prepared as described previously (*27*). Surface binding of ^{125}I-β-VLDL was measured in monolayers of transfected cells at 4°C as described (*27*), except that dextran sulfate was replaced by suramin to release bound lipoproteins (*8*). Assaying of the internalization and degradation of ^{125}I-β-VLDL was carried out at 37°C as described (*27*). The levels of cell surface VLDLR expression were determined using ^{125}I-labeled monoclonal antibody IgG 6B6-2b for the transfected cells as described (*29*).

Metabolic Labeling and Immunoprecipitation—VLDLR proteins were labeled *in vivo* by growth of cells with [^{35}S]-PRO-MIX (>1,000 mCi/mmol [^{35}S]methionine and L-[^{35}S]cysteine, Amersham). The specific conditions for the labeling experiments are given in the figure legends. After the labeling period, the monolayers were chilled at 4°C, washed twice with phosphate-buffered saline, and then lysed with 250 μl of ice-cold buffer comprising 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (w/v) Triton X-100, 1 mM methionine, 1 mM cysteine, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. The cells were scraped from the dish with a rubber policeman and then centrifuged at 4°C for 30 min at 150,000 × *g*. The pellet was discarded, and the supernatant was used for the immunoprecipitation reaction.

The labeled VLDLR proteins were immunoprecipitated by incubation with 10 μg of either IgG 6B6-2b or IgG C4 at 4°C for 1 h, followed by the addition of 25 μl of Protein A-Sepharose CL-4B beads (Pharmacia). After incubation on a shaker at 4°C for 30 min, the beads were collected by centrifugation and washed sequentially: once with 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 1% (w/v) Nonidet P-40; four times with 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) Nonidet P-40, and 0.1% (w/v) SDS; and once with 10 mM Tris-HCl, pH 6.8, and 100 mM NaCl. The washed beads were resuspended in 50 μl of SDS loading buffer (*30*) and then boiled for 3 min. The immunoprecipitated proteins were then separated by 8% SDS-PAGE, followed by fluorography with XAR-5 film (Kodak). Prestained marker proteins (Bio-Rad) were used as molecular weight standards.

RESULTS

Expression of Splice Variant mRNAs—We previously showed the presence of two forms of VLDLR transcript in THP-1 cells: one consists of five domains resembling LDLR, and the other is a variant form lacking an *O*-linked sugar region (*2*). Since the *O*-linked sugar region of human VLDLR is encoded by a single exon, the two forms of VLDLR transcript are presumably through an alternative splicing of the pre-mRNA. Hereafter, VLDLR with all five domains is referred to as type-1 VLDLR and the variant lacking the *O*-linked sugar region as type-2 VLDLR. To determine the tissue expression of these two types of splicing variant in mammals, RT-PCR was carried out using total RNAs from various rabbit tissues and a set of primers encompassing the region corresponding to the *O*-linked sugar region. As shown in Fig. 1, the type-1 VLDLR transcript is a major transcript in heart and muscle, while type-2 predominates in cerebrum, cerebellum, kidney, spleen, adrenal gland, testis, ovary, and uterus. Both VLDLR transcripts are co-expressed in brain stem, lung, adipose tissues and THP-1 cells. These results indicate that the relative levels of the two types of VLDLR transcripts vary greatly among different tissues.

Functional Analysis of the Two Types of VLDLR—To analyze the function of the *O*-linked sugar region of VLDLR, human VLDLR cDNA (*2*) with or without the *O*-linked sugar region was transfected into *ldlA*-7 cells, a line of mutant Chinese hamster ovary cells, isolated by Kingsley and Krieger (*24*), that do not express cell surface LDLRs. Transfection was carried out with pSV2-Neo and G418-resistant clones were selected. The pooled population of G418-resistant cells from each transfection was assayed for VLDLR expression by incubation with fluorescent β-VLDL and used for all subsequent experiments.

To determine the levels of cell surface VLDLR expression, type-1 and -2 VLDLR-transfected cells were incubated with increasing concentrations of ^{125}I-labeled monoclonal antibody IgG 6B6-2b for 2 h at 4°C. As shown in Fig. 2A, the level of IgG 6B6-2b bound to type-2 VLDLR-transfected cells was approximately 48% of that to type-1 VLDLR-transfected cells. These values were used to correct for variations in cell surface receptor expression between type-1 and -2 VLDLR-transfected cells.

Monolayers of the transfected cells were incubated with increasing concentrations of ^{125}I-β-VLDL for 2 h at 4°C to determine the surface binding. Nonspecific binding was determined either by assaying pSV2-Neo-transfected cells with increasing concentrations of ^{125}I-β-VLDL or by incubating type-1 and -2 VLDLR-transfected cells with excess unlabeled β-VLDL: subtraction from total binding gave specific ^{125}I-β-VLDL binding. As shown in Fig. 2B, cells transfected with type-2 VLDLR bound β-VLDL with relatively low affinity compared with type-1 VLDLR-transfected cells: the calculated K_d value of type-2 VLDLR for β-VLDL was 1.4 μg/ml; and that of type-1 VLDLR-transfected cells was 0.5 μg/ml (Fig. 2C).

Although type-2 VLDLR-transfected cells exhibited relatively low affinity for β-VLDL compared with type-1 VLDLR-transfected cells, the internalization of ^{125}I-β-VLDL into both types of transfected cells was not impaired: the internalization index for ^{125}I-β-VLDL (*i.e.* the amount internalized divided by the amount bound on the

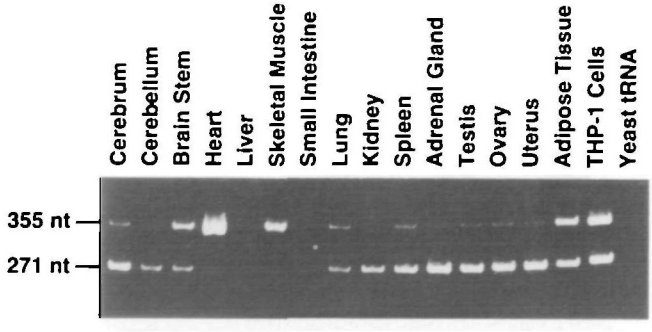

Fig. 1. **RT-PCR analysis of alternative splicing in the *O*-linked sugar region of VLDLR.** Total RNAs (10 μg) from various rabbit tissues and human THP-1 cells were used for RT-PCR (see "EXPERIMENTAL PROCEDURES"). The resulting PCR products were separated on a 2% agarose gel and then stained with ethidium bromide. The figure is representative of four independent experiments.

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surface) was not reduced in either type of transfected cell (Fig. 2, D and E). Figure 3 shows the degradation of internalized ^{125}I-β-VLDL in type-1 and -2 VLDLR-transfected cells. Both types of transfected cells were incubated with ^{125}I-labeled β-VLDL at 4°C to allow cell surface binding, and then the cells were washed and warmed to 37°C for various periods, and then the amount of surface-bound β-VLDL and the amount that had been degraded to trichloroacetic acid-soluble material were measured. In both types of cells, approximately half of the bound β-VLDL was lost from the cell surface within 30 min and degraded within 2 h, suggesting that the degradation of the bound β-VLDL was not impaired in type-2 VLDLR-transfected cells.

The intracellular degradation of LDL taken up by LDLR is inhibited by chloroquine, a weak base that increases the

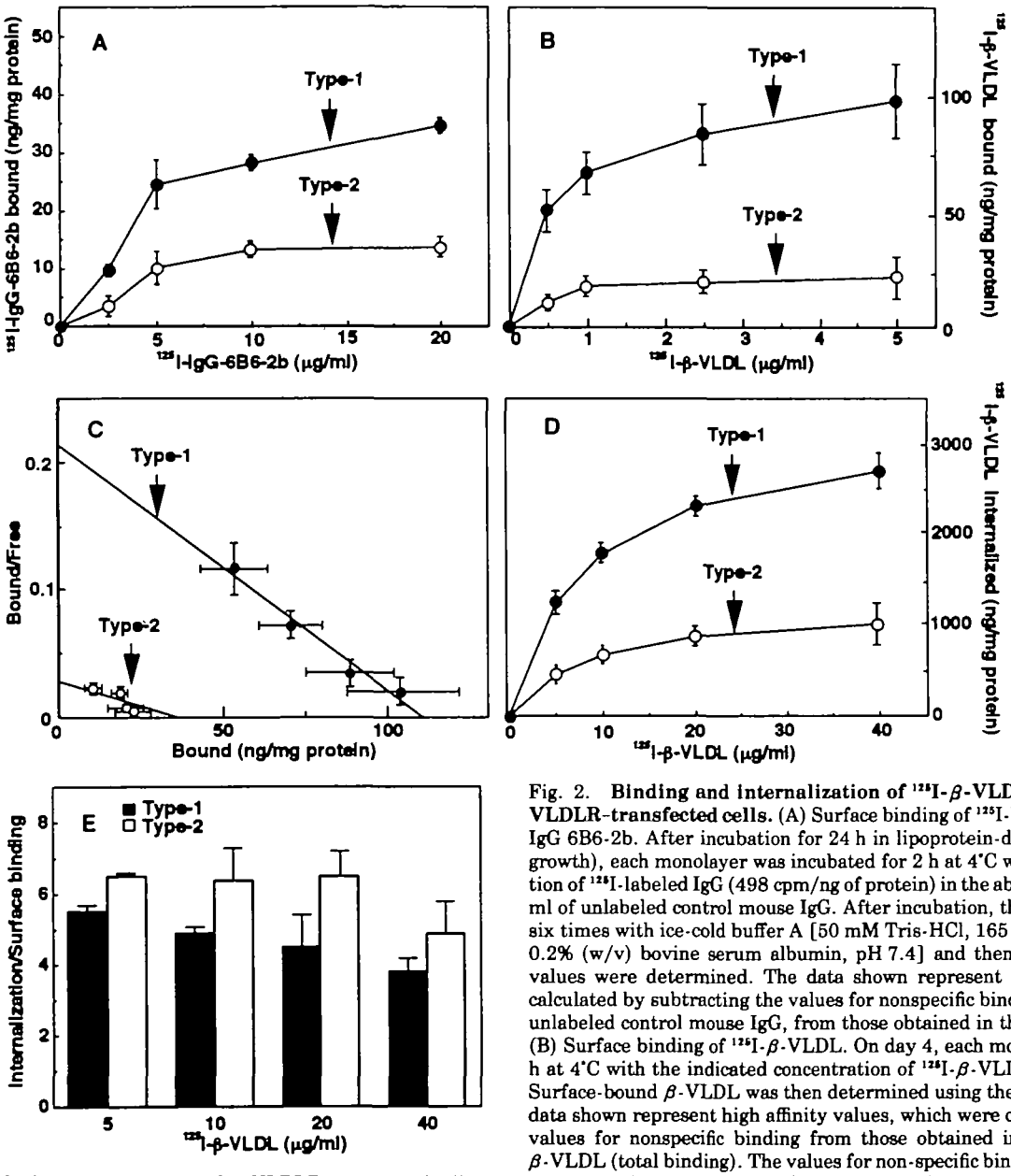

Fig. 2. **Binding and internalization of ^{125}I-β-VLDL in type-1 (●) and -2 (○) VLDLR-transfected cells.** (A) Surface binding of ^{125}I-labeled monoclonal antibody IgG 6B6-2b. After incubation for 24 h in lipoprotein-deficient serum (day 4 of cell growth), each monolayer was incubated for 2 h at 4°C with the indicated concentration of ^{125}I-labeled IgG (498 cpm/ng of protein) in the absence or presence of 350 μg/ml of unlabeled control mouse IgG. After incubation, the cells were washed rapidly six times with ice-cold buffer A [50 mM Tris-HCl, 165 mM NaCl, 3 mM $CaCl_2$, and 0.2% (w/v) bovine serum albumin, pH 7.4] and then the bound ^{125}I-labeled IgG values were determined. The data shown represent specific values, which were calculated by subtracting the values for nonspecific binding, obtained in presence of unlabeled control mouse IgG, from those obtained in the absence of unlabeled IgG. (B) Surface binding of ^{125}I-β-VLDL. On day 4, each monolayer was incubated for 2 h at 4°C with the indicated concentration of ^{125}I-β-VLDL (215 cpm/ng of protein). Surface-bound β-VLDL was then determined using the suramin release assay. The data shown represent high affinity values, which were calculated by subtracting the values for nonspecific binding from those obtained in the absence of unlabeled β-VLDL (total binding). The values for non-specific binding were determined either by incubating type-1 and -2 VLDLR-transfected cells with the indicated concentration of ^{125}I-β-VLDL in the presence of 500 μg/ml of unlabeled β-VLDL, or by incubating SV2-neo transfected cells with the indicated concentration of ^{125}I-β-VLDL. Either method gave essentially the same nonspecific values. (C) Scatchard analysis of the data in (B). (D) Internalization of ^{125}I-β-VLDL. Transfected cells were incubated for 3 h at 37°C with the indicated concentration of ^{125}I-β-VLDL (198 cpm/ng of protein), after which the values for internalized ^{125}I-β-VLDL were determined. (E) Internalization Index. Transfected cells were incubated for 3 h at 37°C, as described above, and then the total amounts of surface-bound and internalized ^{125}I-β-VLDL were determined. The internalization index was calculated by dividing the total amount of internalized ^{125}I-β-VLDL by the total amount of surface-bound ^{125}I-β-VLDL. Specific values were calculated by subtracting values obtained with pSV2-Neo-transfected cells from those obtained with type-1 and -2 VLDLR-transfected cells. Corrections were made for variations in the level of receptor protein expression by normalizing the amount of specifically bound ^{125}I IgG anti-receptor monoclonal antibody (IgG 6B6-2b). Each data point represents the mean ± SD of triplicate incubations. The data shown are representative of three independent experiments.

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pH of lysosomes (*31*). At concentrations below 200 μM chloroquine, inhibits the degradation of LDL, while the internalization and recycling of LDLR are not affected (*31, 32*). To determine whether or not the *O*-linked sugar region of VLDLR has any influence on this chloroquine sensitivity, we compared the degradation of internalized ^{125}I-labeled β-VLDL in type-1 and -2 VLDLR-transfected cells treated with chloroquine (Fig. 4). In both types, the addition of chloroquine led to inhibition of the degradation of ^{125}I-labeled β-VLDL; the responses were indistinguishable.

We next analyzed the influence of monensin, a carboxycyclic ionophore that prevents the acidification of endosomes. This drug prevents the internalized LDLR from returning to the cell surface and thereby causes a loss of LDLR on the cell surface (*32*). As shown in Fig. 5A, the surface binding of ^{125}I-labeled β-VLDL of both type-1 and -2 VLDLR-transfected cells decreased with increasing amounts of monensin. In both types, the degradation of ^{125}I-labeled β-VLDL was inhibited in proportion to the loss of surface receptors (Fig. 5B).

Biosynthesis and Processing of VLDLR-Transfected Cells—Both type-1 and -2 VLDLR-transfected cells were radiolabeled with ^{35}S-labeled ProMix for 1 h, and then chased for the indicated times in the presence of unlabeled methionine and cysteine (Fig. 6). The cells were solubilized and subjected to immunoprecipitation with monoclonal IgG-6B6-2b.

In cells transfected with type-1 VLDLR, at chase time zero most of the receptors appeared to be precursors with an apparent mass of 135-kDa (Fig. 6A, lane 1), and by 2 h chase about 80% of the precursors were converted into mature receptors with an apparent mass of 155 kDa (Fig. 6A, lane 2). These mature 155-kDa receptors were stable for at least 20 h (Fig. 6A, lanes 3–5, and Fig. 6E).

In contrast, type-2 VLDLR-transfected cells produced receptor precursors with an apparent mass of 125 kDa, most of which had disappeared by 2 h chase (Fig. 6B, lanes 1 and 2, and Fig. 6F). After this, about 60% of the precursors appeared to be mature receptors with an apparent mass of 130 kDa, subsequently decreasing to approximately 20% of the amount at chase time zero after 20 h chase (Fig. 6B, lanes 2–5, and Fig. 6F). This decrease in the amount of 130-kDa mature receptors was accompanied by the accumulation of immunoprecipitable proteins with an apparent mass of 115 kDa (Fig. 6, D and F): approximately 35% of the initial radioactivity was recovered in the medium as the 115 kDa form. The 115-kDa protein was not immunoprecipitated by IgG C4 (a monoclonal antibody against the C-terminal cytoplasmic domain of VLDLR), although the antibody recognized both the precursor and mature forms of the receptor in type-2 VLDLR-transfected cells (data not shown). We also detected immunoprecipitable proteins in the culture medium of type-1 VLDLR-transfected cells, but their level was less than 5% of the amount at chase time zero after 20 h chase (Fig. 6, C and E).

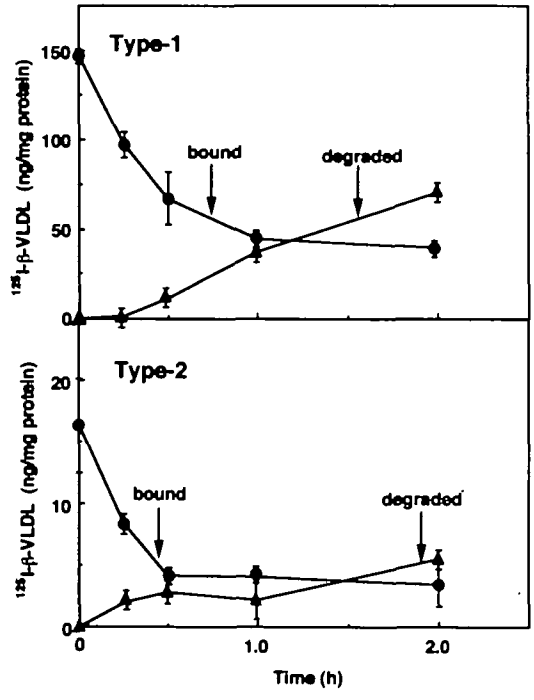

Fig. 3. **Degradation at 37°C of ^{125}I-labeled β-VLDL previously bound to VLDLR at 4°C in type-1 and -2 VLDLR-transfected cells.** The time course for labeled β-VLDL that was surface-bound (●) or degraded (▲). On day 4 of cell growth, triplicate monolayers of type-1 and -2 VLDLR-transfected cells received 2 ml of ice-cold medium supplemented with 10 μg/ml of ^{125}I-labeled β-VLDL (106 cpm/ng protein). After 2 h at 4°C, each monolayer was rapidly washed six times with ice-cold buffer A. Each dish then received 2 ml of warm medium, and all dishes were incubated at 37°C. After the indicated times, groups of dishes were rapidly chilled to 4°C, and then the medium was removed and its content of ^{125}I-labeled trichloroacetic acid soluble (▲) was determined. Each monolayer was washed rapidly six times with ice-cold buffer A and then surface bound ^{125}I-labeled β-VLDL was determined (●). The specific values were determined by subtracting the values obtained with pSV2-Neo-transfected cells from those obtained with type-1 and -2 VLDLR-transfected cells. The experimental data were corrected for variations in the level of receptor protein expression, as described in the legend to Fig. 2. Each data point represents the mean ± SD of triplicate incubations. The data shown are representative of two independent experiments.

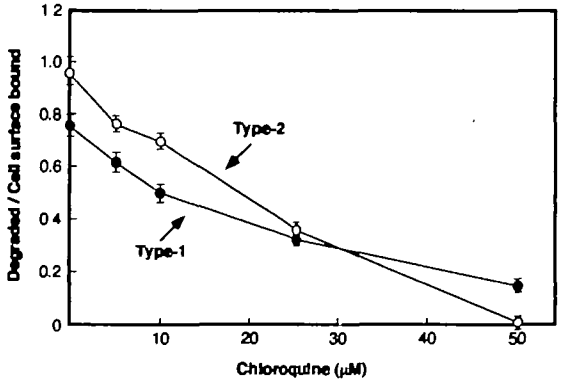

Fig. 4. **Effects of various concentrations of chloroquine on the degradation of ^{125}I-labeled β-VLDL in type-1 (●) and -2 (○) VLDLR-transfected cells.** After incubation for 24 h in lipoprotein-deficient serum (day 4 of cell growth), each monolayer received 2 ml of medium containing lipoprotein-deficient serum, the indicated concentrations of chloroquine and 10 μg of protein/ml of ^{125}I-β-VLDL (176 cpm/ng protein). After incubation for 5 h at 37°C, the total amounts of surface-bound and degraded ^{125}I-β-VLDL were determined. The corrected ^{125}I-β-VLDL degradation values were calculated by dividing the amount (ng/mg protein) of specifically degraded ^{125}I-β-VLDL by that of specifically bound ^{125}I-β-VLDL. Each data point represents the mean ± SD of triplicate determinations. The data shown are representative of two independent experiments.

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Analysis of VLDLR Protein Glycosylation—To analyze the *O*-linked oligosaccharide chains of the two types of VLDLR protein, type-1 and -2 VLDLR-transfected cells were cultured in the presence or absence of tunicamycin (a potent inhibitor of *N*-linked glycosylation), immunoprecipitated and then subjected to treatment with neuraminidase and *O*-glycanase, or both. In the absence of tunicamycin, most type-1 VLDLR was in the 155-kDa mature form (Fig. 7A). When the cells were cultured in the presence of tunicamycin, there was some delay in matura-

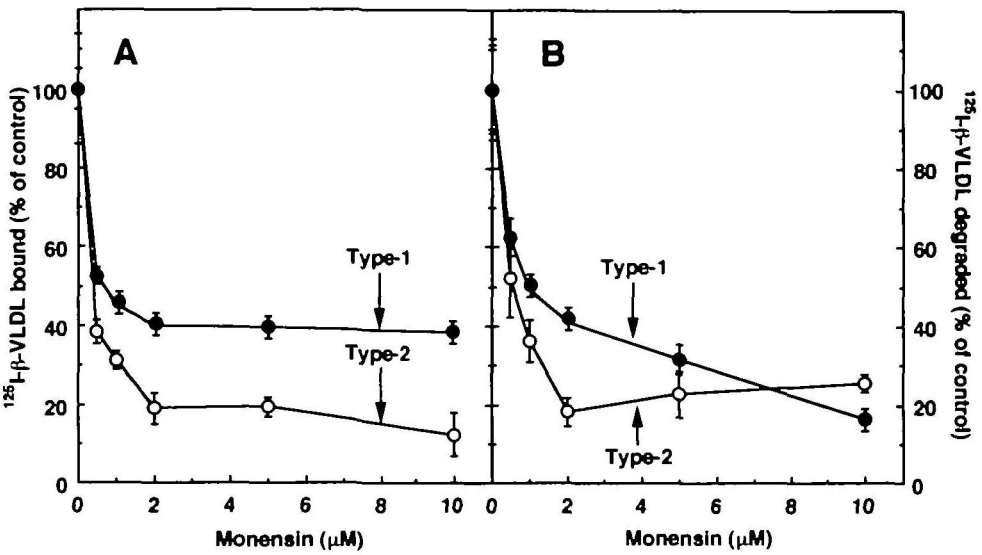

Fig. 5. **Effects of various concentrations of monensin on the surface binding (panel A) and degradation (panel B) of ^{125}I-labeled β-VLDL in type-1 (●) and -2 (○) VLDLR-transfected cells.** After incubation for 24 h in lipoprotein-deficient serum (day 4 of cell growth), each monolayer received 2 ml of medium containing lipoprotein-deficient serum, the indicated concentrations of monensin and 10 μg of protein/ml of ^{125}I-β-VLDL (146 cpm/ng protein). After incubation for 5 h at 37°C, the total amounts of surface-bound and degraded ^{125}I-β-VLDL were determined. The "100% of control" values were as follows for type-1 and -2 VLDLR-transfected cells, respectively: surface binding of ^{125}I-β-VLDL, 2,770 and 590 ng/mg; degradation of ^{125}I-β-VLDL, 2,100 and 560 ng/mg. Each data point represents the mean ± SD of triplicate determinations. The data shown are representative of two independent experiments.

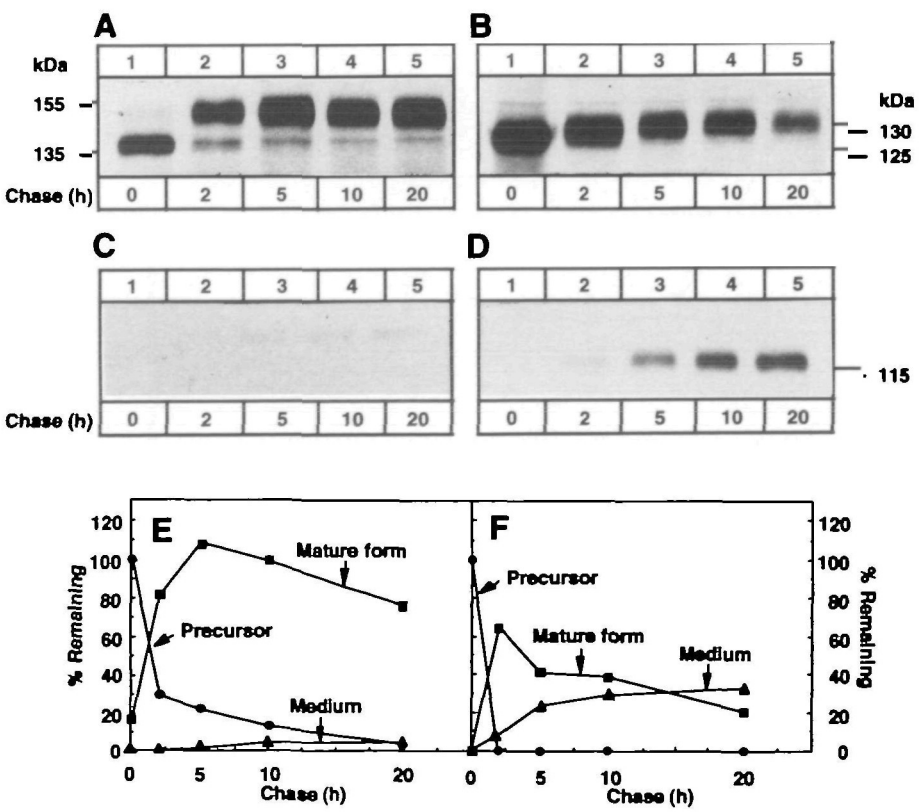

Fig. 6. **Biosynthesis and degradation of ^{35}S-labeled human VLDLR proteins in type-1 and -2 VLDLR-transfected cells.** On day 0, 5×10^4 cells were seeded into each Petri dish (60 × 15 mm) as described under "EXPERIMENTAL PROCEDURES." On day 5 of cell growth, the cells were pulse-labeled with 200 μCi/ml [^{35}S]-ProMix for 1 h in methionine- and cysteine-free Dulbecco's Modified Eagle, Medium supplemented with 10% lipoprotein-deficient serum, and then chased for the indicated times in medium containing 0.2 mM unlabeled methionine and cysteine. Detergent-solubilized cell extracts (A, B) and culture medium (C, D) of the VLDLR-transfected cells were immunoprecipitated with IgG 6B6-2b, and then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography (exposed to XAR-5 film for 27 h). The migration positions of the precursor (135 kDa) and mature form (155 kDa) in type-1 VLDLR transfected cells and of the precursor (125 kDa) and mature forms (130 kDa) in type-2 VLDLR-transfected cells are indicated. The autoradiograms in panels A–D show long exposures for clarity, while the scans in panels E and F were quantified with a BAS2000 image analyzer with different exposure times. The % remaining radioactivity of the precursor (●), mature (■), and soluble (▲) receptor bands is plotted as a function of chase time (E, F). The 100% value represents the intensity of the precursor receptor band at chase time zero. See the text for discussion regarding the 115 kDa protein (D) detected in the culture medium of type-2 VLDLR-transfected cells. Each value in panels E and F represents the average of duplicate experiments. The experiment was repeated three times with similar results.

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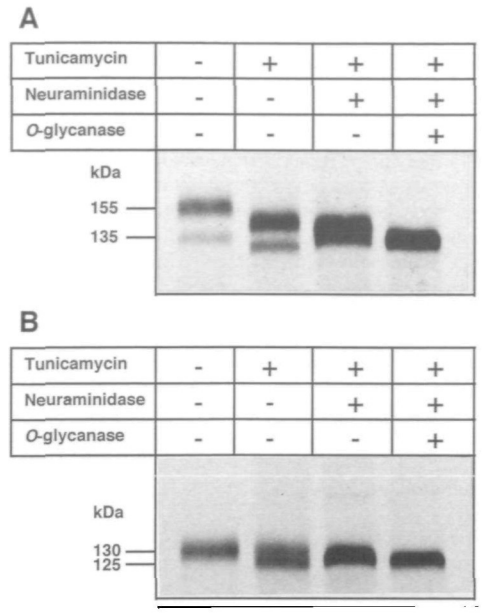

Fig. 7. **Analysis of VLDLR protein glycosylation as to sensitivity to neuraminidase and *O*-glycanase.** On day 5 of cell growth, each cell monolayer was incubated for 3 h in the absence or presence of 2.5 μg/ml tunicamycin, as indicated. The cells were then pulse-labeled for 120 min in the absence or presence of 2.5 μg/ml tunicamycin with 200 μCi/ml of [^{35}S]ProMix, as described under "EXPERIMENTAL PROCEDURES," after which they were washed once with phosphate-buffered saline and then incubated for a further 60 min in Dulbecco's Modified Eagle's Medium containing unlabeled methionine and cysteine supplemented 10% lipoprotein-deficient serum. Detergent-solubilized extracts were immunoprecipitated with IgG 6B6-2b as described under "EXPERIMENTAL PROCEDURES." The immunoprecipitates were divided into three aliquots, each of which was resuspended in 50 μl of buffer comprising 20 mM sodium citrate and 20 mM Tris-malate (pH 6.0) in the absence or presence of 1 unit/ml neuraminidase, as indicated. All reaction mixtures were incubated at room temperature for 18 h. Where indicated, 4 milliunits of *O*-glycanase was then added in a volume of 12 μl, and then incubations were continued for a further 24 h. SDS-polyacrylamide gel electrophoresis was carried out as described under "EXPERIMENTAL PROCEDURES," the gels being exposed to XAR-5 film for 7 days. The migration positions of the precursor (135 kDa) and mature forms (155 kDa) in type-1 VLDLR-transfected cells (A) and of the precursor (125 kDa) and mature form (130 kDa) in type-2 VLDLR-transfected cells (B) are indicated. The figures shown are representative of three independent experiments.

tion so that the precursor with an apparent molecular mass of 130 kDa and the mature form with an apparent molecular mass of 150 kDa were observed; the precursor and mature forms were both about 5 kDa smaller in apparent molecular mass as compared with those in untreated cells. Both neuraminidase and *O*-glycanase treatments of the receptor proteins in tunicamycin-treated cells reduced the molecular mass of the mature form by 20 to 130 kDa, but the molecular mass of the precursor remained the same (130 kDa).

In type-2 VLDLR-transfected cells, tunicamycin treatment also reduced the processing of the precursor to the mature form, but the molecular masses of both the precursor and mature forms were not altered significantly (Fig. 7B). Neuraminidase treatment and subsequent addition of *O*-glycanase reduced the molecular mass of the mature form to 120 kDa, but the molecular mass of the precursor was unaffected by either treatment. These data indicate that the mature forms of type-1 and -2 VLDLR contain two forms of carbohydrate, *N*-linked and *O*-linked sugars, and suggest that the serine- and threonine-rich region of type-1 VLDLR is the major site for the addition of *O*-linked carbohydrate.

DISCUSSION

In the current study, we have examined the expression and functional differences of VLDLR variants, with or without the *O*-linked sugar region, generated through alternative splicing. Unexpected findings in this study were that, when expressed in hamster fibroblasts, VLDLR lacking the *O*-linked sugar region (i) binds with relative low affinity to β-VLDL and (ii) is degraded rapidly, resulting in the accumulation of the receptor in the culture medium.

In chicken, a very high level of VLDLR lacking the *O*-linked sugar region is expressed in growing oocytes, whereas chicken somatic cells and tissues (in particular granulosa cells, heart, and skeletal muscle) predominantly express VLDLR with the *O*-linked sugar region, albeit at much lower levels than oocytes (*20*). RT-PCR of VLDLR mRNAs from various rabbit tissues revealed that the transcript for VLDLR with the *O*-linked sugar region (type-1 VLDLR) is the major form in heart and muscle, while the transcript for VLDLR lacking the *O*-linked sugar region (type-2 VLDLR) predominates in non-muscle tissues (Fig. 1). This distinct tissue specificity suggests that the two types of VLDLR in mammals may play different roles in different tissues. The expression of type-2 VLDLR in the mammalian ovary is of particular interest, since chicken oocytic VLDLR plays a key role in the uptake of yolk precursors into growing oocytes.

The ligand binding study revealed that cells expressing type-2 VLDLR bound with relatively lower affinity to β-VLDL than ones expressing type-1 VLDLR (Fig. 2), although the internalization, dissociation and degradation of the ligand were not significantly impaired (Figs. 2-5). This reduced affinity of type-2 VLDLR for β-VLDL suggests that the *O*-linked sugar region of VLDLR may contribute to the ligand binding function of the receptor. The *O*-linked oligosaccharides of glycoproteins are usually clustered within heavily glycosylated regions of the peptide chain, and the *O*-glycosylation of cell surface proteins is believed to induce their peptide cores to adopt a stiff and extended conformation (*33*). It is therefore suggested that the *O*-glycosylation of the *O*-linked sugar region of VLDLR may extend the ligand binding domain well above the cell surface, allowing it to interact more freely with its ligands. Alternatively, type-2 VLDLR may bind ligands other than apoE-containing lipoproteins, since chicken oocytic VLDLR lacking the *O*-linked sugar region binds two structurally different ligands *in vivo*: VLDL and vitellogenin (*17, 34*).

Although deletion of the *O*-linked sugar region of LDLR has no effect on the LDLR function in transfected fibroblasts *in vitro* (*21*), it is associated with the clinical symptoms of familial hypercholesterolemia (*35-37*). Patients lacking the *O*-linked sugar region of LDLR exhibit mild hypercholesterolemia, presumably due to the poor expression of LDLR on the hepatic cell surface (*37*). Similarly, in hamster fibroblasts, deletion of the *O*-linked sugar region resulted in the poor surface expression of VLDLR proteins

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(Fig. 6). This unstable expression of type-2 VLDLR is accompanied by the accumulation of the receptor in the culture medium, suggesting that the soluble receptor is generated through proteolytic cleavage from the cell surface. Although we could not determine the cleavage site, the following observations suggest that the type-2 VLDLR protein is cleaved after reaching the cell surface. Firstly, receptor proteins in the culture medium of type-2 VLDLR-transfected cells were detected by an antibody against the extracellular region (IgG 6B6-2b), but not by an antibody against the cytoplasmic region (IgG C4). Secondly, both IgGs 6B6-2b and C4 recognized the receptor proteins associated with the type-2 VLDLR-transfected cells, as revealed on immunoblotting (data not shown). Thirdly, ^{125}I-labeled IgG 6B6-2b labeled the receptor proteins on the surface of type-2 VLDLR-transfected cells.

Our present results suggest that the *O*-linked sugar region of VLDLR plays a role in the stability of the receptor by protecting a site that is sensitive to proteases in hamster fibroblasts. In this regard, it is important to note that CHO cells lacking UDP-galactose/UDP-*N*-acetylgalactosamine 4-epimerase produce very unstable LDLR (*38*). This mutant cell line cannot produce enough UDP-Gal and UDP-GalNac to allow normal synthesis of *N*-linked and *O*-linked sugars on LDLR as well as on other glycoproteins. Both the general glycosylation defect and the LDLR negative phenotype can be corrected by growth of the mutant cells with exogenous galactose and GalNac, which are converted to the respective sugar nucleotides through a salvage pathway. Under conditions in which GalNac and galactose cannot be incorporated into *O*-linked sugars, LDLR is synthesized but is broken down rapidly (*38*). Together with the data obtained in the present study, this suggests that the *O*-linked sugar chains play a crucial role in maintaining the stability of VLDLR expressed in hamster fibroblasts. The predominant expression of type-1 VLDLR mRNA in muscle cells may indicate that the *O*-linked sugar is required for the stable expression of VLDLR in muscle cells also.

In chicken oocytes, VLDLR lacking the *O*-sugar region is present throughout the cytoplasm of previtellogenic oocytes, and upon the onset of oocyte growth it is translocated to the plasma membranes (*39*). This translocation event may occur in mammals and require protein factor(s) that stabilize(s) the receptor protein. Further characterization of the translocation and stabilization of type-2 VLDLR in mammalian cells will provide clues for understanding the functional role of *O*-glycosylation.

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