Cytoplasmic Sequence Required for Basolateral Targeting of LDL Receptor in Livers of Transgenic Mice

Masayuki Yokode,* Ravindra K. Pathak,‡ Robert E. Hammer,§ Michael S. Brown,* Joseph L. Goldstein,* and Richard G. W. Anderson‡

Departments of *Molecular Genetics, ‡Cell Biology and Neuroscience, §Biochemistry, and §Howard Hughes Medical Institute, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

Abstract. When expressed in livers of transgenic mice, the human low density lipoprotein (LDL) receptor is specifically targeted to the basolateral (sinusoidal) surface of hepatocytes as determined by immunofluorescence and immunoelectron microscopy. The COOH-terminal cytoplasmic domain of the receptor (residues 790-839) contains a signal for this targeting. A mutant receptor truncated at residue 812 was localized exclusively to the apical (bile canalicular) surface. A mutant receptor terminating at residue 829 showed the normal basolateral distribution, as did a receptor in which alanine was substituted for serine 833, which was previously shown to be a site for phosphorylation in vitro. These data localize the basolateral targeting signal to the 17-residue segment between residues 812 and 828. A 10-amino acid stretch within this segment shows a 4/10 match with a sequence within a previously identified basolateral sorting motif for the receptor for polymeric IgA/IgM in MDCK cells. The four shared residues are spaced at intervals of three, raising the possibility that they all face the same side of an α-helix. We conclude that this 10-amino acid stretch may contain a signal that directs certain proteins, including the LDL receptor and the polymeric IgG/IgM receptor, to the basolateral surface of polarized epithelia.

THE low density lipoprotein (LDL)[1] receptor takes up cholesterol-carrying lipoproteins by receptor-mediated endocytosis in coated pits (Goldstein et al., 1985). In cultured fibroblasts, where the process has been studied most extensively, 60–80% of LDL receptors are located in coated pits at all times. LDL receptors migrate to these pits, internalize, and recycle spontaneously without a requirement for LDL binding. In the body the majority of LDL receptors are found on hepatocytes where they mediate the removal of intermediate density lipoproteins and LDL from blood (Brown and Goldstein, 1986; Havel and Hamilton, 1988; Spady et al., 1986). To perform this function, the receptors must be located on the basolateral (sinusoidal) surfaces of hepatocytes, and it is presumed that they must also migrate to coated pits.

More than 100 different mutations in the gene for the human LDL receptor impair lipoprotein clearance in the liver and produce the syndrome of familial hypercholesterolemia with its attendant atherosclerosis (Hobbs et al., 1990). Three of these mutations alter the cytoplasmic domain of the receptor, which contains the signal that mediates clustering in coated pits. In fibroblasts these receptors bind LDL but they cannot cluster in coated pits, and therefore they cannot carry LDL into the cell. In the body these mutations raise plasma LDL to the same extent as do gross deletions in the LDL receptor gene, indicating that coated pit clustering is crucial for the function of the LDL receptor in hepatocytes as well as in fibroblasts (Goldstein and Brown, 1989).

Despite this circumstantial evidence for coated pit involvement in LDL receptor function in liver, this conclusion has been difficult to validate experimentally. Under normal conditions the number of receptors in liver is too small to be localized directly with immunocytochemical techniques. When the LDL receptor is overexpressed, either as a result of induction with 17 α-ethinyl estradiol (Havel and Hamilton, 1988; Handley et al., 1983) or by transcription from a strong promoter in transgenic mice (Pathak et al., 1990), most of the receptors are found in a diffuse distribution on microvilli at the basolateral surface, and only a few are found in coated pits. Nevertheless, these receptors mediate the rapid uptake of LDL into hepatocytes. These findings have led to the conclusion that the entry of LDL receptors into coated pits in liver may be slower than occurs in fibroblasts (Handley et al., 1983; Pathak et al., 1990). In fact, this localization might occur only in response to the binding of LDL, in a fashion that resembles the ligand-triggered endocytosis of the receptor for epidermal growth factor (Schlessinger, 1988). If such regulation exists, it is likely to be mediated by sequences in the cytoplasmic tail of the

1. *Abbreviations used in this paper*: LDL, low density lipoprotein; pIgR, polymeric IgA/IgM receptor.

receptor, which is responsible for clustering in coated pits (Chen et al., 1990).

The cytoplasmic tail of the LDL receptor comprises the 50 COOH-terminal amino acids (residues 790–839). Residues 802–807 comprise the signal for entry into coated pits, FDNPVY (Chen et al., 1990). Studies with nuclear magnetic resonance spectroscopy have shown that a synthetic peptide containing this sequence forms a tight reverse turn in solution (Bansal and Gierasch, 1991). A tight turn containing a tyrosine is a frequent, if not universal, signal for entry of receptors into coated pits (Collawn et al., 1990, 1991). Truncation of the LDL receptor at residue 812, which leaves the internalization signal intact, does not affect the rapid coated pit-mediated internalization of LDL receptors, nor its recycling in fibroblasts (Davis et al., 1987; Chen et al., 1990). Although the COOH-terminal 28 amino acids of the LDL receptor are dispensable for function in fibroblasts, these residues are nearly 100% conserved in species as remote as *Xenopus laevis* and humans (Mehta et al., 1991). Considered together, these findings raise the possibility that the COOH-terminal 28 amino acids might contain a signal that regulates the distribution of the LDL receptor in polarized epithelial cells such as hepatocytes.

The localization of the LDL receptor to specific surfaces of polarized epithelia has been examined in transgenic mice overexpressing the normal human LDL receptor (Pathak et al., 1990) and in cultured MDCK cells (Li et al., 1991; Hunziker et al., 1991). In the transgenic mice the LDL receptor was present in a diffuse distribution on microvilli at the basolateral surface of hepatocytes and intestinal epithelial cells, but surprisingly in renal epithelial cells it was present on the apical surface where it appeared to cluster in coated pits and endocytic vesicles (Pathak et al., 1990). In sharp contrast, in MDCK cells the wild-type LDL receptor was located primarily on the basolateral surface (Li et al., 1991; Hunziker et al., 1991). Elimination of the tyrosine internalization signal prevented rapid internalization of the receptor in MDCK cells, but it did not alter its location on the basolateral surface. However, when the receptor was truncated so as to eliminate the cytoplasmic tail, most of the receptors were found at the apical surface (Hunziker et al., 1991). These results were interpreted to indicate that the cytoplasmic tail contains a signal, separate from the tyrosine internalization signal, that directs it to the basolateral surface of MDCK cells.

As yet, there are no reliable experimental systems to study polarized expression of proteins in cultured hepatocytes. Accordingly, in the current studies we have compared the cellular distribution of normal and mutant human LDL receptors in livers of transgenic mice. The results suggest that the cytoplasmic tail of the receptor contains a signal for basolateral sorting in hepatocytes and that this signal is located between residues 812 and 828.

Materials and Methods

Materials

All reagents and chemicals used for immunofluorescence and immunogold labeling were obtained from previously described sources (Pathak et al., 1990; Pathak and Anderson, 1991). We obtained rabbit anti-mouse IgG and goat anti-rabbit IgG labeled with FITC from Zymed Laboratories (San Francisco, CA); sheep anti-rabbit IgG from Organon Teknika-Cappel (Malvern, PA); monoclonal mouse anti-dinitrophenol IgG from Oxford Biomedical (Oxford, MI); and goat anti-rabbit IgG conjugated to gold (10 nm) from Energy Sciences (Woburn, MA). A polyclonal rabbit IgG directed against the LDL receptor was prepared by immunizing a New Zealand white rabbit with purified bovine LDL receptor as previously described (Russell et al., 1984). A preimmune rabbit IgG was also prepared from the same rabbit.

Construction of Metallothionein Promoter-mutant LDL Receptor Minigenes

An expression plasmid containing the mouse metallothionein-I promoter fused to a human LDL receptor minigene (designated transgene 2) was constructed as previously described (Pathak et al., 1990). This wild-type transgene was modified by oligonucleotide-directed mutagenesis (Zoller and Smith, 1984) to create three mutant versions of the LDL receptor: one contained a termination codon at amino acid residue 812 (designated transgene 4); the second contained a termination codon at residue 829 (designated transgene 5); and the third contained a single amino acid substitution (Ser → Ala) at residue 833 (designated transgene 6).

To engineer LDL receptor transgene 4 (hereafter designated Stop 812 receptor), a single-stranded M13 DNA template containing a 2.4-kb EcoRI-SmaI fragment from pLDLR-2 (Yamamoto et al., 1984) was hybridized with a mutagenic 27-mer oligonucleotide. After hybridization, primer extension, and ligation, the DNA was transformed into *Escherichia coli* TG1 cells. Clones carrying the desired mutation were identified by hybridization and sequencing as described by Davis et al. (1987). A 1.1 kb BglII-SmaI fragment encoding the membrane-spanning and cytoplasmic domains of the mutated LDL receptor was excised from the replicative form of M13 DNA and then subcloned into pSLH16, a plasmid that harbors LDL receptor transgene 2 (Pathak et al., 1990). To create LDL receptor transgene 5 (Stop 829 receptor) and transgene 6 (Ala 833 receptor), a 1.1-kb BglII-SmaI fragment that encodes the same domains of the LDL receptor as described for LDL receptor transgene 4 was excised from pLDLR4 Stop 829 and pLDLR4 Ala 833, two previously described plasmids encoding mutant receptors (Davis et al., 1987), and subcloned into pSLH16.

The mutated region of each reconstructed plasmid was sequenced by the method of Sanger et al. (1977), and the plasmids were checked for expression by transient transfection into hamster *ldlA7* cells using a modified calcium phosphate precipitation method (Davis et al., 1986). The length of LDL receptor transgenes 4, 5, and 6 was ~14.7 kb. NotI sites at the 5′ and 3′ ends of the transgenes were used to excise the DNA from the plasmid vector before microinjection into fertilized mouse eggs.

Transgenic Mice

A total of 909 eggs were microinjected with the Stop 812 transgene and transferred into pseudopregnant females (Brinster et al., 1985). Among 99 offspring, 24 (24%) contained the transgene as determined by dot hybridization of DNA obtained from tail homogenates (Hofmann et al., 1988). These founder mice were treated with 25 mM ZnSO4 in the drinking water for 7-d to induce the metallothionein promoter (Palmiter et al. 1983), and three mice were shown to overexpress the transgene-derived mutant LDL receptor in the liver as determined after partial hepatectomy and immunoblot analysis (see below). These mice were bred to C57Bl/6J × SJL Fl mice to establish three lines of Stop 812 mice (321-4, 340-1, and 343-2).

A total of 555 eggs were microinjected with the Stop 829 transgene and transferred to recipients. A total of 45 offspring was obtained, seven of which (16%) contained the transgene. After treatment with 25 mM $ZnSO_4$ in drinking water for 7 d, three mice were shown to express the transgene-derived mutant LDL receptor in the liver as determined by immunoblot analysis. Two of these mice were bred to C57Bl/6J × SJL Fl mice to establish one line of Stop 829 mice (403-1). The second male mouse (399-5), which expressed at the highest level, was infertile, and therefore we could not establish a line.

A total of 579 eggs were microinjected with the Ala 833 transgene and transferred to recipients. Of 56 offspring obtained, 26 (46%) contained the transgene. After treatment with 25 mM $ZnSO_4$ in the drinking water for 7 d, 11 mice were shown to express the transgene-derived mutant LDL receptor in the liver as determined by immunoblot analysis. These mice were bred to C57Bl/6J × SJL Fl mice, and one line of the Ala 833 mice (369-2) was established.

Two lines of transgenic mice (192-2 and 188-1) that express transgene 2 encoding the metallothionein-driven wild-type human LDL receptor minigene were established as previously reported (Pathak et al., 1990).

Where indicated in the figure legends, mice were treated either with $ZnSO_4$ (25 mM) in the drinking water for 7 to 10 d or with $CdSO_4$ (1 mg/kg) intraperitoneally 18 and 6 h before the experiment.

Immunofluorescence Microscopy

Tissues obtained from perfusion-fixed mice (Pathak et al., 1990) were refixed for 2 d in a fixative that contained 10% (vol/vol) glacial acetic acid, 30% (vol/vol) 1,1,1-trichloroethane, and 60% (vol/vol) absolute methanol, dehydrated in ethanol, embedded in paraffin, and sectioned (10-μm thickness) with a steel knife. Sections were mounted on glass slides, and the paraffin was removed by incubating slides in xylene, followed by rehydration through a descending series of ethanol. Sections were sequentially incubated at room temperature as follows: (*a*) buffer A (20 mM [vol/vol] Tris-HCL at pH 9.0, 200 mM NaCl, 0.2% [wt/vol] BSA, and 0.01% [vol/vol] NaN_3) for 30 min; (*b*) either 50 μg/ml anti-LDL receptor IgG or 0.1 mg/ml preimmune IgG in buffer A overnight; (*c*) 25 μg/ml FITC-labeled goat anti-rabbit IgG in buffer A for 2 h. Each incubation was followed by three successive 5 min washes in buffer A. Finally, sections were rinsed in distilled water for 2 min and mounted in DABCO (90% [vol/vol] glycerol, 50 mM Tris-HCL at pH 9.0, 25% [wt/vol] 1, 4-diazadicyclo-[2.2.2]-octane). The samples were viewed and photographed with a Zeiss photomicroscope III using either a 63 or 25 neofluar oil immersion objective and the appropriate filter package for fluorescein.

Immunoelectron Microscopy

Liver samples from perfusion-fixed mice were dissected and kept overnight in the perfusion fixative. Vibratome sections (60–80-μm thick) were prepared and washed in 100 mM sodium phosphate buffer at pH 7.8 containing 50 mM ammonium chloride for 30 min and then transferred to buffer B (buffer A containing 0.05% [wt/vol] saponin) for 1 h. The primary antibodies, rabbit anti-LDL receptor IgG and the preimmune IgG, were diluted in buffer B to a final concentration of 50 μg/ml. Groups of six to eight sections were incubated overnight with each of the antibodies. This was followed by a 3-h incubation with 20 μg/ml of sheep anti-rabbit IgG conjugated with dinitrophenol in buffer B (Pathak and Anderson, 1989). After each incubation sections were washed three times for 5 min each in buffer B. After a final wash, sections were rinsed twice in 100 mM sodium phosphate buffer (pH 7.8) and fixed with 1.33% (vol/vol) glutaraldehyde for 2 h in the same buffer. Tissue sections were postfixed with 1% (vol/vol) osmium tetroxide in phosphate buffer, dehydrated, embedded in Epon, sectioned, and processed to localize dinitrophenol groups by immunogold labeling using mouse anti-dinitrophenol IgG followed by rabbit anti-mouse IgG and goat anti-rabbit IgG conjugated to gold (10 nm) (Pathak and Anderson, 1991).

Immunoblot Analysis

Mouse liver membranes were prepared and subjected to immunoblot analysis as described by Pathak et al. (1990). The extracts (125 to 250 μg of protein per lane) were subjected to 7% SDS-PAGE under either reducing or nonreducing conditions (with or without 10% [vol/vol] 2-mercaptoethanol) and transferred to nitrocellulose filters. After transfer the filters were incubated with 5 μg/ml of polyclonal rabbit anti-LDL receptor IgG followed by ^{125}I-labeled goat anti-rabbit IgG (5×10^6 cpm/ml) and then subjected to autoradiography.

Lipoprotein Turnover Studies

Transgenic mice (22–27-g body weight) were treated with $CdSO_4$ (1 mg/kg) intraperitoneally 18 and 6 h before the experiment. After anesthesia with sodium pentobarbital (90 mg/kg), each mouse received an intravenous bolus via the external jugular vein of 0.1 ml of 150 mM NaCl containing BSA (2 mg/ml) and 15 μg of ^{125}I-labeled human LDL (600 cpm/ng protein) (Goldstein et al., 1983). Blood was collected by retro-orbital puncture and placed in EDTA-coated tubes at the times indicated, and a portion of the plasma (10 μl) was counted for its ^{125}I content. The amount of ^{125}I-labeled LDL remaining in the circulation was calculated as the percent of control. The "100% of control" value represents the mean value for ^{125}I-radioactivity present in the plasma of four nontransgenic normal mice at 1 min after injection with the same preparation of ^{125}I-LDL.

Results

Fig. 1 shows the amino acid sequence of the cytoplasmic tail of the human LDL receptor and indicates the position of the FDNPVY internalization signal as well as the three mutations that were studied in the current paper. Stop 812 and Stop 829 are premature termination codons. Ala 833 contains an alanine in place of a serine at a site near the COOH terminus that was previously shown to be phosphorylated in vitro by a casein kinase II-like enzyme isolated from adrenal cortex (Kishimoto et al., 1987). All of these mutations were created by in vitro mutagenesis techniques in a plasmid that contains a minigene comprising the entire coding region of the human LDL receptor as well as several introns near the

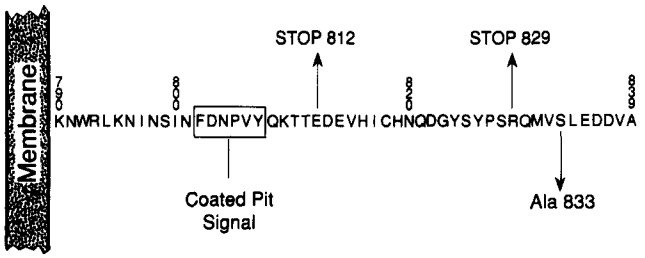


Figure 1. Amino acid sequence of the cytoplasmic domain of the human LDL receptor. The signal for coated pit localization is boxed (residues 802-807). The four LDL receptor transgenes used in the current study are designated in the text as follows: wild-type receptor (transgene 2), Stop 812 receptor (transgene 4), Stop 829 receptor (transgene 5), and Ala 833 receptor (transgene 6).

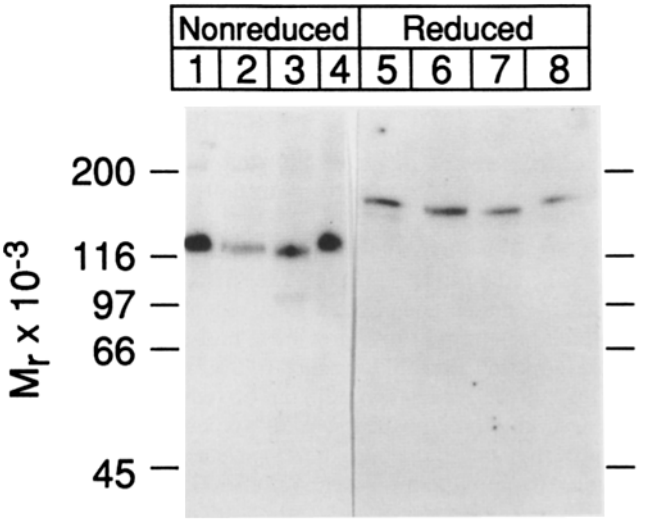


Figure 2. Immunoblot analysis of human LDL receptors in livers of transgenic mice. Transgenic mice were treated with $CdSO_4$ as described in Materials and Methods, after which aliquots of the 100,000 *g* membrane fraction from liver were subjected to 7% SDS-PAGE under either nonreducing (lanes *1* to *4*) or reducing (lanes *5* to *8*) conditions and transferred to nitrocellulose filters. The filters were immunoblotted with a rabbit anti-LDL receptor IgG, probed with ^{125}I-labeled goat anti-rabbit IgG, and exposed to XAR-5 film with an intensifying screen at −70°C for either 6 h (lanes *1* to *4*) or 24 h (lanes *5* to *8*). (Lanes *1, 4, 5,* and *8*) Membranes from a mouse expressing the wild-type receptor (125 μg protein per lane); (lanes *2* and *6*) membranes from a mouse expressing the Stop 829 truncation (205 μg protein per lane); (lanes *3* and *7*) membranes from a mouse expressing the Stop 812 truncation (250 μg protein per lane).

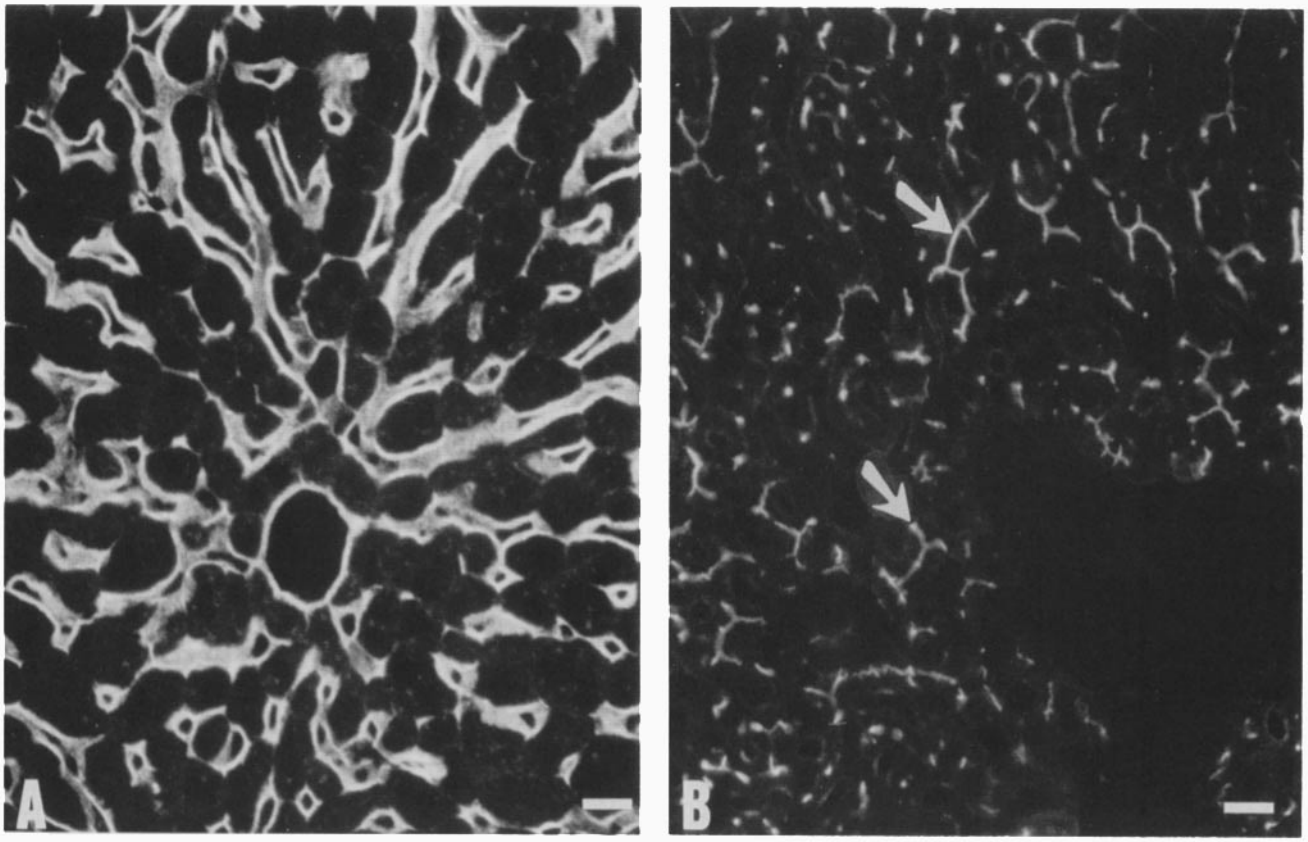


Figure 3. Immunofluorescence localization of human LDL receptors in liver from transgenic mice expressing either the wild-type receptor (*A*) or Stop 812 receptor (*B*). Mice were treated with $ZnSO_4$, after which liver samples were processed for immunofluorescence as described in Materials and Methods. (*B*) Arrows denote canaliculi that are stained with the anti-LDL receptor IgG. Bar, 10 μm.

5′ end (see Fig. 1 in Pathak et al., 1990). The promoter was derived from the mouse metallothionein-I gene, and all mice were treated with either zinc or cadmium to induce maximal expression of the receptor.

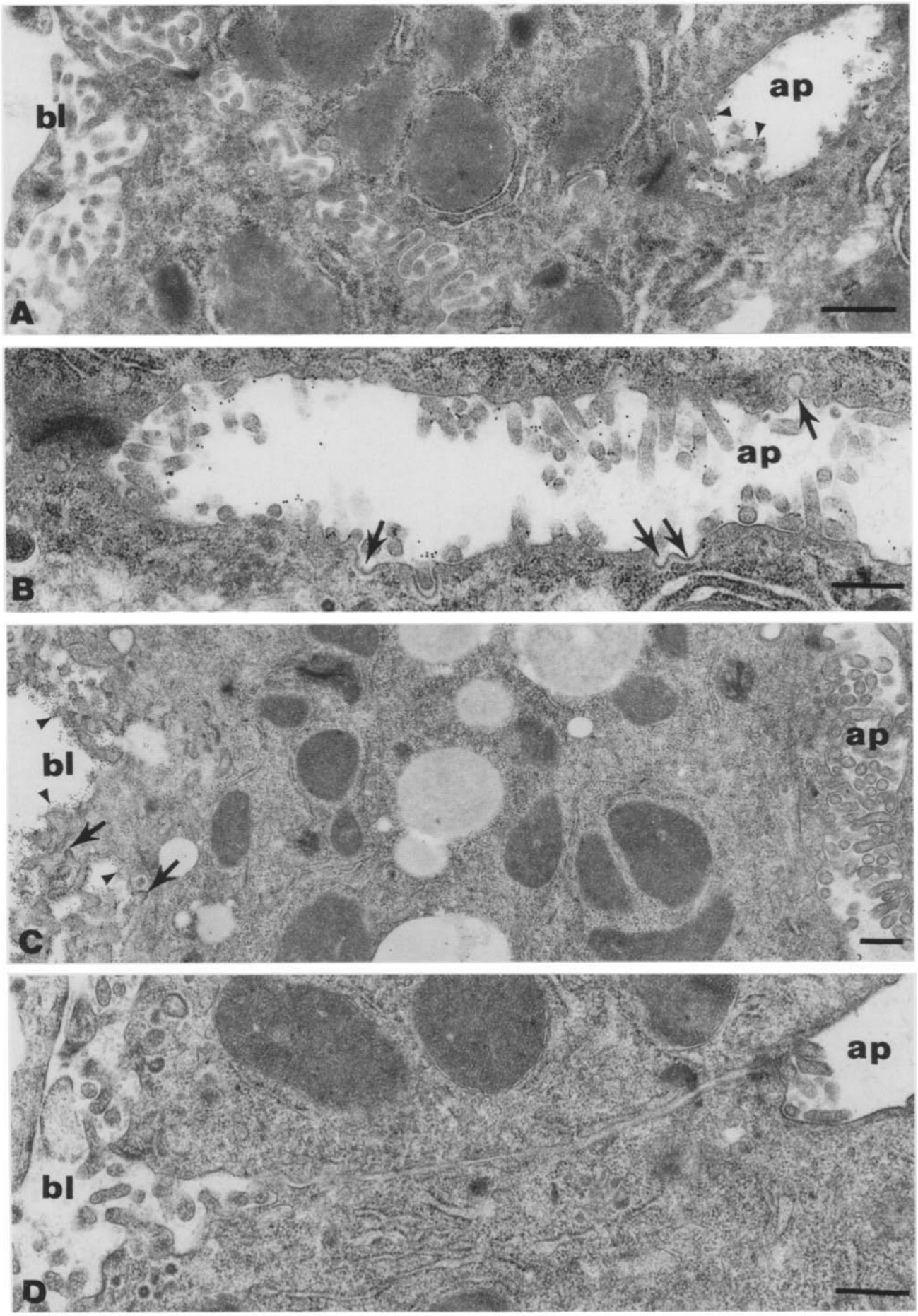
Fig. 2 shows immunoblots of the wild-type, Stop 829, and Stop 812 human LDL receptors after SDS gel electrophoresis of crude membrane pellets from livers of transgenic mice. All three receptors were expressed at high levels after heavy metal induction, and all three showed the expected sizes after electrophoresis under nonreducing or reducing conditions.

As reported previously, the wild-type human LDL receptor was confined to the basolateral surface of hepatocytes as revealed by immunofluorescence studies (Fig. 3 *A*). In striking contrast, the Stop 812 receptor was located exclusively at the bile canalicular (apical) surface (Fig. 3 *B*). Distributions similar to those in Fig. 3 were observed by immunofluorescence in livers from eight transgenic mice from two separate mouse lines expressing the wild-type receptor and from 16 transgenic mice from 2 separate lines expressing the Stop 812 receptor.

Immunoelectron microscopy using gold-labeling techniques confirmed that the Stop 812 receptor was located at the apical surface of hepatocytes (Fig. 4 *A*). When examined by higher power, the mutant receptors were observed to be in a diffuse distribution on microvilli of the apical surface with no apparent concentration in coated pits (Fig. 4 *B*). In contrast, the wild-type human LDL receptor was found exclusively at the basolateral surface of hepatocytes in the transgenic mice, and again there was no tendency for concentration in coated pits (Fig. 4 *C*). Fig. 4 *D* shows a control experiment from a nontransgenic mouse showing that the antibody does not detect the mouse receptor.

As would be expected from its apical distribution, the Stop 812 receptor was unable to mediate the rapid clearance of LDL from plasma. Fig. 5 shows an experiment in which we injected ^{125}I-labeled LDL intravenously into transgenic

Figure 4. Immunogold localization of LDL receptors in hepatocytes from a nontransgenic normal mouse (*D*) and from transgenic mice that express either the wild-type receptor (*C*) or the Stop 812 receptor (*A* and *B*). All animals were treated with $ZnSO_4$, after which liver samples were prepared for immunogold labeling as described in Materials and Methods. (*A*) Low magnification view of hepatocytes: *bl*, basolateral surface that lines the sinusoidal space; *ap*, apical surface that lines the canalicular space; arrowheads denote microvilli that are labeled with anti-LDL receptor IgG-gold. (*B*) High magnification view of the apical surface: *ap*, apical surface and canalicular space; arrows denote clathrin-coated pits that are devoid of immunogold labeling. (*C*) Low magnification view of hepatocytes: *bl*, basolateral surface; arrows denote clathrin-coated pits; arrowheads denote microvilli; *ap*, apical surface. (*D*) Low-magnification view of hepatocytes from a nontransgenic mouse; *bl*, basolateral surface; *ap*, apical surface. Bar, 0.5 μm.


bl
ap
A
ap
B
bl
ap
C
ap
bl
D

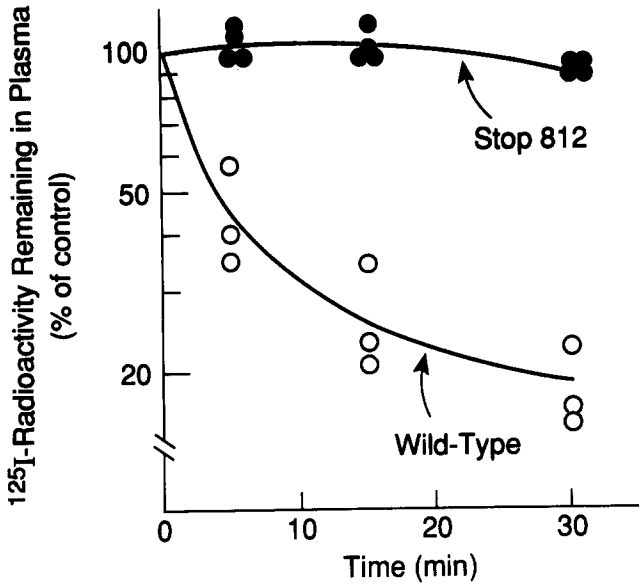


Figure 5. Removal of ^{125}I-LDL from plasma in transgenic mice. Nonfasted transgenic mice expressing the wild-type (○) or Stop 812 (●) versions of the human LDL receptor were treated with $CdSO_4$ and then injected intravenously with 15 μg of ^{125}I-LDL (600 cpm/ng protein) as described in Materials and Methods. At the indicated time, blood was collected, and the plasma content of ^{125}I was measured as described in Materials and Methods. Each value represents an individual animal.

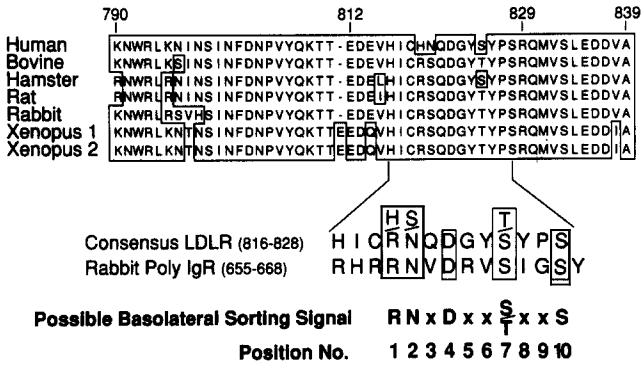


Figure 7. Alignment of cytoplasmic domain sequences implicated in basolateral sorting of the LDL receptor and the polymeric IgA/IgM receptor. LDL receptor (LDLR) sequences from Mehta et al. (1991). Rabbit polymeric IgA/IgM receptor (*poly IgR*) sequence from Mostov et al. (1984).

mice and measured the radioactivity remaining in blood after varying intervals up to 30 min. In agreement with previous observations (Hofmann et al., 1988), transgenic mice expressing the wild-type human LDL receptor cleared LDL rapidly from the circulation with a half-time of less than 5 min. In contrast, the animals expressing the Stop 812 receptor showed no tendency for accelerated clearance of LDL. The rate of clearance was similar to the rate observed in nontransgenic mice studied previously (Hofmann et al., 1988).

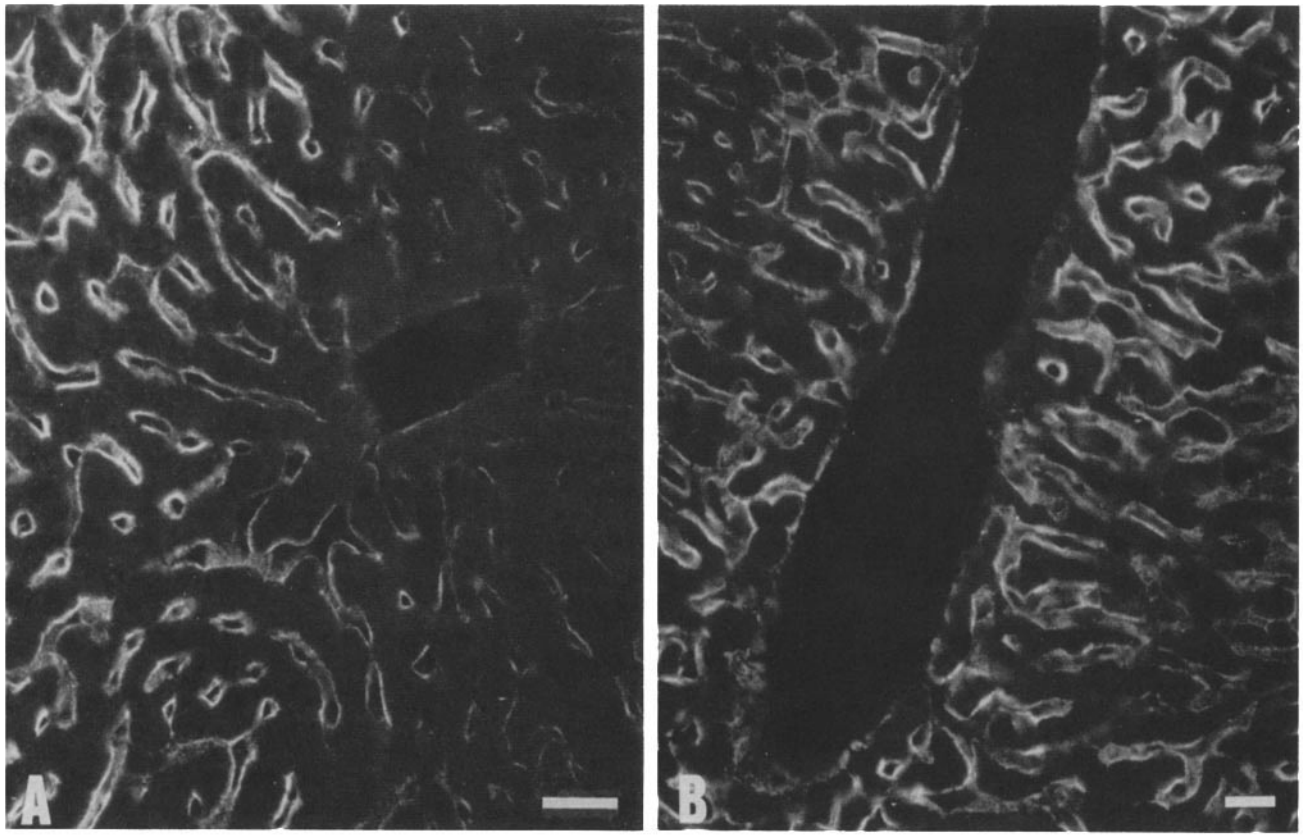


Figure 6. Immunofluorescence localization of human LDL receptors in livers of transgenic mice expressing either the Stop 829 receptor (*A*) or the Ala 833 receptor (*B*). Mice were treated with $ZnSO_4$, after which liver samples were processed for immunofluorescence as described in Materials and Methods. Results similar to these were obtained in a total of two mice that express the stop 829 receptor (one established line and one founder), and from 11 mice expressing the Ala 833 receptor (one established line and 10 founders). Bar, 10 μm.

The results with the Stop 812 mutation suggest that the LDL receptor contains a signal distal to residue 812 that is responsible for retention on the basolateral surface of hepatocytes. In an initial attempt to localize this signal, we studied two additional mutant receptors in transgenic mice. One of these receptors contains an alanine substituted for a serine at residue 833. This serine was previously shown to be a site of phosphorylation by an enzyme resembling casein kinase-II that was isolated from adrenal cortex and shown to phosphorylate the LDL receptor with extremely high affinity (Kishimoto et al., 1987). By immunofluorescence the Ala 833 receptor showed the same basolateral distribution as did the wild-type receptor (Fig. 6 *B*). The same normal distribution was also observed for a receptor that was terminated prematurely at position 829 (Fig. 6 *A*), which eliminates the last coding exon of the LDL receptor gene (Südhof et al., 1985). Although the amino acid sequence encoded by this exon is essentially 100% conserved in humans versus *Xenopus laevis* (Mehta et al., 1991), elimination of this exon did not affect the steady-state distribution of LDL receptors in livers of transgenic mice. This result suggests that the important signal for basolateral localization resides between residues 812 and 828.

Discussion

The current data suggest that residues 812-828 of the cytoplasmic tail of the LDL receptor contain a signal that causes the receptor to be localized to the basolateral surface of hepatocytes in vivo. This conclusion is suggested by the observation that truncation of the receptor at position 812 redirects the receptor to the apical surface, whereas truncation at position 829 allows the receptor to remain in its normal location at the basolateral surface.

The mechanisms that direct proteins to the apical or basolateral surfaces of hepatocytes are unknown, but they appear to differ from the mechanisms in cultured MDCK cells. Using pulse–chase labeling and cell fractionation techniques in livers of intact rats, Bartles et al. (1987) provided evidence that proteins destined for the apical surface travel first to the basolateral surface, and only later are transported apically, presumably as a result of transcytosis. In MDCK cells, which have been studied extensively in vitro, proteins appear to be directed primarily to the apical or basolateral surfaces, apparently as a result of sorting in the *trans*-Golgi network (Simons and Wandinger-Ness, 1990; Bomsel and Mostov, 1991).

Although basolateral targeting in MDCK cells was formerly considered to be a nonspecific "default" pathway, this view has been challenged recently. Casanova et al. (1991) identified a 14-residue segment of the cytoplasmic domain of the polymeric IgA/IgM receptor (pIgR) whose deletion specifically redirects the receptor to the apical surface. When this segment was transferred to alkaline phosphatase, a protein that is normally targeted apically, the protein became basolateral. Hunziker et al. (1991) showed that deletion of the entire cytoplasmic domain of the LDL receptor caused it to be restricted to the apical surface of MDCK cells, a finding that also suggests the presence of a specific basolateral targeting signal in the cytoplasmic domain of this receptor.

A 10-amino acid segment between residues 812 and 828 of the LDL receptor resembles 10 residues in the 14-residue sequence implicated in the basolateral sorting of the pIgR in MDCK cells. Fig. 7 shows the sequence of the cytoplasmic tail of the LDL receptor in six animal species, including the two *Xenopus* gene products. The sequence of the 812-828 region is compared with the basolateral sorting sequence in the pIgR. Within a 10-residue segment, there is a series of identical amino acids spaced at intervals of three. This shared sequence (RNxDxxS/TxxS) is shown in Fig. 7 with the positions numbered 1–10. It is interesting that the sequences of the various LDL receptors match the sequence of the pIgR at position 1 or 2, but not both. All of the LDL receptors except the human contain an Arg at position 1, which matches the sequence of the pIgR. These receptors all contain a Ser instead of Asn at position 2. The human LDL receptor contains a His instead of an Arg at position 1, but restores the match with the pIgR by containing an Asn at position 2. Thus, all sequences match at positions 1 or 2, 4, 7, and 10 (allowing the interchangeability of Ser and Thr), ie., all show a four out of 10 match in this region. The regular spacing of the matching residues at intervals of three suggests that they might all face the same side of an α-helix. Although the sequences include glycine and proline residues, which are often helix breakers, one cannot rule out a helix from primary sequence alone.

The similarity in these two sequences implies that polarized epithelial cells contain a traffic-directing protein that binds to the conserved residues and thereby directs the receptors to the basolateral surface of the cells. Surprisingly, the recognition sequence appears to be the same in hepatocytes and MDCK cells, even though previous data, reviewed above, suggests that the mechanism for sorting differs in these two cell types.

Casanova et al. (1990) showed that the serine at position 7 of the targeting sequence in the pIgR is phosphorylated and that this negative charge is necessary for the receptor to undergo transcytosis from the basolateral to the apical surface, a normal event in the transcellular transport of the polymeric IgA ligand. When this serine was changed to an alanine, the pIgR continued to be targeted to the basolateral surface of MDCK cells, but it no longer underwent transcytosis. Rather, it appeared to recycle back to the basolateral surface. On the other hand, when this serine was replaced with a negatively charged aspartic acid, it underwent transcytosis normally. These data would suggest that the conserved serine at position 7 is not essential for basolateral targeting of the pIgR, but rather it plays a role in transcytosis. If the corresponding serine in the LDL receptor were to be phosphorylated, it might allow the receptor to transport LDL across the cell to the biliary surface. Evidence for a low level of such transport has been obtained (Kleinherenbrink-Stins et al., 1990).

A cell surface endocytic receptor that shares structural and functional features with the LDL receptor is the LDL receptor-related protein (LRP)/α_2-macroglobulin receptor. The external domain of this receptor resembles a four-fold replicated LDL receptor (Herz et al., 1988; Brown et al., 1991). The cytoplasmic domain has 100 amino acids (twice the size of that of the LDL receptor) that includes two copies of a putative internalization sequence (FTNPVY and IGNPTY), but little other sequence identity with the LDL receptor. The T in the former sequence is the final T in the sequence DKPTNFT, which resembles residues 4–10 of the putative basolateral sorting sequence. The functional significance of this similarity, if any, remains to be determined.

Preliminary analysis of the sequence of several other pro-

teins known to be targeted to the basolateral surface of MDCK cells did not reveal any sequences that resemble the postulated targeting sequence shown in Fig. 7. The proteins examined included the G protein of vesicular stomatitis virus, the Fc receptor, and the transferrin receptor. These proteins might have basolateral sorting signals that are similar in three-dimensional structure but not in primary sequence. A precedent for this type of recognition has been established for the sorting mechanism that directs receptors to coated pits. An adaptor protein in coated vesicles (Glickman et al., 1989) recognizes tyrosine residues in the context of a tight turn, a shape that can be achieved by many different primary sequences (Bansal and Gierasch, 1991; Collawn et al., 1990, 1991).

An important unresolved question in the present study is whether the Stop 812 receptor is targeted directly to the apical surface of hepatocytes or whether it must first reach the basolateral surface and then migrate to the apical surface by transcytosis according to the pathway described by Bartles et al. (1987). One way to test this hypothesis is to produce a Stop 812 receptor that lacks the tyrosine signal for coated pit internalization. If the coated pit pathway is required for transcytosis, such a receptor would be expected to remain on the basolateral surface.

This work was supported by research funds from the National Institutes of Health (HL 20948), the Perot Family Foundation, and the Moss Heart Fund.

Received for publication 6 December 1991.

References

Bansal, A., and L. M. Gierasch. 1991. The NPXY internalization signal of the LDL receptor adopts a reverse turn conformation. *Cell.* 67:1195–1201.

Bartles, J. R., H. M. Ferracci, B. Stieger, and A. L. Hubbard. 1987. Biogenesis of the rat hepatocyte plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. *J. Cell Biol.* 105:1241–1251.

Bomsel, M., and K. Mostov. 1991. Sorting of plasma membrane proteins in epithelial cells. *Curr. Opin. Cell Biol.* 3:647–653.

Brinster, R. L., H. Y. Chen, M. E. Trumbauer, M. K. Yagle, and R. D. Palmiter. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. Natl. Acad. Sci. USA.* 82:4438–4442.

Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science (Wash. DC).* 232:34–47.

Brown, M. S., J. Herz, R. C. Kowal, and J. L. Goldstein. 1991. The low-density lipoprotein receptor-related protein: double agent or decoy? *Curr. Opin. Lipidology* 2:65–72.

Casanova, J. E., P. P. Breitfeld, S. A. Ross, and K. E. Mostov. 1990. Phosphorylation of the polymeric immunoglobulin receptor required for its efficient transcytosis. *Science (Wash. DC).* 248:742–746.

Casanova, J. E., G. Apodaca, and K. E. Mostov. 1991. An autonomous signal for basolateral sorting in the cytoplasmic domain of the polymeric immunoglobulin receptor. *Cell.* 66:65–75.

Chen, W.-J., J. L. Goldstein, and M. S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* 265:3116–3123.

Collawn, J. F., M. Stangel, L. A. Kuhn, V. Esekogwu, S. Jing, I. S. Trowbridge, and J. A. Tainer. 1990. Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell.* 63:1061–1072.

Collawn, J. F., L. A. Kuhn, L-f. Sue-Liu, J. A. Tainer, and I. S. Trowbridge. 1991. Transplanted LDL and mannose-6-phosphate receptor internalization signals promote high-efficiency endocytosis of the transferrin receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3247–3253.

Davis, C. G., M. A. Lehrman, D. W. Russell, R. G. W. Anderson, M. S. Brown, and J. L. Goldstein. 1986. The J. D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors. *Cell.* 45:15–24.

Davis, C. G., I. R. van Driel, D. W. Russell, M. S. Brown, and J. L. Goldstein. 1987. The LDL receptor: identification of amino acids in cytoplasmic domain required for rapid endocytosis. *J. Biol. Chem.* 262:4075–4082.

Glickman, J. N., E. Conibear, and B. M. F. Pearse. 1989. Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1041–1047.

Goldstein, J. L., and M. S. Brown. 1989. Familial hypercholesterolemia. *In* The Metabolic Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Publishing Co., New York. 1215–1250.

Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of LDL in cultured cells. *Methods Enzymol.* 98:241–260.

Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* 1:1–39.

Handley, D. A., C. M. Arbeeny, and S. Chien. 1983. Sinusoidal endothelial endocytosis of low density lipoprotein-gold conjugates in perfused livers of ethinyl-estradiol treated rats. *Eur. J. Cell Biol.* 30:266–271.

Havel, R. J., and R. L. Hamilton. 1988. Hepatocytic lipoprotein receptors and intracellular lipoprotein catabolism. *Hepatology.* 8:1689–1704.

Herz, J., U. Hamann, S. Rogne, O. Myklebost, H. Gausepohl, and K. K. Stanley. 1988. Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:4119–4127.

Hobbs, H. H., D. W. Russell, M. S. Brown, and J. L. Goldstein. 1990. The LDL receptor locus and familial hypercholesterolemia: mutational analysis of a membrane protein. *Annu. Rev. Genet.* 24:133–170.

Hofmann, S. L., D. W. Russell, M. S. Brown, J. L. Goldstein, and R. E. Hammer. 1988. Overexpression of low density lipoprotein (LDL) receptor eliminates LDL from plasma in transgenic mice. *Science (Wash. DC).* 239:1277–1281.

Hunziker, W., C. Harter, K. Matter, and I. Mellman. 1991. Basolateral sorting in MDCK cells requires a distinct cytoplasmic domain determinant. *Cell.* 66:907–920.

Kishimoto, A., M. S. Brown, C. A. Slaughter, and J. L. Goldstein. 1987. Phosphorylation of serine 833 in cytoplasmic domain of LDL receptor by a high molecular weight enzyme resembling casein kinase II. *J. Biol. Chem.* 262:1344–1351.

Kleinherenbrink-Stins, M. F., J. van der Boom, H. F. Bakkeren, P. J. M. Roholl, A. Brouwer, Th. J. C. van Berkel, and D. L. Knook. 1990. Light- and immunoelectron microscopic visualization of *in vivo* endocytosis of low density lipoprotein by hepatocytes and Kupffer cells in rat liver. *Lab. Invest.* 64:73–86.

Li, C., S. Stifani, W. J. Schneider, and M. J. Poznansky. 1991. Low density lipoprotein receptors on epithelial cell (Madin-Darby canine kidney) monolayers. *J. Biol. Chem.* 266:9263–9270.

Mehta, K. D., W-J. Chen, J. L. Goldstein, and M. S. Brown. 1991. The low density lipoprotein receptor in *Xenopus laevis*: I. Five domains that resemble the human receptor. *J. Biol. Chem.* 266:10406–10414.

Mostov, K. E., M. Friedlander, and G. Blobel. 1984. The receptor for transepithelial transport of IgA and IgM contains multiple immunoglobulin-like domains. *Nature (Lond.).* 308:37–43.

Palmiter, R. D., G. Norstedt, R. E. Gelinas, R. E. Hammer, and R. L. Brinster. 1983. Metallothionein-human GH fusion genes stimulate growth of mice. *Science (Wash. DC).* 222:809–814.

Pathak, R. K., and R. G. W. Anderson. 1989. Use of dinitrophenol-IgG conjugates to detect sparse antigens by immunogold labeling. *J. Histochem. Cytochem.* 37:69–74.

Pathak, R. K., and R. G. W. Anderson. 1991. Use of dinitrophenol IgG conjugates: immunogold labeling of cellular antigens on thin sections of osmicated and epon-embedded specimens. *In* Colloidal Gold: Principles, Methods, and Applications. M. A. Hayat, editor. Academic Press, Inc., New York. 223–241.

Pathak, R. K., M. Yokode, R. E. Hammer, S. L. Hofmann, M. S. Brown, J. L. Goldstein, and R. G. W. Anderson. 1990. Tissue-specific sorting of the human LDL receptor in polarized epithelia of transgenic mice. *J. Cell Biol.* 111:347–359.

Russell, D. W., W. J. Schneider, T. Yamamoto, K. L. Luskey, M. S. Brown, and J. L. Goldstein. 1984. Domain map of the LDL receptor: sequence homology with the epidermal growth factor precursor. *Cell.* 37:577–585.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Schlessinger, J. 1988. The epidermal growth factor receptor as a multifunctional allosteric protein. *Biochemistry.* 27:3119–3123.

Simons, K., and A. Wandinger-Ness. 1990. Polarized sorting in epithelia. *Cell.* 62:207–210.

Spady, D. K., J. B. Meddings, and J. M. Dietschy. 1986. Kinetic constants for receptor-dependent and receptor-independent low density lipoprotein transport in the tissues of the rat and hamster. *J. Clin. Invest.* 77:1474–1481.

Südhof, T. C., J. L. Goldstein, M. S. Brown, and D. W. Russell. 1985. The LDL receptor gene: a mosaic of exons shared with different proteins. *Science (Wash. DC).* 228:815–822.

Yamamoto, T., C. G. Davis, M. S. Brown, W. J. Schneider, M. L. Casey, J. L. Goldstein, and D. W. Russell. 1984. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell.* 39:27–38.

Zoller, M. J., and M. Smith. 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *DNA.* 3:479–488.