Use of Antipeptide Antibodies to Demonstrate External Orientation of the NH₂-Terminus of the Low Density Lipoprotein Receptor in the Plasma Membrane of Fibroblasts

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ABSTRACT The low density lipoprotein (LDL) receptor is a member of a class of receptors that bind macromolecules at the cell surface and facilitate their cellular uptake by receptor-mediated endocytosis. The orientation of the LDL receptor in the plasma membrane is unknown. In the current studies the sequence of amino acids at the NH₂-terminus of the bovine adrenal LDL receptor was determined, and a synthetic peptide corresponding to amino acids 1-16 was prepared. Antibodies against this peptide were raised in rabbits and were shown by immunoblotting analysis to react specifically with the bovine LDL receptor. The anti-receptor peptide antibodies also bound to the LDL receptor on the outer surface of the plasma membrane of intact human fibroblasts, as visualized by indirect immunofluorescence. Specificity of this binding reaction was confirmed by the observation that the anti-receptor peptide antibodies did not bind to mutant fibroblasts from a patient with homozygous familial hypercholesterolemia that lack LDL receptors. These data demonstrate that the LDL receptor is oriented in the plasma membrane with its NH₂-terminus facing the extracellular surface.

Certain proteins on the surface of animal cells serve as receptors that facilitate the uptake of extracellular macromolecules by receptor-mediated endocytosis. Among these proteins is the receptor for plasma low density lipoprotein (LDL),¹ the major cholesterol transport protein in human plasma (1). After LDL binds to its receptor, the ligand/receptor complex enters the cells in coated pits and coated vesicles. The LDL is subsequently delivered to lysosomes, where it is digested; this liberates its cholesterol for metabolic use by the cell. Receptors for a variety of other ligands, including glycoproteins, insulin, transferrin, lysosomal enzymes, and certain lipid-enveloped viruses, also enter cells through coated pits (2, 3). It has been postulated that receptors may enter coated pits by binding directly or indirectly to clathrin, the structural protein that coats the cytoplasmic surface of these pits (2, 4). This hypothesis implies that the cytoplasmic domain of these receptors may share some common determinant that binds to clathrin or some clathrin-associated protein. The nature of such a putative recognition marker is not known.

¹ Abbreviation used in this paper: LDL, low density lipoprotein.

Recent studies have shown that antibodies made against short synthetic peptides can react with the corresponding...
amino acid sequence present in the native protein structure (11, 12). For example, antibodies prepared against short synthetic peptides corresponding to segments of the influenza virus hemagglutinin react with the intact hemagglutinin on the viral envelope (13).

In the current studies, we have used this new immunologic technique to locate the NH2-terminus of the LDL receptor. Although the receptor is only a minor component of total cellular protein, it can be purified from bovine adrenal cortex in milligram amounts (14, 15). It is a single glycoprotein chain of apparent Mr, 160,000, as determined by SDS PAGE. It behaves as an integral membrane protein that spans the plasma membrane. We identified the first 16 amino acid residues from the NH2-terminus of the LDL receptor and prepared a synthetic peptide corresponding to this sequence. Antibodies to the peptide reacted with the extracellular (exposed) portion of the LDL receptor in intact human fibroblasts, demonstrating that the NH2-terminus of the receptor is on the external surface of the plasma membrane.

MATERIALS AND METHODS

Materials: We obtained female New Zealand White rabbits weighing 2 kg from Hickory Hills Rabbitry, Flint, TX; keyhole limpet hemocyanin (cat. no. 374817) from Calbiochem-Behring Corp., La Jolla, CA; goat anti-rabbit IgG (IgG fraction, Cat. No. 0212-0081) from Cappel Laboratories, Inc., Cochranville, PA; goat anti-rabbit IgG (affinity-purified fraction) coupled to fluorescein isothiocyanate (cat. no. 62-6111) from Zymed Laboratories, San Francisco, CA; and [3H]iodoacetic acid (100-300 mCi/mmol) from New England Nuclear, Boston, MA. Disuccinimidyl suberate was synthesized by Dr. J. R. Falk in our Department of Molecular Genetics. A polyclonal antibody directed against the purified bovine adrenal LDL receptor was raised in rabbits, and an IgG fraction was prepared (16). Other materials were obtained as previously reported (15-18).

Purification of LDL Receptor from Bovine Adrenal Cortex: The LDL receptor was purified to homogeneity by chromatography on DEAE-cellulose followed by chromatography on an immunoadfinity column containing a monoclonal antibody directed against the LDL receptor (15).

Preparation of LDL Receptor for Sequencing: Before sequencing, LDL receptor preparations were reduced and alkylated. In a typical experiment, thiolated LDL receptor (1.6-1.8 mg protein) was dissolved in 1 ml of buffer containing 0.5 M Tris-chloride (pH 8.2), 7 M guanidine, 2 mM EDTA, and 30 mM 2-mercaptoethanol. The tube was flushed with N2 and incubated for 2 h at 37°C. [3H]iodoacetic acid (1 mCi dissolved in above buffer without EDTA and 2-mercaptoethanol) was then added. After 30 min in the dark at room temperature, solid unlabeled iodoacetic acid was added to a final concentration of 40 mM. The incubation was continued for 15 min at room temperature in the dark. The solution was diazylated at 4°C against three changes of 6 l each of 0.25 M NaH2O2 and lyophilized. The reduced and 3H-carboxymethylated LDL receptor preparations were subjected to amino acid sequence analysis as described below. For one of the sequence runs, a CNBr digest of reduced and 3H-carboxymethylated LDL receptor was prepared by dissolving 1.8 mg protein in 2 ml of 70% formic acid and adding 25 mg of solid CNBr (19). After incubation for 32 h at room temperature, 8 ml H2O was added, and the solution was lyophilized. The peptides were separated by reverse phase high-performance liquid chromatography using a Brownlee RP 300 column (Brownlee Labs Inc., Santa Clara, CA). 0.25 M and 0.1 M Quadrol programs were employed in conjunction with the nonprotein carrier Polybrene (20). The phencylthiourea glutamic acid amino derivates were identified by high-performance liquid chromatography using both a Waters RCM C18 and an RCM CN columns for each determination. Cysteine residues were identified by radioactivity. Amino acids in the first 16 positions were assigned on the basis of 5 sequencer runs. Yields of the NH2-terminal peptides ranged between 100 and 700 pmol per run. Recovery of phenylthiourea glutamic acid-[3H]cysteine, averaged 92%. The NH2-terminal sequence of the LDL receptor was also determined from a peptide that was fortuitously found among several peptides generated by CNBr digestion and isolated by reverse-phase high-performance liquid chromatography. In the sequencer run on this latter peptide, recovery of the NH2-terminal residue was 600 pmol and the repetitive yield was 89%.

Synthesis of NH2-terminal Peptide of LDL Receptor (Receptor Peptide): The 16-residue peptide corresponding to the NH2-terminal sequence of the LDL receptor (Table 1) was synthesized by Peninsula Laboratories, Inc., Belmont, CA, with the use of solid-phase methods developed by Merrifield and co-workers (see reference 21). Its composition was confirmed by amino acid analysis, and its sequence was confirmed in our laboratory by amino acid sequence analysis. The receptor peptide was coupled to keyhole limpet hemocyanin at room temperature as follows. A solution of hemocyanin in 50% glycerol (80 μl containing 10 mg protein) was mixed with 430 μl of buffer A (0.14 M NaCl, 1.6 mM KCl, 1.1 mM KH2PO4, and 8 mM Na2HPO4 at pH 7.4). A freshly prepared solution (20 μl) of 0.1 M disuccinimidyl suberate in dimethylsulfoxide was added and the mixture was stirred for 10 min. Then, 6.3 mg of solid receptor peptide was added and stirring was continued for 90 min. Finally, 500 μl of buffer A was added and, after stirring for 30 min, the material was divided into 160-μl aliquots and stored at −20°C.

Immunizations: To obtain the anti-receptor peptide antibodies, we injected rabbits subcutaneously on days 0, 14, 28, 39, and 67 with a solution containing 160 μl receptor peptide/hemocyanin, 340 μl of buffer A, and 500 μl of either Freund's complete adjuvant (day 0) or incomplete adjuvant (days 14, 28, 39, and 67). Rabbits were bled on days 35, 46, 74, and 88 by heart puncture. IgG fractions from immunized and nonimmunized rabbits were prepared by affinity chromatography on Protein A-Sepharose (17).

PAGE: One-dimensional electrophoresis was carried out on 6 or 8% polyacrylamide slab gels (14 × 8.5 × 0.15 cm) containing 0.1% (vol/vol) SDS and 10% glycerol, 0.1% bromphenol blue, and 100 mM dithiothreitol. The samples were not heated.

Immunoblotting: Proteins were transferred from SDS slab gels to nitrocellulose paper as described (18) or spotted directly onto nitrocellulose paper. Lanes containing sample proteins were incubated for 30 min at 37°C with 100 ml buffer B (50 mM Tris-chloride, 2 mM CaCl2, and 0.5% (vol/vol) glycerol) containing 5% (wt/vol) BSA and 0.2% (vol/vol) Nonidet P-40. Rabbit IgG fractions were added at the concentration indicated in the legends, and incubations were resumed for 2 h at room temperature. The paper was rinsed twice with 10-50 ml buffer B containing 2% albumin, and incubated twice for 20 min each in buffer B containing 2% albumin and 0.2% Nonidet P-40. The paper was incubated in buffer B containing 5% albumin, 0.2% Nonidet P-40, 1% bovine anti-rabbit IgG (IgG fraction, Cat. No. 0212-0081) from Cappel Laboratories, Inc., Cochranville, PA; and 3H-iodoacetic acid at pH 7.4 containing either nonimmune IgG or anti-receptor peptide IgG at a final concentration of 0.2 mg/ml. After incubation for 2 h at 37°C, the coverslips were washed extensively at 4°C, fixed with 3% paraformaldehyde, and rinsed with 5 ml NMKL (17). After being washed as described above and rinsed twice in buffer B, the paper was dried at 37°C for 45 min, and autoradiograms were obtained (18).

Localization of Anti-Receptor Peptide Binding Sites in Human Fibroblasts by Indirect Immunofluorescence: Skin fibroblasts from a healthy subject and from a subject with the receptor-negative form of homozgyous familial hypercholesterolemia were grown in monolayer culture and set up for experiments according to a standard format (17). On day 7 of cell growth after incubation for 48 h in lipoprotein-deficient serum, cell monolayers grown on glass coverslips were transfused to a fresh Petri dish and then overlaid with 200 μl of ice-cold buffer C (10 mM sodium phosphate, 0.15 M NaCl, and 2 mM MgCl2 at pH 7.4) containing either nonimmune IgG or anti-receptor peptide IgG at a final concentration of 0.2 mg/ml. After incubation for 1 h at 4°C or 2 h at 37°C, the coverslips were washed extensively at 4°C, fixed with 3% paraformaldehyde, and rinsed with 5 ml NMKL (17). After being washed as described above and rinsed twice in buffer B, the paper was dried at 37°C for 45 min, and autoradiograms were obtained (18).

| Table 1 | Amino Acid Sequence of the NH2-terminal Portion of the Bovine Adrenal LDL Receptor |
|---------|---------------------------------------------------------------------------------|
| Ala-Val-Glu-Asp-Asn-Cys-Gly-Arg-Asn-Glu-Phe-Glu-Cys-Gln-(Asp)-Gly |

(1) tentative assignment
FIGURE 1 Immunoblot analysis of purified LDL receptor (left) and LDL receptor peptide (right) with anti-receptor peptide IgG and anti-LDL receptor IgG. Purified LDL receptor from bovine adrenal cortex or chemically synthesized receptor peptide were applied to nitrocellulose paper as follows: The indicated amounts of LDL receptor or receptor peptide were dissolved in 10 μl each of a solution of 5 mg/ml BSA in 0.15 M NaCl and applied to dry nitrocellulose paper. The paper was dried at 4°C for 16 h and incubated with the indicated antibodies (5 μg/ml of nonimmune rabbit IgG, A and A'; 5 μg/ml of anti-receptor peptide IgG, B and B'; or 2.5 μg/ml of anti-LDL receptor IgG, C and C'), followed by incubation with 125I-goat anti-rabbit IgG and visualization by autoradiography.

FIGURE 2 Immunoblot analysis of purified LDL receptor with anti-receptor peptide IgG. Purified LDL receptor from bovine adrenal cortex (2 μg/lane) was subjected to electrophoresis on an 8% SDS polyacrylamide gel, transferred to nitrocellulose paper, incubated with 5 μg/ml of either anti-receptor peptide IgG or nonimmune IgG, followed by incubation with 125I-goat anti-rabbit IgG and visualization by autoradiography. Mr standards are indicated.

FIGURE 3 Immunoblot analysis of partially purified LDL receptor with anti-receptor peptide IgG. Partially purified LDL receptor from bovine adrenal cortex (DEAE-cellulose fraction) (50 μg/lane) was subjected to electrophoresis on a 6% SDS polyacrylamide gel. After electrophoresis, one lane of the gel (A) was stained with Coomassie Blue and photographed, and two lanes were transferred to nitrocellulose paper, incubated with 5 μg/ml of either anti-receptor peptide antibody (B) or nonimmune IgG (C), followed by incubation with 125I-goat anti-rabbit IgG and visualization by autoradiography. Mr standards are indicated.

RESULTS
The LDL receptor from bovine adrenal cortex was purified to homogeneity (15), and the first 16 amino acid residues at the NH2-terminal end of the molecule were determined by automated sequencing methodology (Table I). A 16-amino acid peptide corresponding to this NH2-terminal sequence (hereafter referred to as the “receptor peptide”) was synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits. IgG fractions from the antiserum of three rabbits were prepared, and the resulting IgG fractions were tested for their ability to bind to the purified LDL receptor, as determined by immunoblotting techniques. Two of the three IgGs gave positive reactions. One of these was chosen for further study.

When different amounts of the purified LDL receptor were spotted onto nitrocellulose paper and incubated with either the anti-receptor peptide IgG or an IgG fraction of a polyclonal antiserum directed against the intact receptor, the receptor was stained by both antibodies (Fig. 1, left). On the other hand, when the receptor peptide was spotted onto nitrocellulose, it was stained by the anti-receptor peptide IgG, but not by the anti-receptor IgG (Fig. 1, right). Thus, whereas the anti-receptor peptide IgG recognizes the intact LDL receptor, the anti-receptor IgG does not recognize the receptor peptide.

Further evidence that the anti-receptor peptide IgG reacted with the intact LDL receptor is provided by the data in Fig. 2. Purified LDL receptor from bovine adrenal cortex was subjected to SDS PAGE, transferred to nitrocellulose paper, and incubated with the anti-receptor peptide IgG or control IgG from nonimmunized rabbits. The nitrocellulose paper

![Graph](image)

**M r x 10^-3**

| Stain  | Blot |
|--------|------|
| Immune | A    |
|        | B    |
|        | C    |

Mr standards are indicated.
Figure 4 Indirect immunofluorescence staining pattern of LDL receptors in normal human fibroblasts (A and C) and homozygous familial hypercholesterolemia fibroblasts (B and D) after incubation with anti-receptor peptide IgG at 4°C (A and B) or 37°C (C and D). Monolayers of normal fibroblasts or mutant fibroblasts were grown on glass coverslips and incubated with anti-receptor peptide IgG at either 4°C or 37°C. The coverslips were then washed, fixed in paraformaldehyde, permeabilized in the case of C and D, and processed for indirect immunofluorescence localization of either surface-bound (A and B) or internalized (C and D) binding sites for anti-receptor peptide IgG. A, × 800; B–D, × 750.
was then incubated with an 125I-labeled goat anti-rabbit IgG. The anti-receptor peptide IgG from the immunized rabbit reacted strongly with the LDL receptor; this gave an intense radioactive band at a position corresponding to a protein of 160,000 mol wt. There was no reaction determined by immunoblotting techniques after electrophoresis of the receptor in SDS polyacrylamide gels. The anti-receptor peptide IgG also reacted with the human LDL receptor. When incubated with intact normal human fibroblasts, the anti-receptor peptide IgG bound to the receptor, where it could be visualized by indirect immunofluorescence. Specificity of this reaction was confirmed by the failure of the anti-receptor peptide IgG to bind to intact fibroblasts that lack LDL receptors from a patient with the homozygous form of familial hypercholesterolemia. That the anti-receptor peptide IgG recognizes the intact LDL receptor from the human as well as the cow suggests a conserved amino acid sequence in this region of the molecule.

Fig. 3 shows an experiment designed to test the specificity of the anti-receptor peptide IgG. A crude DEAE-cellulose fraction from an early step in the purification of the bovine adrenal LDL receptor (15) was subjected to SDS gel electrophoresis and transferred to nitrocellulose paper. The receptor, which represents less than 1% of the total protein in this fraction (15), is not visible by Coomassie Blue staining (Fig. 3A). However, when the nitrocellulose paper was incubated with the anti-receptor peptide IgG, the IgG bound specifically to a band corresponding in molecular weight to the LDL receptor (Fig. 3B). Nonimmune IgG did not stain (Fig. 3C).

The specificity of the staining was further demonstrated by showing that fibroblasts from a patient with homozygous familial hypercholesterolemia, which lack LDL receptors, failed to bind the anti-receptor peptide IgG (Fig. 4B). Previous studies with polyclonal (24–26) and monoclonal antibodies (17, 26) directed against the intact receptor. No staining was observed when a nonimmune IgG was substituted for the anti-receptor peptide IgG (data not shown). The specificity of the staining was further demonstrated by showing that fibroblasts from a patient with homozygous familial hypercholesterolemia, which lack LDL receptors, failed to bind the anti-receptor peptide IgG (Fig. 4B).

The specificity of the staining was further demonstrated by showing that fibroblasts from a patient with homozygous familial hypercholesterolemia, which lack LDL receptors, failed to bind the anti-receptor peptide IgG (Fig. 4B). Previous studies with polyclonal (24–26) and monoclonal antibodies (17, 26) directed against the intact receptor have demonstrated that these antibodies are rapidly internalized by cells after they bind to the receptor at 37°C. A similar phenomenon was observed with the anti-receptor peptide IgG. When normal fibroblasts were made permeable and incubated with the fluorescein-labeled goat anti-rabbit IgG, the anti-receptor peptide IgG was visualized as foci of fluorescence within the cell in a distribution that corresponds to endosomes and lysosomes (Fig. 4C). No immunoreactive staining was seen in the mutant fibroblasts that lack LDL receptors (Fig. 4D).

Despite its ability to bind to the LDL receptor of human fibroblasts and bovine adrenal cortex, the anti-receptor peptide IgG did not inhibit receptor binding of either 125I-LDL or of an 125I-labeled monoclonal antibody (IgG-C7) directed against the receptor (17) (data not shown).

DISCUSSION

The intense current interest in endocytosis-mediating receptors has led to a widespread effort to elucidate the structural elements that enable these proteins to concentrate in coated pits. However, with the exception of the hepatic glycoprotein receptors discussed above, little is known of the detailed structures of this class of membrane proteins (5). In the current experiments we prepared a rabbit antiserum against a synthetic peptide corresponding to the 16 amino acids at the NH2-terminus of the bovine LDL receptor. The IgG fraction of this antiserum reacted with the intact bovine receptor as determined by immunoblotting techniques after electrophoresis of the receptor in SDS polyacrylamide gels. The antireceptor peptide IgG also reacted with the human LDL receptor. When incubated with intact normal human fibroblasts, the anti-receptor peptide IgG bound to the receptor, where it could be visualized by indirect immunofluorescence. Specificity of this reaction was confirmed by the failure of the antireceptor peptide IgG to bind to intact fibroblasts that lack LDL receptors from a patient with the homozygous form of familial hypercholesterolemia. That the anti-receptor peptide IgG recognizes the intact LDL receptor from the human as well as the cow suggests a conserved amino acid sequence in this region of the molecule.

The above results also suggest that the NH2-terminus of the LDL receptor is located on the external surface of the plasma membrane. Thus, the orientation of this receptor differs from that of the agalactoglycoprotein receptor of chicken liver (6) and the asialoglycoprotein receptor of rat liver (7), both of whose NH2-termini face the cytoplasmic side of the membrane. Since both the LDL receptor and the hepatic glycoprotein receptors mediate rapid endocytosis of their respective ligands through coated pits (2, 27), the current findings indicate that the NH2-terminal/COOH-terminal orientation of a receptor molecule with respect to the plasma membrane is not a determinant of a receptor's interaction with coated pits. The current results also suggest that the extreme NH2-terminal region of the LDL receptor is not an essential component of the LDL binding domain since the anti-receptor peptide IgG does not block receptor binding of the lipoprotein.

The groundwork for the current studies lies in the pioneering work of Lerner and colleagues (see references 11 and 13) and of Doolittle and his associates (see reference 12). These investigators showed that antibodies against certain short synthetic peptides are capable of reacting with the corresponding amino acid sequences in large proteins. Apparently, short peptides may sometimes assume the same conformations that they assume in large proteins. This powerful observation makes it possible to raise antibodies that react with specified regions of proteins, and this in turn makes it possible to localize these regions with a high degree of accuracy.

The orientation of the COOH-terminus of the LDL receptor is not yet known. If the receptor traverses the plasma membrane only once, then the COOH-terminus must face the cytoplasmic surface. However, it is possible that the receptor crosses the membrane several times; if so, the COOH-terminus might be on either side of the membrane. If the amino acid sequence of the COOH-terminus can be deduced, it should be possible to localize this region with techniques similar to the ones employed in the current study.

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