Immunoblot Analysis of Low Density Lipoprotein Receptors in Fibroblasts from Subjects with Familial Hypercholesterolemia*

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This paper describes a sensitive method for study of the isoelectric point and molecular weight of immunoreactive low density lipoprotein (LDL) receptors of cultured human fibroblasts. The fibroblast receptors are solubilized with Triton X-100, partially purified by batch elution from DEAE-cellulose, and subjected to two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins are transferred electrophoretically to nitrocellulose paper which is then incubated with a mouse monoclonal antibody (IgG-C7) directed against the LDL receptor, followed by an 125I-labeled antibody against mouse IgG. The receptor-bound monoclonal antibody is localized by autoradiography. By this technique, the immunodetectable LDL receptors from normal human fibroblasts migrate as a single spot with an isoelectric point of 4.3 and a M, of ~160,000. In one patient with homozygous familial hypercholesterolemia whose cells fail to bind 125I-labeled IgG-C7, no immunoreactive LDL receptor spot was detected after electrophoresis. We also studied LDL receptors from three homozygotes whose cells bind 125I-IgG-C7, i.e. cross-reacting material-positive mutants. Their immunodetectable receptors were indistinguishable from normal receptors in terms of isoelectric point and molecular weight. Similarly, the receptors from one patient with the internalization-defective form of familial hypercholesterolemia showed normal electrophoretic migration. The immunoblotting technique should prove useful in analyzing structural alterations, if they exist, in LDL receptors from other subjects with cross-reacting material-positive forms of familial hypercholesterolemia.

The LDL* receptor is a cell surface protein that binds LDL, the major cholesterol-transport protein in plasma. Binding leads to cellular uptake of LDL by adsorptive endocytosis in coated pits. The LDL is digested within lysosomes, providing the cell with cholesterol for new membrane synthesis (1). In patients with a disease called homozygous FH, LDL receptor function is diminished. As a result, LDL is not removed normally from the circulation and it accumulates to high levels in plasma, ultimately producing atherosclerosis (2).

Three classes of FH homozygotes have been delineated

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1 The abbreviations used are: LDL, low density lipoprotein; CRM, cross-reacting material; FH, familial hypercholesterolemia; SDS, sodium dodecyl sulfate.

through quantitative analyses of the binding of 125I-LDL to cultured fibroblasts (reviewed in Ref. 3). Cells in the first class, termed receptor-negative, do not bind detectable amounts of 125I-LDL (i.e. less than 2% of the normal amount). It has been suggested that these subjects are homozygous for a mutant allele at the receptor locus that produces either no receptors or else produces nonfunctional receptor molecules. Cells from the parents of these subjects, who are obligate heterozygotes, produce about one-half of the normal amount of functional LDL receptors (3).

Cells from the second class of FH homozygotes, termed receptor-defective, bind 2-20% of the normal amount of 125I-LDL. Cells from their parents bind about one-half of the normal amount of 125I-LDL. These receptor-defective "homozygotes" are believed to constitute a heterogeneous group, composed largely of genetic compounds who have inherited two mutant alleles, at least one of which specifies a receptor that can bind detectable amounts of LDL (3). Cells from the third class of subjects produce an internalization-defective receptor that can bind LDL but cannot migrate to coated pits and, hence, cannot carry the lipoprotein into the cell. The most extensively studied subject in this category, J. D., appears to be a genetic compound who has inherited a receptor-negative allele from his mother and an internalization-defective allele from his father (3, 4). There is now a report from Japan of a subject who is homozygous for the internalization-defective allele. This individual inherited two copies of this rare mutation as a result of parental consanguinity (5).

We have recently found that the receptor-negative and receptor-defective subgroups of FH homozygotes can each be further subdivided into two groups on the basis of the amount of material on the cell surface that reacts with a monoclonal antibody to the LDL receptor (so-called "cross-reacting material" or CRM) (6). The antibody, designated IgG-C7, is produced by cells from a mouse that was immunized with a partially purified preparation of LDL receptors from bovine adrenal cortex. The 125I-labeled antibody binds to normal human fibroblasts in amounts that are equivalent to the molar amounts of 125I-LDL bound (6). In fibroblasts from six receptor-negative FH homozygotes, there was no detectable binding of either 125I-LDL or 125I-IgG-C7. However, two other receptor-negative FH homozygotes whose cells were unable to bind 125I-LDL nevertheless bound a substantial amount of 125I-IgG-C7 (6). Similarly, cells from one receptor-defective homozygote bound much more 125I-IgG-C7 than 125I-LDL. These three individuals were designated as CRM-positive mutants (6).

The current studies were designed to characterize the material in the CRM-positive FH homozygote cells that binds monoclonal 125I-IgG-C7. For this purpose, we have adapted the technique of immunoblotting as recently described by Towbin et al. (7) and Burnette (8). In this method, proteins

13150
are separated by one- or two-dimensional electrophoresis in polyacrylamide gels, transferred electrophoretically to nitrocellulose paper, and incubated with an antibody to one of the proteins. The bound antibody is localized with a second antibody (directed against the first antibody) that is radiolabeled with 125I. We have devised a sensitive protocol by which this technique can be applied to analysis of membrane proteins from cultured human fibroblasts and have been able to study the isoelectric point and molecular weight of the LDL receptor from CRM-positive FH homoygotes as well as normal individuals.

**EXPERIMENTAL PROCEDURES**

**Materials**—We obtained a mixture of insulin, transferrin, and selenium (ITS Premix) from Collaborative Research Inc., Waltham, MA; DEAE-cellulose (DE32) from Whatman; bovine serum albumin (Fraction V powder, A4503), Triton X-100, Nonidet P-40, Amido black 10B, Protein A (soluble and purified from Staphylococcus aureus), and phenylmethylsulfonfluoride from Sigma; Ammonium phosphate (pH 3.5–10) from LKB Instruments Inc., Rockville, MD; urea (ultrapure) and sodium deoxycholate from Schwarz/Mann; Clostridium perfringens neuraminidase (4762) from Worthington; nitrocellulose technique can be applied to analysis of membrane proteins with g for 1 h at 4 °C.

**Antibodies**—A monoclonal antibody (designated IgG-C7) directed against the LDL receptor was prepared from splenic lymphocytes of mice that were immunized with partially purified LDL receptors from bovine adrenal cortex (6). This antibody, which reacts cross with the human LDL receptor, is routinely isolated from ascitic fluid by chromatography on columns of Protein A-Sepharose CL-4B (6). A control monoclonal antibody (designated IgG-2001) directed against an irrelevant antigen (*Hemophilus influenza* type B) was isolated from a hybridoma kindly provided by Stella Robertson, Department of Microbiology, University of Texas Health Science Center at Dallas. Goat anti-mouse IgG and rabbit anti-goat IgG were purchased from Cappel Laboratories, Cochranville, PA. Antibodies were radiola belled with 125I by either of two methods: 1) Iodogen (6), which gave specific radioactivities of ~1,000 cpm/ng; or 2) chloramine-T (9), which gave specific radioactivities of ~15,000 cpm/ng. S. aureus Protein A was radiola belled with 125I by the Iodogen method (6). SDS transformation of fibroblasts was carried out by incubating cells with SV40 virus strain 776, which was kindly provided by Dr. Brad Ozanne, Department of Microbiology, University of Texas Health Science Center at Dallas.

**Electrophoresis**—One-dimensional electrophoresis was conducted on 7% polyacrylamide slab gels (14 × 8.5 × 0.15 or 14 × 8.5 × 0.3 cm) containing 0.1% (w/v) SDS according to the method of Laemmli (11). Samples of DEAE-cellulose-purified adrenal (50–70 µg of protein) or fibroblast (0.19–0.8 mg of protein) extracts were applied in 100–200 µl of buffer B containing 0.5% SDS, 10% (v/v) glycerol, and 0.1% bromphenol blue. No reducing agent was added and no heating was performed since these procedures decreased the immunodetectable receptor band. Electrophoresis was carried out at 25–50 mA/slab gel at 5 °C for 3–4 h.

**Two-dimensional Isoelectric Focusing**—Samples were transferred from SDS gels using polyacrylamide gel electrophoresis was performed by the method of O'Farrell (12) except that the isoelectric focusing tube gels (12 × 5 mm) were prepared with pH 3.5–10 Ammonium and the electrode solutions were 0.1 M HPO4 and 0.1 M NaOH. Samples of DEAE-cellulose-purified adrenal (100 µg of protein) or fibroblast (60–21.4 µg of protein) extracts were applied to 100–400 µl of buffer C containing 20% (v/v) glycerol, and 0.1% bromphenol blue. No reducing agent was added and no heating was performed since these procedures decreased the immunodetectable receptor band. Electrophoresis was carried out at 25–50 mA/slab gel at 5 °C for 3–4 h.

**Two-dimensional Isoelectric Focusing**—Proteins were transferred from SDS gels onto nitrocellulose paper according to the method of Burnette (8). The slab gel was placed on wet nitrocellulose. Whatman 3MM paper and Bio-Rad Scotch Brite 3M pads were placed on each side, and the “sandwich” was placed in a Trans-Blot Cell apparatus (Bio-Rad). The chamber was filled with electrode buffer consisting of 20 mM Tris base, 150 mM glycine and 20% (v/v) methanol. Electrophoresis was carried out at 200 mA for 16 h at 4 °C with the anode on the nitrocellulose side of the sandwich. The section of nitrocellulose paper containing the standard proteins was cut off and stained with Amido black (0.1% w/v in 45% methanol and 10% acetic acid) and destained in 10% acetic acid. The rest of the nitrocellulose paper was incubated with antibodies as described below. In some experiments, the nitrocellulose paper was preincubated for 30 min at room temperature in methanol/water/acidic acid (4:45:45, v/v/v) or in 0.1% (w/v) mouse anti-LDL receptor antibody. The paper was then washed at room temperature with 100 µl of buffer E (10 mM Tris-Cl and 0.15 mM NaCl at pH 7.4) (one rapid wash), 100 µl of buffer F containing 1.0% SDS, 0.2% Nonidet P-40, and 0.25% sodium deoxycholate (two 5–10 min washes each on a rocking platform), and 100 µl of buffer E (one rapid wash). The washed nitrocellulose paper was then incubated for 30 min at room temperature with 50 µl of buffer D containing the indicated amount of 125I-labeled goat anti-mouse IgG.
The paper was again washed as described above and then air-dried. In several of the experiments shown in Fig. 3 (lanes 6-11), the nitrocellulose paper was subjected to an additional incubation with either $^{125}$I-labeled rabbit anti-goat IgG or $^{125}$I-labeled Protein A, followed by washing and air-drying. Autoradiograms were obtained by exposing the dried nitrocellulose paper to Kodak XAR-5 film for 2-16 h at -70 °C with Cronex Lightning Plus enhancing screens (E. I. DuPont) (13).

RESULTS

We previously observed that intact fibroblasts from two of eight receptor-negative FH homozygotes appear CRM-positive in that they are able to bind monoclonal $^{125}$I-lgG-C7, whereas they do not bind $^{125}$I-LDL (6). These results were obtained from screening assays performed at one concentration of each ligand. Fig. 1 shows saturation curves that compare $^{125}$I-labeled LDL and $^{125}$I-labeled IgG-C7 binding to fibroblast monolayers from a normal subject and from two receptor-negative FH homozygotes, one of whom is CRM-positive. Both FH homozygotes (subjects 302 and 388) failed to bind detectable amounts of $^{125}$I-LDL with high affinity (Fig. 1A). However, at saturation, fibroblasts from subject 302 bound approximately 50% of the normal amount of $^{125}$I-IgG-C7, whereas fibroblasts from subject 388 failed to bind detectable amounts of $^{125}$I-LDL, with low affinity (Fig. 1A).

![Fig. 1](image)

**Fig. 1.** Binding at 4 °C of $^{125}$I-LDL (A) and $^{125}$I-labeled monoclonal antireceptor IgG-C7 (B) to monolayers of fibroblasts from a normal subject (△) and from two subjects with receptor-negative homozygous FH (▲, ○). On day 7 of cell growth, each monolayer received 1.5 ml of ice-cold binding medium (6) containing 10% lipoprotein-deficient serum and the indicated concentration of $^{125}$I-LDL in the absence or presence of 1 μM unlabeled LDL (A) or $^{125}$I-IgG-C7 in the absence or presence of 1.3 μM unlabeled IgG-C7 (B). After incubation for 2 h at 4 °C, the total radioactivity bound to the cells was determined. The data represent high affinity binding, which was determined by subtracting the values for $^{125}$I-ligand binding in the presence of unlabeled ligand (nonspecific binding) from that in its absence (total binding). For both $^{125}$I-ligands, nonspecific binding was <10% of the total binding seen in normal cells.

![Fig. 2](image)

**Fig. 2.** SDS-polyacrylamide gel electrophoresis and immunoblotting of LDL receptors from normal fibroblasts. LDL receptors were partially purified from normal human fibroblasts (subject 480) by DEAE-cellulose chromatography as described under "Experimental Procedures." Extracts of fibroblasts (190 μg of protein) were subjected to electrophoresis in 7% slab gels containing 0.1% SDS. The gel in lane 1 was stained with Coomassie brilliant blue. The proteins in lanes 2 and 3 were transferred electrophoretically to nitrocellulose filter paper and incubated with 1.9 μg/ml of one of the following antibodies: lane 2, mouse IgG-C7 (monoclonal antireceptor antibody); and lane 3, mouse IgG-2001 (control monoclonal antibody). Following the first incubation, the samples in lanes 2 and 3 were incubated with 140 ng/ml of $^{125}$I-labeled goat anti-mouse IgG (15,000 cpm/ng). The dried filters were processed for autoradiography as described under "Experimental Procedures."

![Fig. 3](image)

**Fig. 3.** SDS-polyacrylamide gel electrophoresis and immunoblotting of LDL receptors from normal and FH homozygote fibroblasts and from bovine and human adrenal glands. LDL receptors were partially purified by DEAE-cellulose chromatography as described under "Experimental Procedures." Samples containing the indicated amounts of protein were subjected to electrophoresis in 7% polyacrylamide gels containing 0.1% SDS. The proteins were transferred electrophoretically to nitrocellulose paper and incubated with monoclonal anti-LDL receptor antibody (1 μg/ml of mouse IgG-C7) and then incubated with $^{125}$I-labeled proteins as follows: lanes 1-5, 70 ng/ml of $^{125}$I-labeled goat anti-mouse IgG (15,000 cpm/ng); lanes 6-9, 1 μg/ml of $^{125}$I-labeled goat anti-mouse IgG (1,000 cpm/ng) followed by 1 μg/ml of $^{125}$I-labeled rabbit anti-goat IgG (1,000 cpm/ng); and lanes 10 and 11, 1 μg/ml of $^{125}$I-labeled goat anti-mouse IgG (1,000 cpm/ng) followed by 0.5 μg/ml of $^{125}$I-labeled Protein A (1,200 cpm/ng). The dried filters were processed for autoradiography as described under "Experimental Procedures." Molecular weight standards are indicated. The LDL receptor preparations were derived from the following sources: lane 1, bovine adrenal cortex (50 μg of protein); lane 2, normal human fibroblast 480 (0.4 mg); lane 3, fibroblasts from receptor-negative FH homozygote 388 (0.8 mg); lane 4, normal human fibroblast 480 (0.4 mg); lane 5, fibroblasts from receptor-defective FH homozygote 353 (0.8 mg); lane 6, normal human fibroblast 480 (0.4 mg); lane 7, fibroblasts from receptor-defective FH homozygote 101 (0.8 mg); lane 8, normal human fibroblast 480 (0.4 mg); lane 9, fibroblasts from internalization-defective FH homozygote 380 (0.4 mg); lane 10, bovine adrenal cortex (50 μg); lane 11, human fetal adrenal gland (70 μg).
amounts of $^{125}$I-IgG-C7 (Fig. 1B). Subject 302 was one of the receptor-negative individuals who was previously designated CRM-positive on the basis of the screening assay (6).

To characterize further the $^{125}$I-IgG-C7 binding site in subject 302 fibroblasts and in other mutant fibroblasts from FH subjects with CRM-positive receptor mutations, we developed a method in which Triton-solubilized extracts of cells were prepared, partially purified by adsorption to DEAE-cellulose, and then subjected to either one-dimensional or two-dimensional electrophoresis. The location of the receptor was visualized by electrophoretic transfer of the proteins from the polyacrylamide gel to nitrocellulose paper which was then incubated with IgG-C7 followed by an $^{125}$I-labeled goat anti-mouse IgG. When DEAE-cellulose extracts of normal fibroblasts were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis, many proteins were seen in the Coomassie-stained gel, but no major protein was seen in the molecular weight region between 116,000 and 200,000 (Fig. 2, lane 1). When the proteins were transferred to nitrocellulose paper and reacted with the IgG-C7, a single band of antibody-staining activity was seen in the region between the $M_r = 116,000$ and 200,000 markers (Fig. 2, lane 2). No antibody staining was seen when the gels were incubated with a control monoclonal antibody (designated IgG-2001) that contains heavy chains of the same IgG2b subclass as IgG-C7 but is not directed against the LDL receptor (Fig. 2, lane 3).

Fig. 4. Two-dimensional polyacrylamide gel electrophoresis and immunoblotting of LDL receptor from bovine adrenal cortex (A) and normal human fibroblasts (B). After DEAE-cellulose chromatography, extracts of bovine adrenal cortex (100 µg of protein) and human fibroblast 480 (0.8 mg) were subjected to two-dimensional electrophoresis as described under "Experimental Procedures." Proteins were transferred electrophoretically to nitrocellulose paper and incubated with monoclonal anti-LDL receptor antibody (1 µg/ml of mouse IgG-C7) and then with 70 ng/ml of $^{125}$I-labeled goat anti-mouse IgG (15,000 cpm/ng). The dried paper was processed for autoradiography as described under "Experimental Procedures." Molecular weight standards are indicated. The pH gradient in the tube gel was determined by subjecting a parallel gel to isoelectric focusing, after which the gel was cut into consecutive 1-cm slices that were incubated in water for 1 h at room temperature prior to pH measurement.

Fig. 5. Two-dimensional polyacrylamide gel electrophoresis and immunoblotting of LDL receptors from SV40-transformed normal fibroblasts (A) and SV40-transformed fibroblasts from a subject with the receptor-negative form of homozygous FH (B). After DEAE-cellulose chromatography, extracts of normal fibroblast 589 (1.3 mg) and FH homozygote fibroblast 302 (2.1 mg) were subjected to two-dimensional electrophoresis, electrophoretic transfer, and immunoblotting as described in the legend to Fig. 4. The isoelectric point of the visualized LDL receptor protein is at pH 4.5 for A and B. Molecular weight standards are indicated.

Fig. 3 shows a series of one-dimensional immunoblotting experiments performed on fibroblast and adrenal extracts. Lanes 1 and 2 show blots of DEAE-cellulose-purified receptor preparations from the bovine adrenal cortex and normal human fibroblasts, respectively. In both cases, the receptor appeared at a position corresponding to $M_r ~ 160,000$. Estimations of molecular weight by the immunoblotting method are not precise because the lanes containing the molecular weight standards are cut from the paper prior to staining. Previous studies have shown that the bovine adrenal LDL receptor, against which the IgG-C7 was made, migrates as a single protein of $M_r = 164,000$ (10).

Lane 3 of Fig. 3 shows that there was no immunodetectable receptor after electrophoresis of fibroblasts from receptor-negative subject 388, who has no antibody binding activity (see Fig. 1). Fibroblasts from two CRM-positive subjects with the receptor-defective form of homozygous FH are shown in lanes 5 and 7 of Fig. 3. Intact fibroblasts from both of these subjects bound more $^{125}$I-IgG-C7 than $^{125}$I-LDL. The intact fibroblasts from subject 353 (lane 5) had ~20 and 50% of normal binding activities for $^{125}$I-LDL and $^{125}$I-IgG-C7, respectively; the intact fibroblasts from subject 101 (lane 7) had ~5 and 20% of normal binding activities for $^{125}$I-LDL and $^{125}$I-IgG-C7, respectively (data not shown). Fibroblast extracts from both of these subjects showed an immunodetectable band that was reduced in amount, but exhibited the same molecular weight as the normal receptor (lanes 4 and 6). Fibroblasts from a subject whose cells can bind LDL but not internalize the lipoprotein (subject 380, formerly designated J. D.) (3, 4) showed a detectable receptor band that had the same molecular weight (lane 9) as that of normal fibroblasts (lane 8). Extracts from a human fetal adrenal gland (lane 11), obtained
at the time of therapeutic abortion, showed an LDL receptor band that had a molecular weight similar to the bovine adrenal receptor (lane 10). In some of these experiments, a minor immunodetectable spot was seen just below the LDL receptor spot (as in lanes 10 and 11). This lower molecular weight material may result from proteolysis or partial removal of carbohydrate from the receptor during purification (10). In addition, some of the gels showed smudges of faintly stained lower molecular weight material that was not consistently seen (compare Figs. 2 and 3) and that corresponded to areas of the gel that were heavily overloaded with protein (see Fig. 2, lane 1).

The immunoblotting technique was also adapted to analysis

![Image](Fig. 6. Two-dimensional polyacrylamide gel electrophoresis and immunoblotting of LDL receptors from normal human fibroblasts before (A) and after (B) treatment with neuraminidase. After DEAE-cellulose chromatography, extracts from normal fibroblast 480 (0.6 mg of protein) were incubated in buffer B containing either no neuraminidase or 0.15 unit of neuraminidase for 1 h at room temperature. The treated extracts were then subjected to two-dimensional electrophoresis, electrophoretic transfer, and immunoblotting as described in the legend to Fig. 4. Molecular weight standards are indicated.]

![Image](Fig. 7. Two-dimensional polyacrylamide gel electrophoresis and immunoblotting of LDL receptors from normal fibroblasts (A), fibroblasts from a subject with the receptor-defective form of homozygous FH (B), and a mixture of the two strains of fibroblasts (C). After DEAE-cellulose chromatography, the fibroblast extracts were incubated with neuraminidase (0.1 unit/0.4 mg of protein) for 1 h at room temperature. The treated extracts of normal fibroblast 480 (1.0 mg of protein), FH homozygote 101 (1.9 mg), and a mixture of normal (0.7 mg) plus FH homozygote (1.6 mg) were subjected to two-dimensional electrophoresis, electrophoretic transfer, and immunoblotting as described in the legend to Fig. 4. The isoelectric point of the visualized LDL receptor protein is at pH 5.2 for A, B, and C. Molecular weight standards are indicated.

![Image](Fig. 8. Two-dimensional polyacrylamide gel electrophoresis and immunoblotting of LDL receptors from a subject with the internalization-defective form of homozygous FH. After DEAE-cellulose chromatography, an extract of FH homozygote fibroblast 380 (1.1 mg) was subjected to two-dimensional electrophoresis, electrophoretic transfer, and immunoblotting as described in the legend to Fig. 4. The isoelectric point of the visualized LDL receptor protein is at pH 4.5. Molecular weight standards are indicated.

of two-dimensional isoelectric focusing/SDS-polyacrylamide gels performed according to the O'Farrell technique (12). Fig. 4A shows an immunoblot in which the DEAE-cellulose-purified LDL receptor from bovine adrenal cortex was subjected to isoelectric focusing in the horizontal dimension and SDS electrophoresis in the vertical dimension. The receptor was placed on the isoelectric focusing tube gel at the alkaline pole, and it focused near the acidic pole in a region corresponding to a pH of approximately 4.6. In the second dimension, the molecular weight of the bovine adrenal receptor was about 160,000, as expected. The position of the receptor detected by immunoblotting is similar to that previously observed when the 125I-labeled affinity-purified receptor was subjected to two-dimensional electrophoresis (10). The DEAE-cellulose-purified receptor from normal human fibroblasts behaved similarly to that of the bovine adrenal gland, except that the isoelectric point of the fibroblast receptor was slightly more acidic (pH 4.3) (Fig. 4B).

Fig. 5 compares the behavior on two-dimensional electrophoresis of the normal fibroblast receptor and the receptor from FH homozygote 302, who is the CRM-positive receptor-negative mutant shown in Fig. 1. The receptor from this subject (Fig. 5B) behaved similarly to the normal (Fig. 5A) on two-dimensional electrophoresis.

We have previously found that the purified bovine adrenal receptor exhibits an apparent decrease in molecular weight and an increase in isoelectric point after neuraminidase treatment (10). A similar finding was observed with regard to the DEAE-cellulose-purified LDL receptor from human fibroblasts (Fig. 6).

Fig. 7 compares the behavior on two-dimensional electrophoresis of the receptors from normal fibroblasts and from a CRM-positive receptor-defective FH homozygote (subject 101). Both receptors were treated with neuraminidase prior to electrophoresis. The migration of both receptors on two-dimensional gels was similar (Fig. 7, A and B). When the two extracts were mixed and then subjected to electrophoresis, only one spot was seen (Fig. 7C).

Fig. 8 shows an experiment performed with fibroblast extracts from the subject with the internalization-defective form of homozygous FH (subject 380). The receptor in this mutant had the same molecular weight and isoelectric point as did the normal. Moreover, when neuraminidase-treated fibroblast extracts from this mutant were mixed with extracts from normal fibroblasts and then subjected to electrophoresis, the mixture of the two preparations gave a single immunoreactive spot (data not shown).
DISCUSSION

This paper describes a sensitive method for the study of the LDL receptor in crude extracts of cultured cells. The plasma membranes are solubilized in situ with Triton X-100, and the LDL receptor is partially purified by taking advantage of its acidic isoelectric point, which allows it to adsorb to DEAE-cellulose at moderately acidic pH and relatively high ionic strength (10, 14). The receptor is eluted from DEAE-cellulose in a concentrated form and is subjected to either SDS-polyacrylamide gel electrophoresis or to isoelectric focusing followed by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose paper, the receptor is visualized by incubation with a monoclonal antibody to the receptor, followed by incubation with a highly radioactive goat anti-mouse IgG. This method has been used to study LDL receptors in a sample as small as 3 x 10^6 fibroblasts.

The immunoblotting method was used to show that the LDL receptor of human fibroblasts resembles the receptor purified from bovine adrenal cortex. Both proteins have an acidic isoelectric point in the range of 4.3-4.6 and a molecular weight of approximately 100,000. In addition, the human fetal adrenal gland was shown to possess an immunoreactive LDL receptor with a similar molecular weight. Using the technique of radiation inactivation to measure the minimal molecular weight required for LDL binding, Innerarity et al. (15) reported that the functional size of the LDL receptor of human fibroblasts was approximately M = 100,000. The relationship between the functional molecular weight measured by radiation inactivation and the apparent monomeric molecular weight measured by SDS-polyacrylamide gels remains to be determined.

By immunoblotting, the fibroblast receptor, like the bovine adrenal receptor (10), shows a shift toward a more basic isoelectric point and an apparent decrease in molecular weight after treatment with neuraminidase. When the LDL receptors of normal fibroblasts were suppressed by growth of the cells in a mixture of 25-hydroxysterol plus cholesterol (1), the immunodetectable receptor spot was no longer visible after SDS-polyacrylamide gel electrophoresis (data not shown). Moreover, when normal fibroblasts were treated with trypsin under conditions that remove 125I-LDL binding activity from the cell surface (100 μg/ml for 30 min at 4 °C), the immunodetectable receptor spot was markedly diminished (6300 cpm in control cells versus 600 cpm in trypsin-treated cells).

The immunoblotting technique also permitted a comparison of the isoelectric point and molecular weight of the LDL receptor in normal fibroblasts with those in fibroblasts from subjects with various types of homozygous FH. Cells from a receptor-negative homozygote (subject 388) whose intact fibroblasts failed to bind the antibody also failed to show an immunodetectable spot after electrophoresis in either one dimension (Fig. 3, lane 3) or two dimensions (data not shown). Of considerable interest were the findings in three CRM-positive FH subjects whose cells bind more 125I-IgG-C7 than 125I-LDL. The relative amounts of binding of 125I-LDL and 125I-IgG-C7 by intact fibroblasts from these subjects were as follows (data expressed as a percentage of normal binding at saturation for each ligand): cell strain 302, 9/80%; 101, 5/20%; and 353, 20/50%. When these mutant cells were studied by one- or two-dimensional electrophoresis, the immunodetectable receptors were not distinguishable from normal, either on the basis of isoelectric point or molecular weight. This was true whether the receptor was studied in its native form or after extensive treatment with neuraminidase. Similar findings were obtained on a cell strain from a subject with the internalization-defective form of FH. The receptor that was visualized on blotting appeared to have the same molecular

weight and isoelectric point as the normal receptor. All of the electrophoresis experiments shown in this paper were repeated on at least two occasions and the relative behavior of the receptors was consistent.

The finding of normal behavior by two-dimensional electrophoresis does not mean that the receptors from these CRM-positive subjects are structurally identical with the normal LDL receptor. Only about one-third of point mutations result in a charge shift detectable by electrophoresis (16). Moreover, the isoelectric focusing method as currently employed covers a broad pH range (pH 3.5-10). We cannot be certain that a change of a single charge in the receptor would be detectable in this system. When we attempted to perform the isoelectric focusing over a narrower pH range with these crude fibroblast extracts, the intact receptor no longer was clearly visualized, and lower molecular weight bands were often observed on the two-dimensional gels. We believe that proteolysis may have occurred during isoelectric focusing on these narrow range gradients, but pretreatment of the fibroblast extracts with several different types of protease inhibitors failed to prevent the alterations. As a result, we have been restricted to the use of the broad range Ampholine (pH 3.5-10).

Another reason for our inability to detect electrophoretically abnormal receptors in the CRM-positive mutants may relate to the methodology employed. Performance of the immunoblotting procedure requires that the receptors be partially purified by DEAE-cellulose chromatography prior to electrophoresis. A mutant receptor that does not have the strong negative charge of the normal receptor may not be retained in the fraction that adheres tightly to the DEAE-cellulose and would not be detected by the immunoblotting technique. Thus, if fibroblasts are analyzed from an FH homozygote who is a genetic compound with two structurally different types of receptors with differing charge, only the population of receptors that adheres to DEAE-cellulose will appear as an immunoreactive spot in our blotting assay. In addition to the mutant fibroblasts studied in this paper, cells from one other FH homozygote with the internalization defect and cells from at least 12 other homozygotes with apparent CRM-positive receptor mutations are available. The method described here should allow a detailed analysis of these mutant cell lines and may reveal mutations in the structural gene for the receptor, if they exist.

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Immunoblots of LDL Receptors

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