Identification of a novel Arg→Cys mutation in the LDL receptor that contributes to spontaneous hypercholesterolemia in pigs

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Abstract **We previously carried out genetic and metabolic studies in a partially inbred herd of pigs carrying cholesterol-elevating mutations. Quantitative pedigree analysis indicated that apolipoprotein (apo)B and a second major gene were responsible for the hypercholesterolemia in these animals. In this study, we assessed LDL receptor function by three different methods: ligand blots of liver membranes using β-very low density lipoprotein (VLDL) as a ligand; low density lipoprotein (LDL)-dependent proliferation of T-lymphocytes; and direct binding of ^{125}I-labeled LDL to cultured skin fibroblasts. All three methods demonstrated that LDL receptor ligands bound with decreased affinity to the LDL receptor in these animals. In skin fibroblasts from the hypercholesterolemic pigs, the K_d of binding was about 4-fold higher than in cells from normal pigs. The cDNA of the pig LDL receptor from normal and hypercholesterolemic pigs was isolated and sequenced. We identified a missense mutation that results in an Arg→Cys substitution at the position corresponding to Arg_{94} of the human LDL receptor. The mutation is in the third repeat of the ligand binding domain of the receptor. By single-stranded conformational polymorphism (SSCP) analysis, we studied the relationship between LDL receptor genotype and plasma cholesterol phenotype. In contrast to humans, the hypercholesterolemia associated with the LDL receptor mutation in pigs was expressed as a recessive trait. The LDL receptor mutation made a far more significant contribution to hypercholesterolemia than did the apoB mutation, consistent with observations made in human subjects with apoB mutations. Within each genotypic group (mutated apoB or mutated receptor), there was a wide range in plasma cholesterol. As the animals were on a well-controlled low-fat diet, this suggests that there are additional genetic factors that influence the penetrance of cholesterol-elevating mutations.**—Grunwald, K. A. A., K. Schueler, P. J. Uelmen, B. A. Lipton, M. Kaiser, K. Buhman, and A. D. Attie. **Identification of a novel Arg→Cys mutation in the LDL receptor that contributes to spontaneous hypercholesterolemia in pigs.** ***J. Lipid Res.*** **1999.** 40: **475–485.**

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Genetic factors have been estimated to contribute approximately 50% to the variability in plasma cholesterol in various human populations (1). Genes encoding lipoprotein receptors, lipogenic enzymes, lipid transfer proteins, and apolipoproteins have been shown to influence the plasma concentration of cholesterol as well as its distribution among the various lipoproteins (2).

We have characterized the hypercholesterolemia of an inbred line of pigs originally identified through the use of allele-specific alloantibodies against apoB, the apolipoprotein of low density lipoprotein (LDL) (3). Hypercholesterolemia in these animals was originally detected only in animals carrying plasma reacting with antibodies against an apoB marker termed 'Lpb5' (4). LDL from these animals was shown to be cleared 30% slower than normal LDL from the bloodstream of normal animals (5, 6). Receptor binding studies revealed that these particles bound to the LDL receptor in cultured pig skin fibroblasts with one-sixth the affinity of normal pig LDL (7). The nucleotide sequence encoding the carboxyl-terminal half of the apoB from Lpb5 animals revealed 13 amino acid polymorphisms (8–10). However, the Lpb5 apoB contains a unique haplotype consisting of Asp_{3164} and Ala_{3447} (10). As these two amino acids lie within the putative receptor binding domain of apoB, it is likely that this combination of amino acids is responsible for the lowered receptor binding affinity of Lpb5 apoB.

In vivo, LDL turnover studies suggested that, in addition to an apoB mutation, a subgroup of the pigs (termed 'Lpb5.1') carried a mutation that affected the activity of the LDL receptor (5, 11). When LDL was chemically mod-

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; SSCP, single-stranded comformational polymorphism; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; NFDM, non-fat dry milk; RT, room temperature; LPDS, lipoprotein-deficient serum; DMSO, dimethylsulfoxide.

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ified to abolish LDL receptor binding, its clearance rate was the same as that of native LDL, suggesting an absence of LDL receptor activity in these animals (11). Through selective breeding, we were able to obtain hypercholesterolemic pigs with normal apoB alleles, confirming the presence of an additional cholesterol-elevating mutation that segregates from apoB. Quantitative pedigree analysis predicted that this second locus acted in a recessive manner and was much more influential than the apoB mutation in raising the cholesterol level of the Lpb5.1 pigs (11).

In the present study, we directly analyzed the LDL receptor activity of these animals and found that they express normal quantities of the LDL receptor protein, but that it binds with reduced affinity to its ligands. We isolated the cDNA encoding the normal and mutant LDL receptor and identified a mutation that segregated with the defective LDL binding phenotype through five generations of animals in two separate sub-pedigrees and is therefore likely to be responsible for the defective activity of the receptor protein. From our analysis of the LDL receptor genotype of animals in the pedigrees and its relationship to plasma cholesterol and affinity for LDL, we suggest that additional factors influence the penetrance of LDL receptor mutations in this inbred population of pigs.

MATERIALS AND METHODS

Materials

Anti-rat LDL receptor antiserum was donated by Jeff Ellsworth (Palo Alto Research Foundation). Monoclonal antibody MB47 was provided by Linda Curtiss (Scripps Clinic, La Jolla, CA). CHAPS, alkaline phosphatase-conjugated goat anti-rabbit IgG, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). The Sigma Total Cholesterol Kit #352 was used for plasma cholesterol measurements. Sodium dodecyl sulfate, nitrocellulose, and molecular weight standards for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). Antibodies and reagents for enhanced chemiluminescence were obtained from Amersham (Arlington Heights, IL). All other chemicals were obtained from Fisher Scientific, Inc.

Animals

Pigs used in these studies were aged 5 months to 4 years. All animals were fed a diet containing 0% cholesterol and 5% fat. Genotyping for the apoB allele was performed by polymerase chain reaction (PCR), as previously described (12). Control pigs were defined as those with plasma cholesterol levels <100 mg/dl and carrying non-*lpb*^{5} apoB alleles. Lpb5.1 and Lpb5.2 pigs were distinguished solely by plasma cholesterol levels, with the former exhibiting severe hypercholesterolemia (plasma cholesterol >180 mg/dl) and the latter displaying normal to moderately increased plasma cholesterol levels (<120 mg/dl).

A homozygous *lpb*^{5} animal with a plasma cholesterol level greater than 300 mg/dl (Lpb5.1/5.1) was crossed with an animal homozygous for the *lpb*^{4} allele and with a plasma cholesterol level below 100 mg/dl. The resulting heterozygotes had intermediate plasma cholesterol levels and were inbred to give a second generation of mixed *lpb* genotypes and various plasma cholesterol levels. These animals were analyzed for LDL receptor activity, as were the ensuing third generation of animals. Simultaneously, another high cholesterol Lpb5.1/5.1 animal was crossed with a homozygous *lpb*$^{8/8}$ animal. (Both *lpb*^{4} and *lpb*^{8} are associated with phenotypically normal LDL and normal plasma cholesterol levels.) The progeny from this breeding were analyzed in a second pedigree by the same methods.

Lipoproteins

Lipoproteins were isolated and radiolabeled with ^{125}I as previously described (6). The specific activities were 200–400 cpm/ng. More than 95% of the counts were TCA precipitable and less than 5% were lipid bound. β-VLDL was isolated from a rabbit fed a 0.2% cholesterol, 5% peanut oil diet for 30 days. After an overnight fast, blood was drawn into evacuated tubes containing EDTA (final concentration = 1.5 mg/ml).

Isolation and solubilization of liver membrane proteins

Liver cell membranes were isolated using a modification of the method of Ellsworth, Kraemer, and Cooper (13) as follows. Pigs were anesthetized and the abdominal cavity was opened. Approximately 10 g of the lower left lateral lobe of the liver was removed and placed into 50 ml of ice-cold homogenization buffer containing 20 mm Tris (pH 8.0), 150 mm NaCl, 1 mm $CaCl_2$, 1 mm PMSF, and 2.5 mg/dl leupeptin. The liver chunk was minced and homogenized. The homogenate was centrifuged at 20,000 *g* for 20 min at 4°C. The supernatant was then centrifuged at 35,000 rpm for 1 h at 4°C in an SW41 rotor. Pellets were resuspended in 2 ml solubilization buffer (250 mm Tris-maleate, pH 6.0, 2 mm $CaCl_2$, 1 mm PMSF, and 250 μg/ml leupeptin) and passed twice through a syringe fitted with a 22 ga. needle and then twice through a 25 ga. needle. An equal volume of ice-cold CHAPS solution (60 mm CHAPS, 100 mm NaCl) was added to the homogenized protein and the suspension was vortexed briefly. The solubilized proteins were chilled on ice for 10 min with brief vortexing every 2 min. The suspension was then centrifuged at 100,000 *g* in a Beckman TLA 100.3 rotor, 4°C, for 1 h to pellet any insoluble proteins. The supernatant was frozen at −80°C until use. Protein content was determined by the method of Markwell et al. (14) using bovine serum albumin (BSA) as the standard.

Detection and quantitation of LDL receptor in solubilized pig liver membrane proteins

Solubilized membrane protein preparations were combined with an equal volume of loading buffer (50 mm Tris, pH 6.8, 20% glycerol, and 2% sodium dodecyl sulfate (SDS)) and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) through 6% polyacrylamide gels at room temperature and electroblotted to nitrocellulose. Blots were blocked in 2% nonfat dry milk (NFDM) in Tris-buffered saline containing Tween-20 (TBST: 10 mm Tris, pH 8.0, 150 mm NaCl, and 0.05% Tween-20) for 2 h at room temperature (RT). The blocking solution was replaced with rabbit anti-rat LDL receptor antiserum diluted 1:10,000 in 1% NFDM in TBST for a 2-h incubation at RT. The blots were rinsed 4 times with 1% NFDM in TBST and incubated with anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:10,000 in 1% NFDM in TBST for 1 h at RT. They were rinsed as above, followed by two rinses with Tris-buffered saline (TBS: 10 mm Tris, pH 8.0, and 150 mm NaCl). Bound antibody was detected using enhanced chemiluminescence and exposure to Kodak XAR-5 X-ray film. Immunoreactive LDL receptor protein was quantitated by scanning laser densitometry. LDL receptor protein levels are expressed relative to the immunoreactivity of a rat liver membrane protein standard electrophoresed alongside the pig samples.

Ligand blotting of the LDL receptor

The procedure used is a modification of that of Daniel et al. (15). Liver membrane proteins were solubilized and mixed 1:1 with SDS-PAGE sample buffer, loaded on a 6% polyacrylamide

gel, electrophoresed, and transferred to nitrocellulose as above. The membranes were blocked in buffer A (50 mm Tris, pH 8.0, 90 mm NaCl, 2 mm $CaCl_2$, 50 mg/ml BSA) overnight at 4°C with shaking. The blocking solution was replaced with fresh buffer A containing 5 μg/ml rabbit β-VLDL and incubated RT with shaking for 30 min. The blot was then rinsed with buffer B (50 mm Tris, pH 8.0, 90 mm NaCl, 2 mm $CaCl_2$, 5 mg/ml BSA) once quickly, 3 times for 20 min each with shaking, then quickly again. Fresh buffer A containing a 1:10,000 dilution of the anti-human apoB monoclonal antibody MB47 was added, and the blot was shaken for 1 h at RT. The blot was washed 3 times with buffer B for 10 min each, after which fresh buffer A containing 1:10,000 anti-mouse IgG conjugated to horseradish peroxidase (Amersham) was added. The blot was incubated with shaking for 2 h at RT, then rinsed 3 times for 10 min each with buffer C (50 mm Tris, pH 8.0, 90 mm NaCl, 2 mm $CaCl_2$) with shaking. Bound ligand was visualized by enhanced chemiluminescence. In parallel blots, 5 mm EDTA was added during the incubation with lipoprotein as a control for Ca^{2+}-dependent ligand binding. Further control blots showed no reactivity when either the lipoprotein ligand or the primary antibody were omitted (data not shown).

Assay of functional LDL receptor on pig T-lymphocytes

Mevinolin (Merck, Sharp and Dohme, Rahway, NJ) was dissolved in dimethyl sulfoxide (DMSO) and added directly to cultures. An equivalent volume of DMSO (0.5% v/v) was added to control cultures and had no appreciable effect on cell responsiveness. Peripheral blood mononuclear cells were isolated from 25 ml of anticoagulated venous blood by centrifugation according to modifications of the method described by Cuthbert et al. (16). Cells were cultured in RPMI-1640 medium supplemented with 5% lipoprotein-deficient serum (LPDS). The cells were plated in triplicate microtiter wells with or without phytohemagglutinin (5 μg/ml). Cultures were also treated with mevinolin (0.5 μm) or DMSO as control, and various concentrations of pig LDL (0–10 μg/ml). Lymphocyte DNA synthesis was assessed by measurement of [^{3}H]thymidine incorporation as described by Cuthbert and Lipsky (17). Data are expressed as the percentage inhibition by mevinolin as compared with control cultures treated with just DMSO.

Pig skin fibroblasts

All fibroblast cell lines used in these experiments were grown from biopsy punches taken from our animals and cultured in our lab. Biopsied animals varied in age from 6 months to 4 years. All cell lines were between passage 5 and 20 for binding studies.

Receptor binding experiments

Fibroblasts were passed 1 week prior to the experiment. Each binding experiment included a binding curve from a control animal cell line. Each cell line to be assayed was set up in six, 6-well plates at a density of 2.0–3.5 × 10^4 cells/well. They were fed with complete DMEM media plus human epidermal growth factor. The cells were grown for 5 days prior to up-regulation of the LDL receptor, at which time they were verified to be subconfluent. The LDL receptor was up-regulated 48 h prior to the assay by refeeding the cells with DMEM plus 10% LPDS. The plates were placed at 4°C for 30 min before replacing the media with DMEM in buffer A (25 mm HEPES plus 10% LPDS and 5 mg/ml BSA) containing ^{125}I-labeled LDL, 0–50 μg/ml, in duplicate. The cells were incubated for 4 h at 4°C on a rotary shaker. After incubation, the wells were washed 5 times with buffer B (150 mm NaCl, 50 mm Tris-Cl, 2 mg/ml BSA, pH 7.4) and then once with buffer C (buffer B minus BSA) to eliminate excess protein. The specifically bound LDL was then released by a 1 h incubation at 4°C with 1 ml buffer D (50 mm NaCl, 10 mm HEPES, 4 mg/ml dextran sulfate). Released LDL (750 μl) was removed for quantitation of ^{125}I radioactivity. The washed cells were solubilized in 1 ml 0.2 N NaOH and the protein concentration was determined by the Lowry protein assay (18). The binding curves were fitted to a single site model without non-specific binding.

Mutation analysis

LDL receptor cDNA was prepared from mRNA isolated from fibroblasts pre-incubated in LPDS to up-regulate LDL receptor expression. The cDNAs were sequenced by the method of Lee (19) initially using primers from published sequence (20) and then using primers designed from the 5′ end of our newly obtained sequences. All but the last 200 bp at the 5′ end of the cDNA were obtained.

The SSCP analysis used the method of Orita et al. (21). Briefly, 0.5 μg DNA was combined with 350 ng of each primer, 10 mm Tris (pH 8.0), 1.5 mm $MgCl_2$, 50 mm KCl, 1 mm each dNTP, 1 μl ^{32}P-dCTP, 2 U *Taq* polymerase, overlaid with mineral oil and subjected to 30 cycles of amplification (1 min at 96°C, 3 min at 68°C). One μl of the total reaction was mixed with 30 μl formamide dye (98% formamide, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol, 2.5 mm EDTA), boiled for 5 min and run on a 1× TBE, 10% glycerol, 8% acrylamide gel overnight at 250–300 V. The oligonucleotides used, (5′-CCAAGACGTGCTC CCAAGAT-3′ and 5′-TGCACTCGCCACTGTGGCAGTGGAA-3′), yielded a 317-bp fragment.

RESULTS

LDL receptor activity in T-lymphocytes and cultured fibroblasts

Previous in vivo studies comparing the catabolism of native and chemically-modified LDL suggested that animals with dysfunctional LDL receptors existed within our pig pedigree (11). Ligand blot analysis of immobilized pig liver membrane proteins showed that membrane proteins obtained from an Lpb 5.1 animal bound substantially less β-VLDL than did those from an Lpb5.2 or control (Lpb non-5) pig (**Fig. 1**), suggesting that binding of apoE to the Lpb5.1 pig LDL receptor is impaired. Immunoblots of solubilized membranes probed with a monoclonal antibody to the LDL receptor revealed no decrease in total Lpb 5.1 receptor protein mass as compared to Lpb 5.2 or control animals. An ELISA of solubilized pig liver membrane proteins showed similar levels of LDL receptor protein in Lpb5.1, Lpb5.2, and control pigs (~1.6 ng receptor/μg liver membrane protein).

To assess the functional LDL receptor status in several Lpb5 and control pigs, we mitogen-stimulated isolated peripheral blood lymphocytes to proliferate in the presence of an inhibitor of cholesterol synthesis, mevinolin. Cells expressing functional LDL receptors will proliferate in the presence of LDL and phytohemagglutinin, while those with dysfunctional LDL receptor activity will display a decreased ability to proliferate in response to mitogen stimulation (16). This has been demonstrated in circulating lymphocytes from patients with familial hypercholesterolemia (22) Lymphocytes from 14 pigs were analyzed by this method (**Table 1**). Representative data from an Lpb5.1 and a control pig are shown in **Fig. 2**. In most cases, cells from hypercholesterolemic animals prolifer-

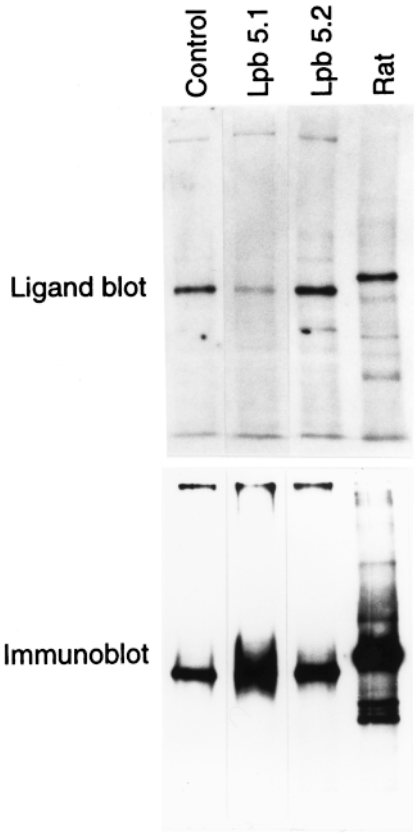

Fig. 1. β-VLDL ligand blot and anti-rat LDL receptor antiserum immunoblot of liver membrane proteins from representative Lpb5.1, Lpb5.2, and control pigs and rat liver membrane proteins. Protein preparations (25 μg protein) were subjected to SDS-PAGE and then blotted onto nitrocellulose. The ligand blot was incubated with 5 μg/ml rabbit β VLDL and probed with anti-apoB monoclonal antibody MB47.

ated less than cells from control animals when exposed to LDL cholesterol levels below 10 μg/ml (Fig. 2). At higher LDL levels, the curves converged, consistent with a defect affecting the affinity for LDL but not the capacity to bind LDL (B_{max}). The data also suggest that the binding defect affects apoB binding in addition to apoE binding. The phenotypes of seven of the animals (designated P* in Table 1) were confirmed through direct binding assays on cultured fibroblasts. The binding parameters are listed in **Table 2**. In two animals, (38-1 and 33-7), the plasma cholesterol phenotype did not correlate with the in vitro assessment of LDL receptor status.

Thirteen pig skin fibroblast cultures were established to measure the equilibrium dissociation constant (K_d) and the maximal binding (B_{max}) of LDL to its receptor in Lpb5.1, Lpb5.2, and control cells. **Figure 3** shows representative data for the binding of LDL to fibroblasts from animals with normal or elevated plasma cholesterol. The mean K_d for LDL binding to the cells from high cholesterol animals was approximately 4-fold higher than normal (22.7 vs. 6.4 μg/ml; Table 2). Maximal binding was not significantly different between the two groups. In addition, binding of ^{125}I-labeled monoclonal antibody (C7)

TABLE 1. Plasma cholesterol, apoB genotype, LDL receptor genotype, and phenotype of pigs

Pig #	Plasma Cholesterol	ApoB Genotype	LDLr Genotype	LDLr Activity	Method
51-1	107	5/5	R/R	+	P
50-9	280	5/5	r/r	−	P
50-6	111	5/5	R/r	+	P
49-9	266	5/5	r/r	−	P*
49-7	225	8/8	r/r	−	P*
42-3	202	5/8	r/r	−	L_2
42-1	230	5/8	r/r	−	L_1
45-1	117	5/5	R/R	+	L_2
45-9	125	5/8	R/R	+	L_2
45-5	121	5/5		+	L_1
47-2	120	4/4	R/r	+	P*
48-2	357	4/4	r/r	−	P
43-5	205	5/4	r/r	+	L_1
44-1	111	4/4	r/r	−	L_2
44-3	246	5/4	R/r	−	L_2
44-2	130	5/4	r/r	+	P
44-5	128	5/4	R/r	+	L_1
38-4	227	4/4	r/r	−	P*
38-2	213	4/4	r/r	−	P*
38-1	112	4/4	R/r	−	P*
35-7	180	5/4	r/r	−	L_1
33-7	123	5/4	R/r	−	P
33-5	197	5/4	r/r	−	P*
51-5	109	non5	R/R	+	P

The table lists animals included in this study whose LDL receptor activity was assayed by either ligand blot (L_1: pig LDL as ligand; L_2: rabbit β-VLDL as ligand), T-cell proliferation (P), or T-cell proliferation and ^{125}I-labeled LDL binding to cultured skin fibroblasts (P*). LDL receptor activity: (+) denotes normal LDL receptor activity; (−) denotes defective receptor activity.

to the LDL receptor plateaued at the same B_{max} in both groups (data not shown). Thus, the defect in the LDL receptor affects binding to the ligand but not transport of the receptor to the cell surface.

The relationship between an animal's K_d for LDL binding to fibroblasts and its plasma cholesterol level stratified into distinct clusters (**Fig. 4**). Four animals, some with apoB mutations, had K_d values below 10 μg/ml and cholesterol levels below 120 mg/dl. Seven animals with K_ds > 20 μg/ml had cholesterol levels spanning a broad range from 160–280 mg/dl. However, two animals with a high K_d did not have an elevated cholesterol (animals 38-1 and 35-1, Table 2). Even though this is a highly inbred pedigree and all the animals are on an identical low-fat diet devoid of cholesterol, there was a wide range in plasma cholesterol values even within groups with functionally defective LDL receptors.

Identification of an LDL receptor mutation and its segregation with the hypercholesterolemia phenotype

We obtained LDL receptor cDNA (by RT-PCR) from Lpb5.1 (#33-5) and control (#51-2 and #51-5) pig fibroblast RNA. The sequence precisely aligns with the five discrete domains that have been described in the LDL receptor from other mammalian species (**Fig. 5**). The amino acid sequence is 84% identical to the human LDL receptor sequence. There is a 13-amino acid segment absent in the pig receptor that corresponds to part of the O-linked sugar domain (amino acids 722–734). This domain is the most poorly conserved domain of the LDL receptor.

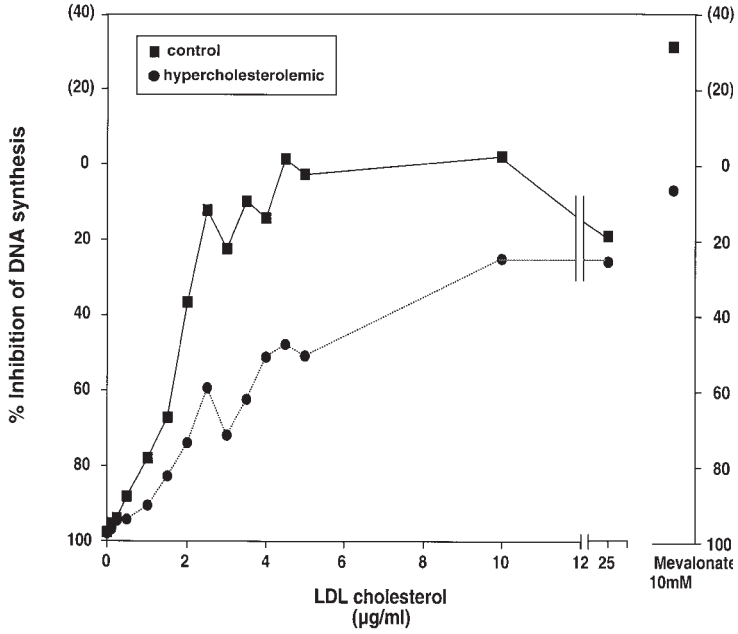

Fig. 2. T-cell proliferation assay for LDL receptor status. T-lymphocytes were incubated in 0.5 µm mevinolin so that LDL was their sole source of cholesterol. T-cell proliferation was quantitated in terms of [^{3}H]thymidine incorporation into DNA. The graph represents degree of rescue by LDL cholesterol from the mevinolin block. Cells receiving 10 mm mevalonate display the maximal growth rate attainable with complete rescue.

The cDNA from cells with a binding defect shows a missense mutation in a single base pair (C→T), leading to an Arg→Cys substitution at the position corresponding to Arg_{84} in the pig LDL receptor (Arg_{94} in the human LDL receptor). This amino acid substitution is located next to an existing cysteine residue in the third repeat of the LDL receptor, Cys_{95}, thus giving rise to two neighboring cysteine residues.

We developed an SSCP assay to allow rapid determination of the LDL receptor genotypes of animals from which we had preserved genomic DNA. The assay readily distinguishes wild type, heterozygous, and homozygous mutant animals. The mutation was traced through two different sub-pedigrees (**Fig. 6**). The SSCP analysis of animals in both sub-pedigrees showed that the LDL receptor mutation is tightly linked to the hypercholesterolemia phenotype, which segregates as a recessive trait.

TABLE 2. Plasma cholesterol and fibroblast LDL receptor binding parameters

Pig #	Plasma Cholesterol B_{max}	ApoB Genotype	LDLR Genotype	K_d	
51-2	110	non5	R/R	6.4	96
49-9	266	5/5	r/r	18.1	70
50-5	107	5/5	R/r	7.2	51
43-2	188	5/4	r/r	22.1	95
49-7	225	8/8	r/r	22.8	188
47-2	120	4/4	R/r	5.7	58
58-2	93	3/4	R/r	8.1	207
38-4	227	4/4	r/r	22.1	190
38-2	213	4/4	r/r	17.9	231
38-1	112	4/4	R/r	19.0	121
35-3	160	5/5	r/r	35.4	132
35-1	94	4/4	R/r	21.7	120
33-5	197	5/4	r/r	30.1	133

Relationship of LDL receptor genotype to receptor binding phenotype

Of the thirteen pig skin fibroblast cultures assayed for direct binding of LDL, seven cell lines displayed a high K_d for LDL (Table 2). All seven were from high-cholesterol animals and were homozygous for the LDL receptor mutation. Three of the animals whose K_d and plasma cholesterol were normal were heterozygous for the LDL receptor mutation (#50-5, #47-2, and #58-2, Table 2). There were two striking anomalies in the relationship between genotype and phenotype. Pig 44-3 had elevated plasma cholesterol level (>240 mg/dl) while it is merely a heterozygote for the LDL receptor mutation (Fig. 6A). The LDL receptor activity was shown to be normal by the T-cell proliferation assay. Pig 44-2 had only a modest elevation in plasma cholesterol while being homozygous for the LDL receptor mutation (Fig. 6A). The LDL receptor activity was shown to be defective by ligand blot analysis.

Relative contributions of apoB and the LDL receptor to hypercholesterolemia

We analyzed more than 200 animals to evaluate the contributions of apoB allele and the LDL receptor allele to hypercholesterolemia. Animals with the normal LDL receptor allele (R/R) and the lpb^5 allele of apoB had higher mean cholesterol levels than non-lpb^5 animals (**Fig. 7**, left panel). The mean total plasma cholesterol levels in $lpb^{non\text{-}5}$, R/R pigs was 81 mg/dl, in heterozygous $lpb^{5/non\text{-}5}$ R/R pigs, 98 mg/dl, and in homozygous $lpb^{5/5}$, R/R animals, 118 mg/dl. These results support the predictions made in our earlier quantitative pedigree analysis, which was based solely on the plasma cholesterol phenotypes and the apoB genotypes (11).

The effect of the LDL receptor Arg→Cys mutation on plasma cholesterol was much greater than that of apoB. Heterozygotes for the receptor mutation (R/r) had mean

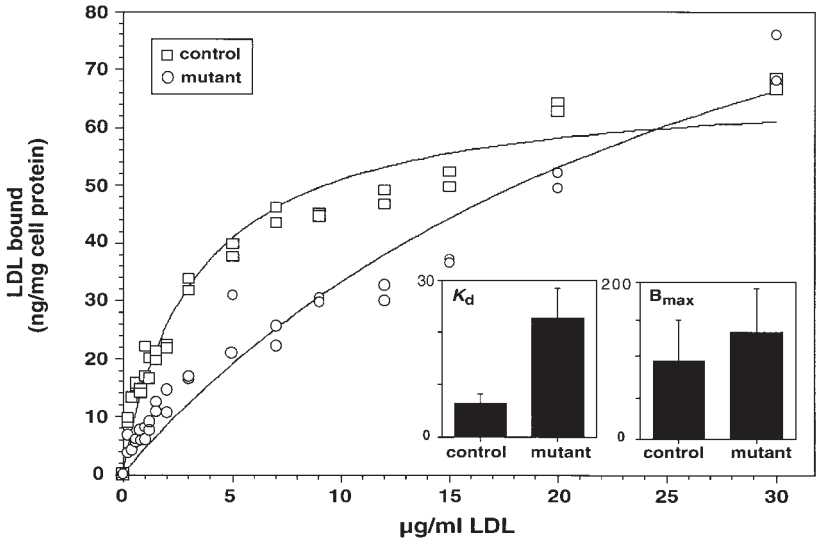

Fig. 3. Binding of LDL to cultured pig skin fibroblasts. Representative binding study showing the binding of normal LDL to fibroblasts from a control (#51-2) and a homozygous mutant pig (#35-3). The binding study was performed at 4°C on cells incubated for 48 h in media containing 10% LPDS. The calculated binding parameters are: control cells: K_d = 8.6 µg/ml LDL, B_{max} = 96.2 ng/mg protein bound; mutant cells: K_d = 35.4 µg/ml LDL; B_{max} = 132 ng/mg protein bound. Inset: Binding parameters for all the animals assayed by direct binding. The bar graphs display the mean and standard deviations from all of the binding experiments. For animals with normal binding affinity (n = 11) the mean K_d = 6.6 ± 1.8, the mean B_{max} = 98.5 ± 50; for animals with reduced binding affinity (n = 10), the mean K_d = 22.72 ± 5.8, the mean B_{max} = 135 ± 56.

cholesterol levels of 122 mg/dl and homozygotes, 237 mg/dl (Fig. 7, middle panel). Among homozygotes for both mutations, there were several animals with plasma cholesterol levels well above those of any other group (Fig. 7, right panel). However, as a group, their cholesterol levels did not differ significantly from animals homozygous for the receptor mutation with normal apoB.

DISCUSSION

The identification of an LDL receptor mutation in our inbred herd of hypercholesterolemic pigs enabled us to study the cholesterol-elevating effects of variations in apoB and the LDL receptor, either alone or in combination with one another. Our data show that the LDL receptor Arg→Cys mutation described here has a much greater effect on plasma cholesterol than the presence of the *lpb*[5] apoB allele. This is in accord with studies in human patients of a much more severe apoB mutation, apoB Arg_{3500}→Gln (23). ApoB carrying this mutation is essentially devoid of LDL receptor binding activity, yet the affected patients are still not as hypercholesterolemic as those with dysfunctional LDL receptors (24).

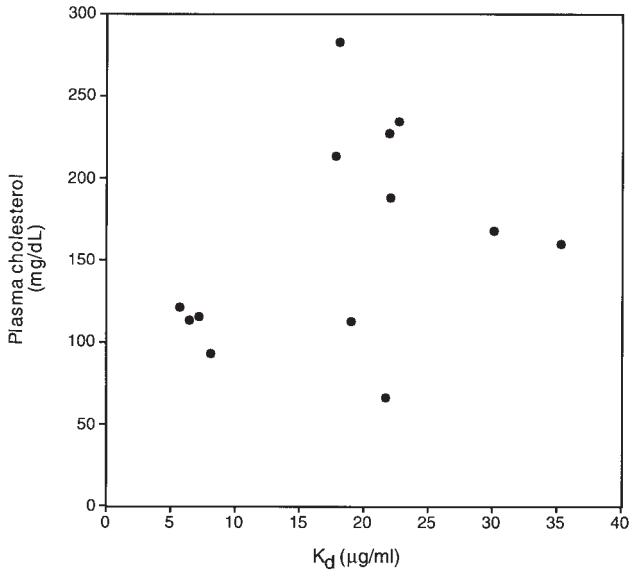

Fig. 4. K_d versus total plasma cholesterol. Each K_d value represents a calculated value from a fibroblast binding experiment, as shown in Fig. 3.

After this manuscript was first submitted for publication, a report was published on the sequence of the LDL receptor from animals obtained from the same kindred as ours (25). The Arg→Cys mutation was the only one found in the entire coding sequence, supporting our conclusion that this mutation is responsible for defective binding of the receptor to its ligands. This report failed to address the major discrepancy between their new results and much of their prior work. During the initial characterization of the Lpb5.1 pig, we addressed the possibility that LDL receptor defects contributed to the severe hypercholesterolemia associated with this animal model (4). Skin fibroblasts were obtained from one normocholesterolemic and one hypercholesterolemic pig and assessed for their ability to degrade normal pig LDL at 37°C. This experiment revealed no difference in LDL degradation between the control and Lpb5.1 pig fibroblasts, a result consistent with normal LDL receptor activity in the hypercholesterolemic pig. The results reported here directly conflict with that conclusion, and we believe this might be due to at least two factors. First, in this report we measured direct binding of LDL to fibroblasts at 4°C, whereas the earlier experiments assessed degradation of LDL at 37°C. Our ligand blot, T-lymphocyte, and direct binding assays all indicate that the Arg→Cys mutation results in an LDL receptor that can bind LDL, albeit with a reduced affinity for lipoproteins when compared to the normal pig LDL receptor. Although we have not assessed the ability of normal versus R/r and r/r pig fibroblasts to degrade LDL in these experiments, it is possible that LDL degradation would not be affected to the same extent as direct binding

Fig. 5. Protein sequence of the pig LDL receptor aligned with the human LDL receptor. The sequence is derived from the cDNA sequence, except for 57 bp of the 5′-end, which was not determined. The position of the R→C substitution in the mutant pigs is indicated. At the carboxy terminus, we did not determine the end of the pig sequence as the sequencing was begun using primers from limited published sequence (19) in this segment. The Genbank Accession number is AF 118147.

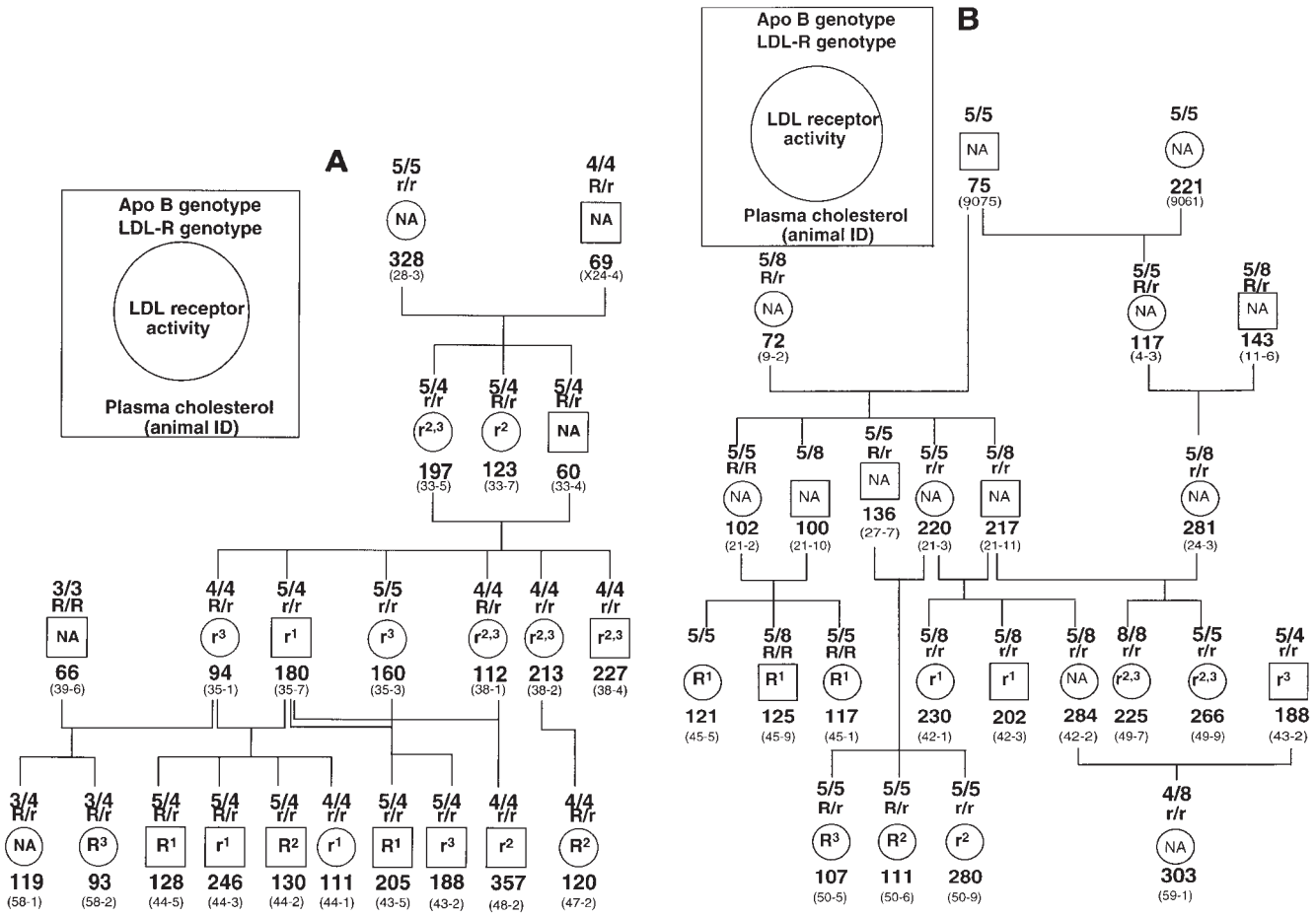

Fig. 6. Pig sub-pedigrees. A: *lpb*$^{4/4}$ sub-pedigree; B: *lpb*$^{8/8}$ sub-pedigree. For each animal, the information given is the pig's apoB genotype and the LDL receptor genotype (R = wild-type allele; r = mutant allele). The information contained within the squares and circles is the LDL receptor phenotype (R = normal binding activity, r = reduced binding activity, NA = not assayed). The superscript following the receptor status symbol indicates the method by which the receptor activity was assayed (1 = ligand blot, 2 = T-cell proliferation, 3 = direct binding). The first line of information below the symbol indicates the total plasma cholesterol (mg/dl). The last line of information is the animal's identification number.

of LDL to the LDL receptor, and in some cases, might appear to be normal. Second, in an attempt to explain the discrepant results between this study and our earlier report, we obtained genomic DNA from fibroblasts preserved from the pigs used in that earlier degradation experiment and determined their LDL receptor genotypes. SSCP analysis of the normocholesterolemic control pig's genomic DNA showed that the animal was heterozygous for the Arg→Cys mutation, although it did not have hypercholesterolemia. The hypercholesterolemic animal was homozygous for the Arg→Cys mutation. Because LDL receptor mutations were presumed not to lead to hypercholesterolemia in heterozygotes, it did not seem possible at that time that animals with normal cholesterol could harbor LDL receptor mutations, as studies in human FH patients indicated a semi-dominant, rather than recessive mode of inheritance. In this study, we measured K_ds for fibroblasts from five R/r heterozygous pigs and found that they ranged from levels typical for r/r cells (e.g., 21.7 μg/ml, pig #35-1) to values found in normal R/R pig fibroblasts (e.g., 5.7 μg/ml, pig #47-2; Table 1). Thus, heterozygosity for the Arg→Cys mutation can be associated with normal or altered LDL binding affinity. In addition, as shown in Fig. 4, altered binding affinity is not always associated with increased plasma cholesterol.

The Arg→Cys missense mutation in the pig LDL receptor partially impairs binding to its ligands, apoB and apoE. The LDL receptor ligand binding domain consists of seven imperfect repeats plus an additional domain with growth factor homology, repeat A (26). Each of the repeats contains six cysteine residues. Repeat 1 is not required for LDL binding, but repeats 2, 3, 5, and 7 plus repeat A are required for LDL binding (27, 28). In studies by Esser and co-workers (27, 28), deletion of repeats 1–3 resulted in a receptor with about 70% loss of LDL binding activity and about 30% loss of binding to β-VLDL, a ligand that binds to the receptor with apoE as the ligand. As deletion of repeat 1 did not impair binding to LDL, it was inferred that repeats 2 and 3 are required for LDL binding (27, 28). Our results provide further evidence of the importance of repeat 3 for LDL binding and also suggest that repeat 3 is also involved in β-VLDL binding.

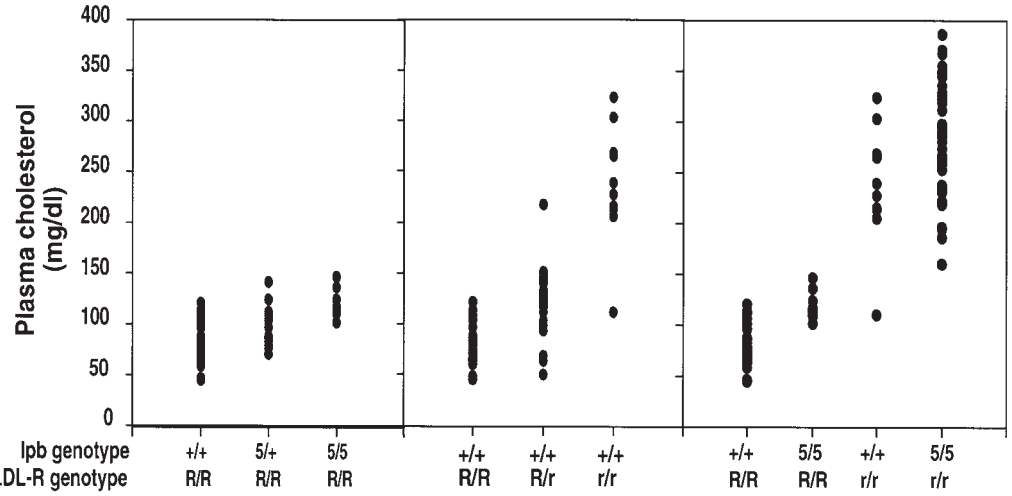

Fig. 7. Plasma cholesterol versus apoB and LDL receptor genotype. Left panel, independent effect of apoB genotype on plasma cholesterol. Middle panel, independent effect of LDL receptor genotype on plasma cholesterol. Right panel, combined effects of apoB and LDL receptor genotypes on plasma cholesterol.

The three-dimensional structure of the LDL receptor has not yet been solved. However, the structure of a recombinant polypeptide corresponding to the first repeat of the ligand binding domain was recently determined by nuclear magnetic resonance spectroscopy (29). The disulfide cross-linking pattern involves three disulfide linkages: Cys_6–Cys_{18}, Cys_{13}–Cys_{31}, and Cys_{25}–Cys_{42}. The mutant pig LDL receptor we describe in this study has two adjacent cysteine residues, Cys_{94} and Cys_{95}. The extra cysteine could very likely cause misfolding of the protein by forming a non-native disulfide bond, which could drastically alter the conformation of the protein. The Arg→Cys mutation in the pig receptor repeat 3 is in the position corresponding to Arg_5 in the first repeat (using the human LDL receptor numbering system). If the disulfide cross-linking pattern in repeat 3 is the same as that of repeat 1, then Cys_{95} would be expected to be cross-linked with Cys_{113}. There are two additional cysteines in the primary sequence between Cys_{95} and Cys_{113}, which could participate in a non-native disulfide linkage with the new cysteine residue at position 94.

To the best of our knowledge, there have been no previous reports of mutations at human Arg_{94} or in the corresponding position of any of the other LDL receptor repeats or of mutations resulting in new cysteine residues in the LDL receptor ligand binding domain (30). In addition, there are no reports of mutations in Cys_{95} or its likely disulfide bonding partner, Cys_{113}. However, mutations in amino acids occupying the corresponding positions in repeat 4 (Cys_{134} and Cys_{152}) have been reported (30). Cys_{134}→Gly and Cys_{152}→Arg each result in partial but not complete loss of LDL receptor binding activity (30).

Arg_{94} is quite well conserved across species; it is found in the rabbit (31), hamster (32), rat (33), and human (26) but not the xenopus LDL receptor, where that position is occupied by threonine (34). The corresponding position in the rabbit VLDL receptor is also occupied by threonine (35). In all ligand binding domain repeats, except repeat 2, the corresponding position is occupied by glutamine, arginine, histidine, or lysine. In repeat 2, it is occupied by serine in all five aforementioned species. Apart from its ability to alter the disulfide bonding pattern, a cysteine at this position contributes a drastic change in polarity, cysteine having approximately the same dipole moment as alanine (36).

The LDL receptor mutation in our pigs is expressed in a recessive fashion. In humans, LDL receptor mutations are expressed in a semi-dominant fashion. In a rabbit model for hypercholesterolemia, the WHHL rabbit, heterozygous animals have only a slight increase in VLDL and IDL and a 2-fold increase in LDL while homozygotes have a 10-fold and 20-fold increase in VLDL/IDL and LDL, respectively (37). These species differences might result from differences in the level of LDL receptor expression relative to the circulating LDL concentration. Both pigs and rabbits have substantially lower LDL levels than humans. In addition, apoE-mediated IDL clearance might be more efficient in pigs and rabbits than in humans and this process might not saturate the LDL receptor even in heterozygous mutant animals. Finally, there might be a compensatory up-regulation of expression of the LDL receptor that results in high levels of wild-type receptor protein in the heterozygous pigs.

The relationship between LDL receptor genotype and plasma cholesterol phenotype revealed several intriguing results. There was one animal that had a nearly normal plasma cholesterol level despite having two mutant alleles of the LDL receptor. Unlike human populations, these animals are on identical low-fat diets, thus the differences in plasma cholesterol between these animals are likely due to additional genetic factors. This phenomenon resembles a compensatory autosomal dominant trait discovered by Hobbs and co-workers in a human kindred (38). In this kindred, one-third of the individuals who inherited a dysfunctional LDL receptor allele did not have hypercholes-

terolemia. In vivo LDL turnover studies revealed that these subjects compensated for the LDL clearance defect by producing LDL at a reduced rate (39).

In this study, four of the animals we studied were homozygous for the wild-type LDL receptor allele, their fibroblasts bound to LDL with normal affinity, and the animals had normal plasma cholesterol levels. Nine animals were heterozygous for the LDL receptor mutation, and of these, eight had low plasma cholesterol. Among the eight normocholesterolemic heterozygotes, three had a decreased affinity for LDL and five had normal affinity. One heterozygous animal had elevated plasma cholesterol and a commensurate decreased binding affinity for the LDL receptor. Fifteen animals were homozygous for the LDL receptor mutation. The ligand blot studies also revealed an animal whose genotype and LDL receptor status did not seem to agree. Pig 43-5 was a homozygous mutant with plasma cholesterol levels in excess of 200 mg/dl. The ligand blot for this animal's liver was indistinguishable from those of normal animals. As ligand blots do not measure binding affinity, it is possible that this result was more a measure of receptor abundance than of receptor affinity. There were two distinct LDL receptor binding phenotypes in LDL receptor heterozygotes. Three cell lines showed normal binding affinity for the LDL receptor and two other cell lines bound LDL with a much lower affinity (Table 2). Clearly, the relationship between plasma cholesterol and receptor binding affinity is not a simple linear function (Fig. 4).

The differences in LDL binding to different cells expressing the same LDL receptor mutation could be due to variability in the ability of the LDL receptor to fold correctly despite the presence of a significant mutation. The contribution of modified protein folding defects to disease phenotype has been studied in cystic fibrosis. Despite the fact that the majority of affected cystic fibrosis patients have inherited the same mutation, a deletion at Phe_{508}, there is a wide spectrum of disease phenotypes (40, 41). The ΔF508 mutation causes misfolding and inefficient transport to the plasma membrane of the cystic fibrosis transmembrane conductance regulator (CFTR) (42). In vitro studies have shown that manipulations of cultured cells from affected patients can partially reverse the misfolding phenotype. For example, culturing the cells at lower temperature (43) or incubating the cells in media containing glycerol (44) promotes normal folding of the CFTR protein. It is therefore plausible that genetic variation in the severity of the ΔF508 mutation is due to the participation of agents within the cell that influence protein folding.

Once all of the major single-gene inherited disorders are characterized, a major challenge will be to understand the genetic and epigenetic factors that modify phenotypes induced by single-gene disorders and to better understand diseases that result from much more complex gene–gene interactions. The fact that this pedigree was not completely inbred illustrates the usefulness of studying well-characterized mutations within outbred populations. Through these studies, one can discover and characterize the basis for phenotypic heterogeneity.

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