Primary structure comparison of the proposed low density lipoprotein (LDL) receptor binding domain of human and pig apolipoprotein B: implications for LDL-receptor interactions

David L. Ebert,[*] Nobuyo Maeda,[†] Scott W. Lowe,[*] Judith Hasler-Rapacz,[†,] Jan Rapacz[†,**] and Alan D. Attie[*,††]**

Departments of Biochemistry,[*] Genetics,[†] Meat and Animal Science,[**] and Comparative Biosciences,[††] University of Wisconsin-Madison, Madison, WI 53706

Abstract Apolipoprotein B (apoB) is the predominant protein in low density lipoprotein (LDL) and is responsible for LDL binding to the LDL receptor. Although the primary amino acid sequence of human apoB has been determined, little is known about the structural domains involved in mediating apoB binding to the LDL receptor. Amino acid sequence comparisons across species lines provide a means of defining structures that are essential for function. We have sequenced a 1.1 kb fragment of pig apoB genomic DNA, corresponding to a 363 amino acid segment proposed to mediate human apoB binding to the LDL receptor. In human apoB this domain contains two regions enriched in positively charged amino acids flanking two disulfide-linked cysteine residues. The pig amino acid sequence shared 72% identity with the human sequence. However, there were differences that have significant structural and functional implications. Human apoB arginine-3,359 corresponds to a critical arginine (position 142) residue in the apoE LDL receptor binding domain. In the pig, this arginine residue was not conserved. Also, the two disulfide-linked cysteine residues found near the proposed apoB binding domain were not conserved in the pig. Despite these differences, pig LDL had a higher affinity than human LDL for both the pig and human LDL receptor. Thus, these features are not required for high affinity binding of pig LDL to the LDL receptor, and may not be necessary for the binding of human LDL to the LDL receptor.—**Ebert, D. L., N. Maeda, S. W. Lowe, J. Hasler-Rapacz, J. Rapacz, and A. D. Attie.** Primary structure comparison of the proposed low density lipoprotein (LDL) receptor binding domain of human and pig apolipoprotein B: implications for LDL-receptor interactions. *J. Lipid Res.* 1988. **29:** 1501–1509.

Supplementary key words apoB genomic DNA • normal human apoE • variant human apoE

Two ligands for the low density lipoprotein (LDL) receptor have been identified: apolipoproteinB-100 (apoB) and apolipoproteinE (apoE). ApoE is associated with very low density lipoproteins (VLDL) and chylomicrons. Shortly after entering the circulation, VLDL and chylomicron triglyceride is lipolyzed, giving rise to remnant lipoproteins. ApoE mediates the binding of these remnant particles to the LDL receptor (1). ApoB is the predominant protein component of LDL and mediates LDL binding to its receptor (2).

The receptor-binding domain of apoE has been characterized. Innerarity and coworkers (3) identified a single peptide fragment (residues 126–218) that, when combined with a phospholipid vesicle, bound the LDL receptor. Modification of the positively charged amino acid residues, arginine and lysine, abolished apoE binding (4,5). Through the use of naturally occurring and in vitro-generated apoE variants, a small amino acid stretch between residues 130 and 160, enriched in positively charged amino acids, was unambiguously shown to be the domain responsible for apoE binding to the LDL receptor (6–10). The sequencing of the LDL receptor cDNA revealed that the ligand-binding domain of the receptor is rich in negatively charged amino acids (11). These observations support a model involving the electrostatic interaction between a positively charged domain in apoE and a negatively charged domain in the LDL receptor.

Unlike apoE, apoB has not been amenable to the identification of domains necessary for binding to the LDL receptor. ApoB has an unusually large molecular mass (550,000 daltons) and, when dissociated from lipid-containing particles, is insoluble in water (12,13). Chemical modification studies have helped to identify the amino acid residues involved in receptor binding. Like apoE, the binding of apoB to the LDL receptor is abolished by the chemical modification of arginine and lysine residues (3,4), suggesting that the apoB

receptor binding domain(s) might also be enriched in lysine and arginine. However, when proteolytic peptides of apoB were individually reconstituted into lipoprotein particles, all interacted with the LDL receptor (14). These studies were therefore unable to confine the receptor-binding domain(s) of apoB to one contiguous peptide fragment.

Recently, the human apoB cDNA sequence and the derived amino acid sequence were determined (15–18). Of particular interest are two regions enriched in positively charged amino acids (residues 3147–3157 and 3359–3367). Monoclonal antibodies that bind near these regions block the interaction of apoB with the LDL receptor (16). Residues 3359–3367 are nearly identical to the LDL receptor binding domain of apoE (19). Additionally, a synthetic peptide corresponding to residues 3345–3381, when recombined with trypsin-inactivated VLDL, reduced 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) activity (20). These observations suggest a role for one or both of these sequences in the binding of LDL to the LDL receptor.

We are currently engaged in research aimed at defining apoB domains that participate in receptor binding. As a first step, we sequenced a 1.1 kb fragment of pig apoB genomic DNA encoding the two clusters of positively charged amino acids suggested to comprise the human apoB receptor-binding domain (16,20). Despite 72% amino acid sequence identity, there were significant differences between human and pig apoB within this domain. In addition to the apoB structural differences, pig LDL had a much higher affinity than human LDL for the LDL receptor.

METHODS

Animals

Genomic DNA and LDL were obtained from pigs whose apoB alleles were identified as described by Rapacz (21). The pigs chosen for this study had apoB alleles, Lpb^2 and Lpb^4, associated with normal cholesterol levels. Human LDL was obtained from an individual with normal cholesterol levels.

ApoB cloning

Complete *Bam* HI digests of genomic DNA from a pig with the genotype $Lpb^{2/3}$ were ligated into a Charon 35 phage vector (22). The DNA was packaged *in vitro* according to the method of Hohn (23). These phage libraries were screened without amplification using a DNA probe specific for the pig apoB gene. This probe, a 500 bp *Xba*I/*Kpn*I fragment, was obtained from the 3′ end of the Lpb^5 gene. The Lpb^5 partial gene clone was identified as a portion of the pig apoB gene using a human apoB probe from the plasmid pB8 (24), kindly provided by Dr. Jan L. Breslow (Rockefeller University, New York, NY). The 10 kb Lpb^2 *Bam*HI fragment recognized by the 500 bp probe can be distinguished from the same fragment from Lpb^3 by a *Hind*III restriction fragment length polymorphism (25). The 1.1 kb *Eco* RI fragment from Lpb^2 was subcloned into M13 mp19 in preparation for sequencing.

DNA sequence analysis

DNA sequencing was carried out using the dideoxy chain termination method (76) with deoxyadenosine 5′-[α-[^{35}S]thiotriphosphate (Amersham) and electrophoresed in 6% acrylamide gels containing 50% (w/v) urea, 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.3. Sixty percent of the sequence was determined from both strands. The entire *Eco* R1 fragment (corresponding to human nucleotides 9,597–10,687) was sequenced at least twice. *E. coli* DNA polymerase I, all nucleotides, and the oligonucleotide M13 primer were purchased from IBI (New Haven, CT). DNA sequences were analyzed using software from the University of Wisconsin Genetics Computer Group (University of Wisconsin Biotechnology Center).

Lipoprotein preparation

Normocholesterolemic human subjects and pigs were fasted overnight. Blood was collected aseptically into either solid EDTA (final concentration, 2 mg/ml) or a phosphate-buffered EDTA cocktail (final concentrations: 8mM sodium and potassium phosphates, 2 mg/ml EDTA, pH 7.4). After low-speed centrifugation to remove red blood cells, the plasma was combined with the following at the indicated final concentrations: 1.0 mM phenylmethylsulfonylfluoride, 20 μg/ml chloramphenicol, 0.05% (w/v) glutathione, 100 units/ml aprotinin. Lipoprotein isolation was carried out immediately.

LDLs were isolated by sequential ultracentrifugation (27) and further purified according to the following procedure. LDLs were layered beneath three volumes of a NaBr solution (ρ 1.019, 1 mM EDTA) and centrifuged in a Beckman SW 41 Ti rotor for 36 hr at 37,000 rpm, 15°C. The ρ<1.019 g/ml fractions were collected from the top of the tube and discarded. The infranatants were adjusted to approximately ρ 1.08 g/ml with NaBr, layered beneath three volumes of a NaBr solution (ρ 1.063, 1 mMEDTA), and centrifuged in a Beckman Ti50.2 rotor for 24 hr at 40,000 rpm, 15° C. LDLs were collected from the top of the tube.

All LDL preparations were delipidated (28), subjected to electrophoresis on 8% SDS-polyacrylamide

gels (29), and stained with Coomassie blue R-250 or silver. Greater than 94% of the protein was apoB-100 and less than 0.5% had a molecular weight equivalent to apoE. All LDL preparations tested negative for bacterial contamination when streaked on agar plates.

LDLs were radioiodinated as described previously (27). The specific radioactivities ranged from 100 to 400 cpm/ng. Relative electrophoretic mobility on agarose gels (Paragon lipoprotein gels, Beckman, Fullerton, CA) was checked before and after iodination. Iodination did not alter LDL mobility. The LDLs were dialyzed against phosphate-buffered saline (PBS) (0.9% (w/v) NaCl, 8 mM sodium and potassium phosphate, pH 7.4, 0.01% (w/v) EDTA) and then passed through a sterile 0.2 μm filter (Gelman, Ann Arbor, MI) and stored at 4°C. LDLs were used within 2 weeks of iodination.

Cell culture

Normal human fibroblasts (GM969A) and normal pig fibroblasts (AG8113) were obtained from the NIA Aging Cell Culture Repository (Camden, NJ). The cultures were maintained on Dulbecco's Modified Eagle's medium (DMEM; GIBCO, Grand Island, NY); 12.5% (v/v) fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin/100 μg/ml streptomycin (P/S, GIBCO) in a 37°C incubator equilibrated with an atmosphere containing 5% CO_2. Between passage numbers 5 and 25, fibroblasts were seeded into 35-mm wells (Falcon). When the cells reached subconfluence, they were washed twice with PBS and the medium was replaced with DMEM containing 5% lipoprotein-deficient serum (LDS). LDS was prepared from human serum by adjusting the serum density to 1.215 g/ml and centrifuging for 48 hr at 40,000 rpm and 15°C in a Beckman Ti 50.2 rotor. The $\rho<1.21$ g/ml fraction was removed from the top of the tube and discarded. The infranatant was dialyzed against PBS. The cholesterol concentration of the infranatant was less than 5 mg/dl. The protein concentration was adjusted to 50 mg/ml by dilution with PBS. Forty-eight hours after the addition of LDS the cells were washed twice with PBS and used as described below.

Competition experiments

Proteolytic degradation of ^{125}I-labeled LDL was determined according to the method of Goldstein, Basu, and Brown (30) except that silver nitrate (1%, final concentration) was used to precipitate free iodide. Radioactivity in the samples was determined in a Packard Model 5001 gamma-counter. Cells were dissolved in 0.2 N NaOH and cell protein was determined by the method of Lowry et al. (31). Cell protein ranged from 30 to 70 μg/dish between experiments.

Binding experiments

Subconfluent human fibroblasts were cooled to 4°C before replacing the medium with chilled medium containing various concentrations of ^{125}I-labeled pig or human LDL in DMEM, 25 mM HEPES, 5 mg/ml bovine serum albumin, pH 7.4. Cells were incubated for 4 hr at 4°C on a rotary shaker (60 rpm). After the incubation period, cells were washed and cell surface-bound LDL was released according to the method of Goldstein et al. (30) using 4 mg/ml dextran sulfate (32). Samples were counted in a Packard gamma counter until a counting error of 1 σ was achieved.

The binding constants were determined using the weighted, nonlinear least squares curve-fitting program, LIGAND (33), as modified by G. A. McPherson (Elsevier-BIOSOFT, Cambridge, UK). The data were fitting using a one-site model: $[B]=((B_{max})\ [L]/K_d+[L]))+N[L]$, where [B] is the concentration of bound ligand, B_{max} is the amount of ligand bound at receptor saturation, [L] is the concentration of free ligand, K_d is the dissociation constant, and [N] is a constant that describes nonspecific (nonsaturable) binding. The method of Munson and Rodbard (33) was used to test for significant differences between the dissociation constants describing pig and human LDL binding to the LDL receptor. First, the binding data were analyzed separately, so that independent values for the dissociation constants for pig and human LDL were obtained. Second, the data were analyzed together such that the pig and human LDL dissociation constants were forced to share the same value. A significant reduction in the goodness of fit using the second procedure indicates that the dissociation constants are different (33). The F-test was used to measure the significance of the difference in pig and human LDL dissociation constants (34).

The binding data were fit to alternative models describing multiple classes of binding sites. Statistical tests were used to determine whether any of these models described the model better than the single-site model (34). A more detailed description of this analysis with regard to LDL binding to the LDL receptor is described elsewhere (35).

RESULTS

Amino acid sequence of pig apoB

A 1,094-base segment of pig apoB genomic DNA, corresponding to human apoB bases 9556 through 10,646, was sequenced. Seventy-two percent of the derived pig apoB amino acid sequence was identical to the human apoB sequence (**Fig. 1**). When the amino

acids were grouped into classes with similar chemical characteristics (i.e., neutral, acidic, basic, hydrophobic, aromatic, and cross-link forming), the similarity was 85%.

The human apoB segment proposed to be the LDL receptor binding domain (13) contains two segments enriched in positively charged amino acids. The corresponding pig segments were conserved in 9 of 11 positions (amino acids 3,147–3,157) and in 7 of 9 positions (amino acids 3,359–3,367). In the first segment, a histidine at position 3,155 in human apoB is replaced with the negatively charged aspartate, and lysine was substituted for arginine at position 3,156. Thus this pig apoB segment has a net positive charge of four, compared to five in the corresponding human apoB segment. The second positively charged segment of both pig and human apoB is very similar to the LDL-receptor binding domain of apoE. In this region there was a substitution of a positively charged amino acid (arginine—3,359 in humans) for a neutral amino acid (serine), as well as a threonine in humans for a methionine in pigs. Thus, the net charge of this region was also reduced by one in pig apoB.

Another difference between the 363 amino acids in this region was the absence in pig apoB of two cysteine residues corresponding to residues 3167 and 3297 in human apoB. In the pig they were replaced by tyrosine and glycine, respectively. However, like the human apoB region between residues 3211 and 3305, the corresponding pig apoB segment was highly enriched in proline residues.

Competition for degradation

Pig LDL was a better competitor for ^{125}I-labeled human LDL degradation in human fibroblasts than human LDL **Fig. 2a**). The concentration of unlabeled pig LDL required to inhibit ^{125}I-labeled human LDL degradation by 50% was 4.7 ± 0.4 μg/ml, compared to 16.1 ± 1.1 μg/ml for human LDL. In similar experiments using pig fibroblasts, unlabeled pig LDL was again a better competitor for degradation than was human LDL (Fig. 2b). When ^{125}I-labeled pig LDL was used instead of labeled human LDL, similar results were obtained (data not shown). Acetyl-LDL is not taken up and degraded by fibroblasts (36) and did not compete, as expected.

```
                .         .         .         .         .
  Pig  EFLKTTKQSFDLSVKAQYKKNKDKHSIPNPLGVFYAFINRNINSFNRHF
       |||||||||||||||||||||||  ||| ||| |   ||   | || |||
Human  EFLKTTKQSFDLSVKAQYKKNKHRHSITNPLAVLCEFISQSIKSFDRHF 3181
                .         .         .         .         .
  Pig ETVRDKALDFFTESYNEIKITFDKYKVEKPLDQQPRTFQIPGYTIPVINI
      |  |  |||| | |||| || ||||| ||  |  |||||||||| || |
Human EKNRNNALDFVTKSYNETKIKFDKYKAEKSHDELPRTFQIPGYTVPVVNV 3231
                .         .         .         .         .
  Pig EVSPFTVKMLTFGYVIPKEISTPNITILGSGISVPSYTLGLQFLELPALD
      ||||||  |  |||| ||  | |   ||||   |||||| |  |||| |
Human EVSPFTIEMSAFGYVFPKAVSMPSFSILGSDVRVPSYTLILPSLELPVLH 3281
                .         .         .         .         .
  Pig VNRPILQISLPELAVSGTSNKILIPAMGNITYDFSFKSSVITLNTNVGLY
      | |  |  |||       |   | ||||||||||||||||||||||  |
Human VPRN.LKLSLPHFKELCTISHIFIPAMGNITYDFSFKSSVITLNTNAELF 3330
                .         .         .         .         .
  Pig NQSNIVAHFLTFSSSVIDALQYKLEGTSSLMRKRGLKLATALSLSNKFME
      |||||||| |  |||||||||||||||  | |||||||||||||||| |
Human NQSDIVAHLLSSSSSVIDALQYKLEGTTRLTRKRGLKLATALSLSNKFVE 3380
                .         .         .         .         .
  Pig GNHDSTISFTKKNVDASLTTTARVQLPILRMNFKQELKGNTKSKPTISSS
      | | || | | ||   |   |     |||||||||| |||||||| |||
Human GSHNSTVSLTTKNMEVSVAKTTKAEIPILRMNFKQELNGNTKSKPTVSSS 3430
                .         .         .         .         .
  Pig IELKYDFNSPKLHSTAAGAVDHKLSLESLTSYFSIEMSTKGDVKGSILSR
       | |||||  | ||| |||||||||||||||||| |||||||| |||
Human MEFKYDFNSSMLYSTAKGAVDHKLSLESLTSYFSIESSTKGDVKGSVLSR 3480
                .
  Pig VYSGSLASEASTYVN
       |||  |||| || |
Human EYSGTIASEANTYLN                                   3495
```

Fig. 1. Comparison of the amino acid sequence of human apoB residues 3133–3495 with pig apoB. The pig sequence was derived from the nucleotide sequence. The human sequence (16) is shown for comparison. Vertical lines signify identity between the human and pig sequences. A gap in the human sequence between residues 3285 and 3286 is represented by a single dot.

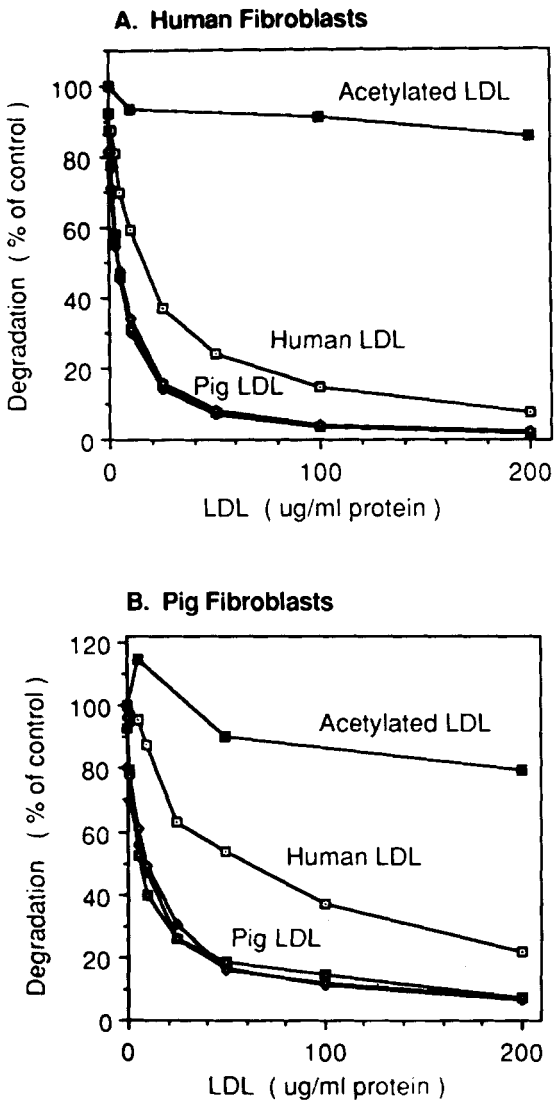

Fig. 2. Competition of unlabeled LDL with ^{125}I-labeled human LDL for degradation in human fibroblasts (A) and pig fibroblasts (B). Human and pig fibroblasts were incubated at 37°C for 5 hr with 10 μg ^{125}I-labeled human LDL protein/ml and the indicated concentrations of unlabeled acetylated human LDL (•), human LDL (□), and pig LDL from three different animals (*Lpb*$^{2/2}$, *Lpb*$^{2/4}$, *Lpb*$^{4/4}$). Proteolytic degradation was determined as described (30). The data shown are from a representative experiment.

Binding of LDL to the LDL receptor

Binding of both pig and human LDL to human fibroblasts is shown in **Fig. 3**. Using the one-site model, the K_d for binding of human LDL to the human LDL receptor was 0.73 ± 0.04, while the K_d for pig LDL was 0.14 ± 0.01. A significant improvement of the fit was obtained using a model describing two classes of binding sites. Pig LDL had a higher affinity for both classes of sites. For simplicity, the data, corrected for nonspecific binding (using a one-site model), are presented graphically in the Scatchard plot shown in the inset to Fig. 3.

DISCUSSION

ApoB is highly polymorphic at both the DNA and protein levels. Several restriction fragment length polymorphisms have been described in the apoB gene (37–39), and a number of allelic immunological variants of the protein have been identified (21,40). Among these is a pig apoB variant that is associated with hypercholesterolemia and premature atherosclerosis (41). LDL taken from animals homozygous for this variant gene was cleared more slowly from the circulation of normal pigs than was normal LDL (27). Furthermore, control pig LDL had a sixfold higher affinity than mutant LDL for pig LDL receptors (35). To date, no changes have been found in the amino acid sequence of the mutant pig apoB that can clearly account for this reduction in affinity for the LDL receptor. Vega and Grundy (42), studying LDL catabolism in vivo in a group of patients with moderately elevated plasma LDL, discovered that 5 of 15 patients cleared their own LDL from the circulation more slowly than LDL from individuals with normal plasma cholesterol. One of these individuals had LDL that competed poorly with normal LDL for binding to the LDL receptor on human fibroblasts (43). These studies provide the first clues that defects in LDL catabolism due to defective LDL, rather than defective LDL receptors, might be a common cause of primary hypercholesterolemia in the human population.

Because of the high degree of polymorphism in apoB, it will be difficult to identify those polymorphisms causally associated with a particular cholesterol phenotype. There is no simple approach currently available to systematically probe functional domains on apoB. LDL is not secreted directly from cells, but is produced in the bloodstream from VLDL through a complex series of lipolytic reactions and lipid transfer processes (44). Thus, unlike many other proteins, it may not be possible to create specific apoB variants and to directly test the function of these variants.

Comparison of apoB sequences and LDL receptor-binding properties from many species provides valuable clues to the importance of particular structures in the binding of LDL to the LDL receptor. This study focused on a portion of pig apoB corresponding to human amino acid residues 3,133 through 3,495. These 363 amino acids include two regions, approximately 200 amino acids apart, enriched in lysine and arginine residues.

In human apoB there is a proline-rich stretch midway between the two regions enriched in positive charge.

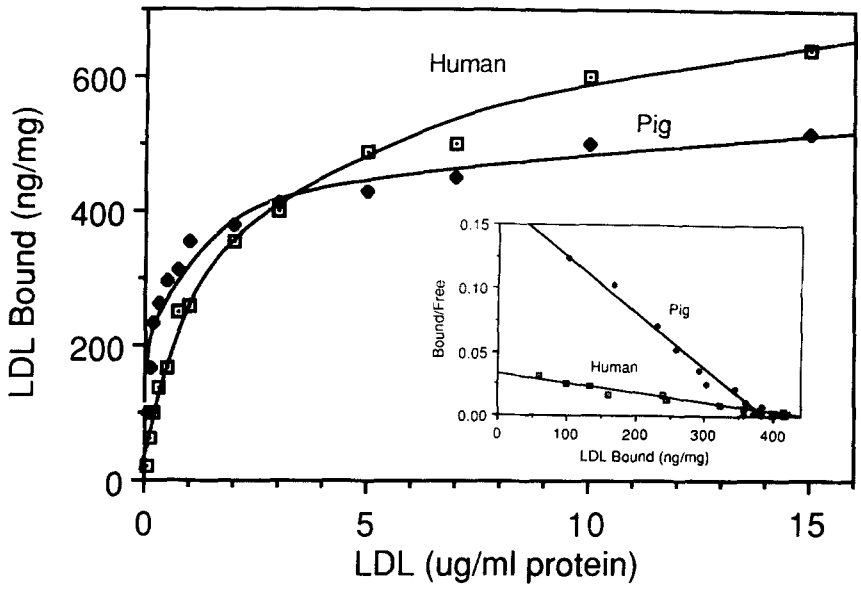

Fig. 3. Concentration-dependent binding of human LDL to human fibroblasts at 4°C. Fibroblast monolayers were incubated for 4 hr at 4°C with either iodinated human LDL (□) or iodinated pig (*Lpb*$^{2/2}$) LDL (♦) at the indicated concentrations. The insert shows a Scatchard plot with the data corrected for nonspecfic binding. Binding parameters were determined as described in Methods. The dissociation constant for mutant LDL was significantly different from normal LDL with $P<0.001$. The data shown are from a representative experiment.

On either side of this proline-rich region and between these two positively charged regions are two cysteine residues that form a disulfide bridge, possibly bringing the two positively charged regions into close proximity (16). Despite an overall sequence identity of 72% between human and pig apoB, we observed a number of differences that may have significant structural and functional implications. Both of the positively charged regions in pig apoB contain one less net positive charge than the corresponding regions in human apoB. The region closer to the amino terminus has five positively charged residues, but also has one negatively charged amino acid. The region closer to the carboxyl terminus and homologous to the apoE binding domain (19) has one less positive charge than human apoB; arg-3359 in human apoB is serine in pig apoB (**Fig. 4**). The presence of cysteine in place of arginine at the corresponding position in apoE from humans with type III hyperlipoproteinemia results in a protein with markedly diminished affinity for the LDL receptor (<20% relative to normal apoE) (6).

Another structural difference is the absence, in pig apoB, of the two disulfide-linked cysteine residues found in human apoB. Cysteines that play an important structural role are usually conserved between proteins with similar function. For example, disulfide-linked cysteines in insulin are not only conserved across species lines (45), but are also conserved in the two homologous proteins, insulin-like growth factors I and II (46). In the immunoglobulin gene family, immunoglobulin molecules are made up of several domains believed to be derived from a common ancestral gene. However, only three residues are invariant among all domains; two are disulfide-linked cysteine residues (47).

Despite the decrease in positive charge and the absence of a disulfide bridge, pig LDL binds with a fivefold higher affinity to human LDL receptors than does human LDL. Furthermore, pig LDL is a better competitor than human LDL for binding to both human and pig fibroblast receptors. Thus, the differences in binding and competition cannot be attributed to species differences between LDL receptors.

Our results suggest certain apoB structures that may have been assumed to play important functional roles in LDL metabolism may not be critical. First, although positively charged amino acids most certainly play a role in the interaction of LDL with the LDL receptor (4,5), not every positively charged amino acid found in the proposed human apoB receptor binding domain was required for high affinity binding of pig LDL to the LDL receptor. Second, the disulfide bridge found in human apoB was not required for high affinity binding of pig LDL to the LDL receptor. We speculate that these structural features may not be required for binding of human LDL to the LDL receptor, since

	Amino Acid Sequence	% Binding
Human apo-B	ArgLeuThrArgLysArgGlyLeuLys	100
Pig apo-B	SerLeuMetArgLysArgGlyLeuLys	520
Normal human apo-E	ArgLysLeuArgLysArgLeuLeuArg	100
Variant human apo-E	CysLysLeuArgLysArgLeuLeuArg	<20

Fig. 4. Comparison of the binding activities of human and pig apoB and of normal and variant human apoE. Part of the apoE receptor-binding domain is shown, along with homologous regions in pig and human apoB. "% Binding" reflects relative association constants of pig and human LDL (Fig. 3) and relative ability of apoE to compete for receptor binding (ref. 6).

functionally important amino acids are usually conserved through evolution. Furthermore, the two positively charged regions may still be in close proximity in pig apoB, perhaps due to the conformational restrictions placed on this domain by the 12 proline residues that lie in a relatively short segment midway between the positively charged domains in both humans and pigs.

Further evidence is needed to conclusively prove that the region of apoB that we have sequenced is actually the LDL receptor binding domain. It may yet be that sequences outside of this domain are required for high affinity binding of LDL to the LDL receptor. In addition to sequence information from apoB variants, further interspecies comparisons will provide valuable clues regarding other important functional domains of apoB.

D.L.E. was supported by postdoctoral fellowship #87-FA-08 from the American Heart Association/Wisconsin Affiliate. A.D.A. is an Established Investigator of the American Heart Association. The work was supported by NHLBI grant HL37251 and grant #2077 from the Council for Tobacco Research. We thank the staff of the University of Wisconsin Veterinary Medicine Teaching Hospital Charmany Instructional Facility for their care of the pigs used in this study, and Dawn Brasaemle for critical review of the manuscript. This is paper number 2967 from the Laboratory of Genetics.

Manuscript received 12 February 1988, in revised form 11 March 1988, and in re-revised form 6 May 1988.

REFERENCES

1. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
2. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
3. Innerarity, T. L., E. J. Friedlander, S. C. Rall, K. H. Weisgraber, and R. W. Mahley. 1983. The receptor-binding domain of human apolipoprotein E. *J. Biol. Chem.* **258:** 12341–12347.
4. Mahley, R. W., T. L. Innerarity, R. E. Pitas, K. H. Weisgraber, J. H. Brown, and E. Gross. 1977. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins. *J. Biol. Chem.* **252:** 7279–7287.
5. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1978. Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *J. Biol. Chem.* **253:** 9053–9062.
6. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.* **257:** 2518–2521.
7. Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, and R. W. Mahley. 1982. Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc. Natl. Acad. Sci. USA.* **79:** 4696–4600.
8. Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, T. P. Bersot, R. W. Mahley, and C. B. Blum. 1983. Identification of a new structural variant of human apolipoprotein E, E2(Lys146 leads to Gln), in a type III hyperlipoproteinemic subject with the E3/2 phenotype. *J. Clin. Invest.* **72:** 1288–1297.
9. Wardell, M. R., S. O. Brennan, E. D. Janus, R. Fraser, and R. W. Carrell. 1987. Apolipoprotein E2-Christchurch (136Arg→Ser) new variant of human apolipoprotein E in a patient with type III hyperlipoproteinemia. *J. Clin. Invest.* **80:** 483–490.
10. Lalazar, A., K. H. Weisgraber, S. C. Rall, Jr., H. Giladi, T. L. Innerarity, A. Z. Ceranon, J. K. Boyles, B. Amit, M. Gorecki, R. W. Mahley, and T. Vogel. 1988. Site specific mutagenesis of human apolipoprotein E. Receptor binding activity of variants with single amino acid substitutions. *J. Biol. Chem.* **263:** 3542–3545.
11. Yamamoto, T., C. G. Davis, M. S. Brown, W. J. Schneider, M. L. Casey, J. L. Goldstein, and D. W. Russell. 1984. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell.* **39:** 27–38.
12. Kane, J. P. 1983. Apolipoprotein B: structural and metabolic heterogeneity. *Annu. Rev. Physiol.* **45:** 637–650.

13. Cardin, A. D., K. R. Witt, C. L. Barnhart, and R. L. Jackson. 1982. Sulfhydryl chemistry and solubility properties of human plasma apolipoprotein B. *Biochemistry.* **21:** 4503–4511.
14. Corsini, A., C. H. Spilman, T. L. Innerarity, K. S. Arnold, S. C. Rall, Jr., J. K. Boyles, and R. W. Mahley. 1987. Receptor binding activity of lipid recombinants of apolipoprotein B-100 thrombolytic fragments. *J. Lipid. Res.* **28:** 1410–1423.
15. Chen, S-H., C-Y. Yang, P-F. Chen, D. Setzer, M. Tanimurea, W-H. Li, A.M. Gotto, and L. Chan. 1986. The complete cDNA and amino acid sequence of human apolipoprotein B-100. *J. Biol. Chem.* **261:** 12918–12921.
16. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
17. Cladaras, C., M. Hadzopoulou-Cladaras, R. T. Nolte, D. Atkinson, and V. I. Zannis. 1986. The complete sequence and structural analysis of human apolipoprotein B-100: relationship between apoB-100 and apoB-48 forms. *EMBO J.* **5:** 3495–3507.
18. Law, S. W., S. M. Grant, K. Higuchi, A. Hospattankar, K. Lackner, N. Lee, and H. B. Brewer, Jr. 1986. Human liver apolipoprotein B-100 cDNA: complete nucleic acid and derived amino acid sequence. *Proc. Natl. Acad. Sci. USA.* **83:** 8142–8146.
19. Knott, T. J., S. C. Rall, Jr., T. L. Innerarity, S. F. Jacobson, M. S. Urdea, B. Levy-Wilson, L. M. Powell, R. J. Pease, R. Eddy, H. Nakai, M. Byers, L. M. Priestly, E. Robertson, L. B. Rall, C. Betsholtz, T. B. Shows, R. W. Mahley, and J. Scott. 1985. Human apolipoprotein B: structure of carboxyl-terminal domains, sites of gene expression, and chromosomal localization. *Science.* **230:** 37–43.
20. Yang, C-Y., S-H. Chen, S. H. Gianturco, W. A. Bradley, W-H. Li, D. A. Sparrow, H. DeLoof, M. Rosseneu, F-S. Lee, Z-W. Gu, A.M. Gotto, Jr., and L. Chan. 1986. Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature.* **323:** 738–742.
21. Rapacz, J. 1978. Lipoprotein immunogenetics and atherosclerosis. *Am. J. Med. Genet.* **1:** 377–405.
22. Loenen, A. M., and F. R. Blattner. 1983. Lambda charon vectors (CH32, 33, 34, and 35) adapted for DNA cloning in recombination-deficient hosts. *Gene.* **26:** 171–179.
23. Hohn, B. 1979. In vitro packaging of lambda and cosmid DNA. *Methods Enzymol.* **68:** 299–309.
24. Huang, L-S., S. C. Bock, S. I. Feinstein, and J. L. Breslow, 1985. Human apolipoprotein B cDNA clone isolation and demonstration that liver apolipoprotein B mRNA is 22 kilobases in length. *Proc. Natl. Acad. Sci. USA.* **82:** 6825–6829.
25. Maeda, N., D. L. Ebert, T. M. Doers, M. Newman, J. Hasler-Rapacz, A. D. Attie, J. Rapacz, and O. Smithies. 1988. Molecular genetics of the apolipoprotein B gene in pigs in relation to atherosclerosis. *Gene.* **70:** 213–229.
26. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
27. Checovich, W. J., W. L. Fitch, R. M. Krauss, M. P. Smith, J. Rapacz, C. L. Smith, and A. D. Attie. 1987. Defective catabolism and abnormal composition of low density lipoproteins from mutant pigs with hypercholesterolemia. *Biochemistry.* **27:** 1934–1941.
28. Utermann, G., K. H. Weisgraber, W. Weber, and R. W. Mahley. 1984. Genetic polymorphism of apolipoprotein E: a variant form of apolipoprotein E2 distinguished by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Lipid Res.* **25:** 378–382.
29. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
30. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–260.
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. L. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
32. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. *Cell.* **7:** 85–95.
33. Munson, P. J., and D. Rodbard. 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107:** 220–239.
34. Motulsky, H. J., and L. A. Ransnas. 1987. Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J.* **1:** 365–374.
35. Lowe, S. W., W. J. Checovich, J. Rapacz, and A. D. Attie. 1988. Defective receptor binding of low density lipoprotein from pigs possessing mutant apolipoprotein B alleles. *J. Biol. Chem.* **263:** 15467–15473.
36. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
37. Berg, K., L. M. Powell, S. C. Wallis, R. Pease, T. J. Knott, and J. Scott. 1986. Genetic linkage between the antigenic group (Ag) variation and the apolipoprotein B gene: assignment of the Ag locus. *Proc. Natl. Acad. Sci. USA.* **83:** 7367–7370.
38. Hegele, R. A., L. S. Huang, P. N. Herbert, C. B. Blum, J. E. Buring, C. H. Hennekens, and J. L. Breslow. 1986. Apolipoprotein B–gene DNA polymorphisms associated with myocardial infarction. *N. Engl. J. Med.* **315:** 1509–1515.
39. Law, A., L. M. Powell, H. Brunt, T. J. Knott, D. G. Altman, J. Rajput, S. C. Wallis, R. J. Pease, L. M. Priestly, J. Scott, G. J. Miller, and N. E. Miller. 1986. Common DNA polymorphism within coding sequence of apolipoprotein B gene associated with altered lipid levels. Lancet. **1:** 1301–1303.
40. Young, S. G., S. J. Bertics, L. K. Curtiss, D. C. Casal, and J. L. Witztum. 1986. Monoclonal antibody MB19 detects genetic polymorphism in human apolipoprotein B. *Proc. Natl. Acad. Sci. USA.* **83:** 1101–1105.
41. Rapacz, J., J. Hasler-Rapacz, K. M. Taylor, W. J. Checovich, and A. D. Attie. 1986. Lipoprotein mutations in pigs are associated with elevated plasma cholesterol and atherosclerosis. *Science.* **234:** 1573–1577.
42. Vega, G. L., and S. M. Grundy. 1986. In vivo evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. *J. Clin. Invest.* **78:** 1410–1414.

43. Innerarity, T. L., K. H. Weisgraber, K. S. Arnold, R. W. Mahley, R. M. Krauss, G. L. Vega, and S. M. Grundy. 1987. Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding. *Proc. Natl. Acad. Sci. USA.* **84:** 6919–6923.
44. Tall, A. R., D. Sammett, G. M. Vita, R. Deckelbaum, and T. Olivecrona. 1984. Lipoprotein lipase enhances the cholesteryl ester transfer protein-mediated transfer of cholesteryl esters from high density lipoproteins to very low density lipoproteins. *J. Biol. Chem.* **259:** 9587–9594.
45. Gammeltoft, S. 1984. Insulin receptors—binding kinetics and structure–function relationship to insulin. *Physiol. Rev.* **64:** 1321–1378.
46. Rinderknecht, E., and R. E. Humbel. 1978. Primary structure of human insulin-like growth factor. *FEBS Lett.* **89:** 283–286.
47. Lesk, A. M., and C. Chothia. 1982. Evolution of proteins formed by β-sheets. II. The core of the immunoglobulin domains. *J. Mol. Biol.* **160:** 325–342.