Pig Apolipoprotein R: A New Member of the Short Consensus Repeat Family of Proteins[†,‡]

Scott T. Cooper and Alan D. Attie*

Departments of Biochemistry and Comparative Biosciences, University of Wisconsin—Madison, Madison, Wisconsin 53706-1569

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ABSTRACT: Apolipoprotein R (apoR) is a 23-kDa protein found on very low-density lipoprotein (VLDL), on chylomicrons, and in the $d > 1.21$ g/mL fraction of pig plasma. The plasma concentration of apoR is 5.1 μg/mL, with 11.5% of apoR found on VLDL. In vitro, apoR can transfer from the $d > 1.21$ g/mL infranatant onto artificial lipid emulsions or human chylomicrons but not onto human VLDL. An apoR cDNA was isolated from a pig liver λgt11 expression library. DNA sequence analysis of the apoR cDNA revealed 67% identity with the 3′-terminal region of human C4b-binding protein α-chain cDNA (C4BPα). C4BPα is a 70-kDa glycoprotein that regulates both the coagulation and the complement cascades. In plasma, C4BPα exists as disulfide-linked multimers consisting of seven C4BPα chains and a single C4BPβ chain. Like C4BP, apoR forms high molecular weight disulfide-linked complexes in plasma. However, unlike C4BPα, apoR complexes do not appear to contain C4BPβ. ApoR mRNA was detected in pig liver, spleen, lung, bone marrow, and lymph node, but was absent in intestine and white blood cells. This distribution is consistent with the production of apoR in terminally differentiated macrophages but not in blood monocytes. ApoR mRNA was not detected in RNA isolated from human liver or lung. ApoR may be a lipoprotein-borne regulator of either the coagulation or the complement cascades.

Apolipoproteins are essential for the biogenesis and metabolism of lipoprotein particles. Lipoproteins are complexes of lipid and apolipoproteins which transport fats through the bloodstream. In humans, lipoproteins termed chylomicrons emerge from the intestine carrying apolipoproteins B-48 (apoB-48),[1] apoA-I, and apo-IV (Bisgaier & Glickman, 1983) while the liver secretes apoB-100, -E, -CII, and -CIII on nascent very low-density lipoprotein (VLDL) (Wu & Windmueller, 1979).

Most apolipoproteins have several isoforms. Cross-immunization experiments by Rapacz generated allo-antibodies that were used to identify apolipoprotein isoforms in pigs (Rapacz et al., 1978). One series of cross-immunization experiments generated allo-antibodies that reacted with a 23-kDa protein (named apoR) which comprises a major proportion of pig VLDL protein (Rapacz et al., 1986b). Two isoforms of apoR were identified and termed apoR-1 and apoR-2. They have similar molecular weights but differ in isoelectric point (Rapacz et al., 1986b). ApoR-2 is the common isoform, with a gene frequency of 0.9 in Chester White pigs (Rapacz et al., 1986a). In the present work, we present primary structural data demonstrating that apoR is a member of the family of proteins that contain short consensus repeats (SCR). Of these SCR-containing proteins, apoR has the most sequence identity with C4b-binding protein (C4BPα).

The SCR motif consists of 55 amino acids with 4 conserved cysteine residues (Hessing, 1991). The cysteines form intradomain disulfide bonds in a 1–3, 2–4 arrangement (Janatova et al., 1989). Many proteins with SCR repeats bind complement components C3b or C4b (Kristensen et al., 1987a). The site at which C3b or C4b attaches to a membrane determines where a membrane attack complex will form, and thus which cells will be lysed. C3b/C4b-binding proteins can either circulate in the blood or reside on the extracellular surface of host tissues. Once C3b or C4b is bound by a C3b/C4b-binding protein, it is degraded by specific proteases. This localizes the complement reaction to the surface of foreign cells, thereby protecting autologous cells and tissues (Hessing, 1991).

C4BP is a circulating complex that consists of disulfide-linked C4BPα (70 kDa) and C4BPβ (45 kDa) chains. The C4BPα chain contains eight SCR domains and binds C4b, allowing it to be degraded by a specific protease. C4BPβ has three SCR domains and binds protein S of the coagulation cascade (Dahlbäck & Stenflo, 1981). Protein S is a vitamin K-dependent cofactor for activated protein C. Activated protein C degrades factor Va and factor VIIIa, thereby blocking thrombin formation. Thus, C4BP can regulate both the coagulation and the complement cascades.

We have sequenced the cDNA encoding apoR and found 67% sequence identity with a segment of C4BPα. In the current work, we describe similarities between apoR and C4BPα in both primary structure and biochemical characteristics.

EXPERIMENTAL PROCEDURES

Animals Used in This Study. New Zealand White rabbits were used for production of antisera. Tissue samples were collected from Chester White pigs between the ages of 3 and 12 months. For purification of apoR, 500-mL blood samples were collected from adult pigs.

Immunoprecipitation of Labeled ApoR from Tissue Homogenates. Incorporation of [^{3}H]leucine (NEN) into apoR,

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[‡] The nucleotide and protein sequences reported in this paper have been submitted to GenBank under Accession Numbers L06820 and J50773, respectively.

* Address correspondence to this author at the Department of Biochemistry, University of Wisconsin—Madison.

[1] Abbreviations: VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; apoR, apolipoprotein R; C4BP, C4b-binding protein; C4BPα, C4b-binding protein α-chain; C4BPβ, C4b-binding protein β-chain; SCR, short consensus repeat; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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apoA-I, and apoA-IV by liver slices and intestinal loops was measured. Tissue samples were prepared and incubated with [^{3}H]leucine as reported by Black and Davidson (1989) with the modification that leucine-free DMEM, prepared using the Select-Amine kit (GIBCO), was used instead of Krebs buffer in the liver slice incubations. The liver slices were incubated with [^{3}-H]leucine for 90 min, and the intestinal segments for 9 min, before homogenization. Homogenates were precleared with 50 μL of 20% protein A–Sepharose for 2 h at room temperature and immunoprecipitated with specific antibodies to apoR, apoA-I, and apoA-IV. Antisera was incubated with homogenates. Then, protein A–Sepharose was added, and the beads were collected by centrifugation and washed 3 times. The immunoprecipitates were solubilized in SDS sample buffer and then subjected to SDS–PAGE on 15% gels. The gels were sliced into 2-mm slices and incubated in 0.3 mL of 6% NH_4OH/30%H_2O_2 overnight at 65 °C to solubilize the gel and protein. Hionic fluor (Packard) was added, and radioactivity was determined using a Beckman 100LS scintillation counter.

Lipoprotein Isolation and Polyacrylamide Gel Electrophoresis. VLDL was isolated from plasma as previously described (Checovich et al., 1988). Protein concentrations were determined by the method of Lowry, as modified for use with lipoproteins (Markwell et al., 1978). The delipidated VLDL samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970; Hunkapiller et al., 1983). For protein sequence determination, proteins were transferred to a poly(vinyl difluoride) membrane (Millipore) in 10 mM CAPS/10% methanol, pH 11, for 0.05 A-h. The membranes were stained with Coomassie R-250, and the 23-kDa band corresponding to apoR was excised. Automated protein sequencing was performed using a gas-phase sequencer by the University of Wisconsin—Madison Biotechnology Center.

ApoR Immunodetection. Polyclonal antisera were raised in a rabbit after inoculation with 100 μg of SDS–PAGE-purified apoR in Freund's complete adjuvant (Hager & Burgess, 1980). The animal was boosted 4 weeks later with 100 μg of pure apoR in Freund's incomplete adjuvant.

For immunoblots, samples were subjected to SDS–PAGE and transferred to nitrocellulose by electroblotting. The membrane was incubated with anti-apoR antisera in Tris-buffered saline containing 0.05% (v/v) Tween-20 and 2% (w/v) nonfat dry milk (TBST). The membrane was then incubated with secondary antisera, linked to alkaline phosphatase. Specific bands were detected following incubation with Nitroblue tetrazolium and bromochloroindolyl phosphate (Sigma).

ApoR concentrations were determined using a competitive enzyme-linked immunosorbent assay (ELISA) in which 0.5 μg of pig VLDL protein was adsorbed to each well of a 96-well microtiter plate (Becton-Dickinson). Equal volumes of sample and a 1:200 dilution of anti-apoR antibody in TBST were added to each well and incubated at room temperature overnight (Young et al., 1986). A VLDL sample of known apoR concentration, as determined by quantitative scanning densitometry of an SDS gel, was used as a standard. Alkaline phosphatase-conjugated secondary antibody was added for 2 h, and enzyme activity was measured with 1 mg/mL *p*-nitrophenyl phosphate (Sigma).

Cloning Strategy and DNA Sequence Analysis. A λgt11 cDNA library prepared from pig liver was kindly provided by Dr. Dennis Black, University of Chicago. Approximately 600 000 plaques were screened at a density of 30 000 plaques per 150-mm dish. Nitrocellulose filters were lifted from each dish and immunoreactive plaques detected as described above for immunoblotting. Single clones were isolated by 2–3 rounds of plaque purification.

Phage DNA was prepared from positive clones by liquid culture lysis followed by poly(ethylene glycol) precipitation and phenol extraction of the phage coat proteins. The cDNA insert was subcloned into pBluescript SK$^+$ (Stratagene) following digestion of the phage DNA with *Kpn*I and *Sac*I endonucleases. Sequencing was performed using primers homologous to the flanking λgt11 sequence (Sanger et al., 1977). The sequencing strategy is outlined in Figure 2. Each sequencing reaction was performed at least twice. DNA sequences were analyzed using the computer program DNA Strider on a Macintosh LC computer. Homology searches were performed using the GenBank sequence library (Pearson & Lipman, 1988). The entire amino acid sequences were aligned using the programs GAP and LINEUP from the University of Wisconsin Genetics Computer Group package on a VAX 4000 (Devereux et al., 1984). A "gap weight" of 3.0 and a "gap length weight" of 0.1 were used.

Two partial cDNA clones, pR6 and pR11, were isolated. To obtain the sequence downstream from these clones, cDNA was made from pig spleen total RNA. Briefly, 5 μg of total RNA was incubated with 200 units of reverse transcriptase (BRL), 0.5 mM each deoxynucleotide, and 500 ng of $(dT)_{12-18}$ for 1 h at 37 °C. This cDNA was then PCR-amplified at an annealing temperature of 45 °C, using a synthetic primer homologous to the pR6 sequence (CCCTCAATGTAAAGC; Figure 2) and an oligo(dT) primer with a flanking *Xba*I linker (Jain et al., 1992). The presence of a *Hin*dIII site immediately 3′ of the primer homologous to pR6 sequence allowed the PCR products to be digested with *Hin*dIII and *Xba*I and cloned into pBluescript SK$^+$. Clones containing the correct insert were detected by colony hybridization using the PCR-amplified sequence from pR6 as a probe. DNA probes were labeled with [α-^{32}P]dCTP (3000 Ci/nmol, New England Biolabs) by the random priming technique (Feinberg & Vogelstein, 1983). Positive clones were confirmed by diagnostic restriction digests using *Hin*dIII and *Rsa*I. Two clones, pR12 and pR19, had 200 bp of overlapping sequence with pR6 (Figure 2). Both clones obtained by PCR amplification were sequenced and shown to be identical.

RNA Hybridizations. Human lung RNA was isolated from frozen tissue obtained through the Cooperative Human Tissue Network, Ohio State University. Human liver RNA was a gift of Dr. John Trawick, University of Colorado. Pig RNAs were isolated from fresh tissues. Tissue samples were homogenized in buffer containing 1% β-mercaptoethanol and 4 M guanidinium thiocyanate, followed by ultracentrifugation over 5.7 M CsCl. The RNA was then subjected to electrophoresis in a formaldehyde gel and transferred to charged nylon. The PCR product from pR6 was used as a probe as described above. The filters were probed overnight at 65 °C in hybridization buffer (Reed & Mann, 1985), washed in 0.04 M NaCl/1% SDS at 65 °C, and subjected to autoradiography. Blots were stripped by repeated washings in boiling 0.04 M NaCl/1% SDS for reprobing.

Lipid-Binding Assay. One-milliliter aliquots of pig infranatant were incubated with each of the following: 0.5 mg of human VLDL, 0.5 mg of human chylomicrons, or 0.5 mL of intralipid (KabiVitrum) for 1 h at 25 °C. The lipoproteins and Intralipid were reisolated as described below. Human plasma chylomicrons were obtained from three volunteers after ingestion of a fatty meal (>80 g of fat). VLDL was obtained

from the same subjects after an overnight fast. Intralipid, an artificial lipid emulsion, was used (Weinberg & Scanu, 1983) to identify any general lipid-binding properties of apoR. Chylomicrons and Intralipid were purified by ultracentrifugation for 35 min at 50 000 rpm in an SW 50.1 rotor at 4 °C. Following ultracentrifugation, all preparations were subjected to gel filtration chromatography on Sepharose 2B in PBS (Imaizumi et al., 1978). Only trace levels of albumin could be detected by Coomassie R-250 stain, demonstrating the removal of loosely-associated proteins. Following incubation with pig infranatant as a source of apoR and reisolation, each sample was delipidated, subjected to SDS–PAGE, and immunoblotted to detect apoR.

Two-Dimensional Nonreducing Gel Electrophoresis. Pig chylomicrons were collected as described above and delipidated. The delipidated protein was dissolved in SDS sample buffer without β-mercaptoethanol. Following SDS–PAGE on a 6% gel, the sample lanes were excised and incubated with sample buffer containing 10% β-mercaptoethanol. The excised lanes from the nonreducing gel were layered over a 12% gel and subjected to electrophoresis. Duplicates gels were stained with Coomassie R-250.

Barium Citrate Precipitation of Plasma. Proteins bound to protein S were precipitated as described by Dahlbäck for the purification of C4BP (Dahlbäck, 1983). Briefly, blood was collected in sterile 3.2% citrate and plasma isolated by centrifugation. Barium chloride was added dropwise to the plasma to a final concentration of 80 mM, and the resulting precipitate was collected by centrifugation. The pellet was redissolved in 0.2 M EDTA and dialyzed overnight. The barium chloride precipitate and the starting plasma were then subjected to SDS–PAGE and immunoblotted with apoR antisera.

RESULTS

Molecular Isolation and Sequence Analysis of an ApoR cDNA. Liver and intestine are responsible for the majority of apolipoprotein biosynthesis. Before a library was screened, the site of apoR synthesis had to be determined. [^{3}H]Leucine was used to label newly-synthesized proteins in both liver slices and intestinal loops. ^{3}H-Labeled apoR was immunoprecipitated from liver but not intestinal cytosolic supernatants (Figure 1). To obtain cDNA encoding apoR, a pig liver λgt11 cDNA library was screened with apoR antisera. Two immunoreactive plaques, pR6 and pR11, were identified and plaque-purified. Subsequent sequence analysis showed that the clones were identical, but incomplete at the 3′ terminus because no polyadenylation site was present. To obtain an overlapping clone containing the 3′ end of the gene, spleen cDNA was PCR-amplified using a specific primer complementary to a segment of pR6 and an oligo(dT) primer. Two clones, pR12 and pR19, were obtained containing a 200 bp overlap with pR6 and pR11 and encoding the carboxy-terminal segment of apoR (Figure 2).

To confirm that the clones obtained from the expression library encoded apoR, the amino-terminal protein sequence was obtained from apoR purified from pig VLDL, yielding 19 amino acids of sequence. There was alignment between the deduced amino acid sequence from the cDNA sequence and the amino-terminal sequence for the first 15 residues (Figure 3). The second amino acid from the amino terminus could not be resolved, but is predicted to be a cysteine from the cDNA sequence.

The apoR genotype of the pig from which the library was produced is not known. Using the program ISOELECTRIC

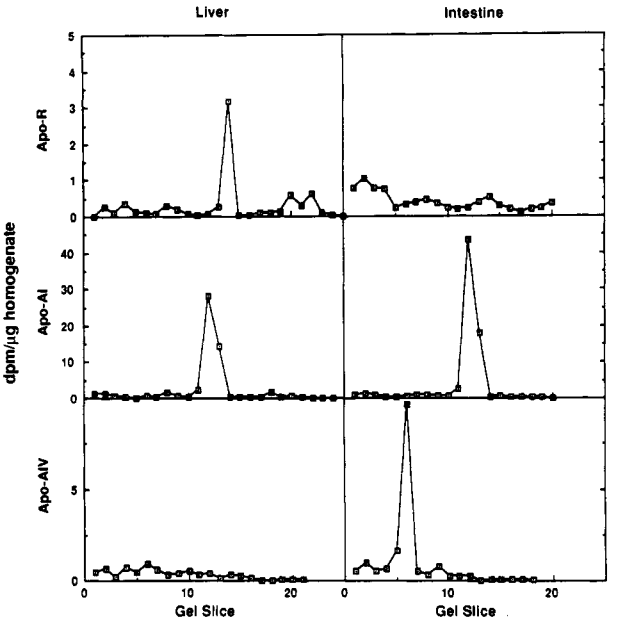

FIGURE 1: Apolipoprotein synthesis by liver slices and intestinal loops. Tissue slices were incubated with [^{3}H]leucine for 90 min (liver slices) and 9 min (intestinal loops), homogenized, and immunoprecipitated with specific antibodies. The immunoprecipitates were subjected to SDS–PAGE, and the radioactivity in the gel segments was determined by scintillation counting.

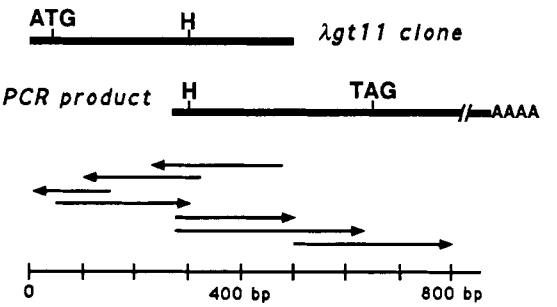

FIGURE 2: Sequencing strategy for apoR cDNA clones. The top diagram represents the cDNA obtained by screening a pig liver λgt11 library with an apoR-specific antibody (clones pR6 and pR11). The bottom diagram represents pig spleen cDNA amplified by the polymerase chain reaction (PCR) using a primer homologous to a segment of pR6, just 5′ of the *Hin*dIII site (H), and a second primer, oligo(dT) with a flanking *Xba*I linker (clones pR12 and pR19). The sites of translation initiation (ATG) and termination (TAG) are indicated. The arrows indicate the direction and extent of sequencing. All four clones were sequenced, and each sequencing reaction was performed at least twice.

from GCG (Devereux et al., 1984), the isoelectric point of the protein encoded by the apoR cDNA sequence was calculated. The calculated p*I* was 5.7, closest to the p*I* of the apoR-2 isoform (Table I). ApoR-2 is also more common than apoR-1, with a gene frequency of 0.9 in Chester White pigs and 1.0 in two other breeds (Rapacz et al., 1986). The cDNA sequence is most likely of the apoR-2 isoform. The discrepancies at amino acids 17 and 19 might have arisen from allelic differences between the protein and DNA sequences. They also might be due to protein sequencing errors.

ApoR has 67% identity with the cDNA sequence of human C4BPα. Pig apoR and human C4BPα have 56% identity and 75% homology in protein sequence (Figure 4). C4BPα is a member of a family of proteins containing a motif known as a short consensus repeat (SCR). Each SCR consists of a 55 amino acid consensus with 4 interdomain disulfide bonds. C4BPα contains 8 of these SCR repeats and has a small, 58 amino acid, carboxy-terminal tail. The protein sequence of

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-12 GGACTCTGCACCATGCCTCCCAATTTACAGAGAATTTTCCCAGCACTGTGCCTC 42
                M  P  P  N  L  Q  R  I  F  P  A  L  C  L

 43 CTTGGGGTCCTGTTTCTCCTGCACTGCACACCTGTCCTGTGTGGTTGCGATAAT 96
    L  G  V  L  F  L  L  H  C  T  P  V  L  C  G  C  D  N
                                                G  X  D  N

 97 CCTCCTGTGGTTGCCCATGGACATCATACACAAATTATTGGGCTATTTGGAATG 150
    P  P  V  V  A  H  G  H  H  T  Q  I  I  G  L  F  G  M
    P  P  V  V  A  H  G  H  H  T  Q  V  I  A  L

151 AAAAAAGATGAGGTTGTATATAAATGTGATGAAGGATACACTCTGGTTGGAGAG 204
    K  K  D  E  V  V  Y  K  C  D  E  G  Y  T  L  V  G  E

205 GACAGACTCTCCTGTCGTTCTTCACGCTGGTCACCTGCAGCCCCTCAATGTAAA 258
    D  R  L  S  C  R  S  S  R  W  S  P  A  A  P  Q  C  K

259 GCATTTGTGTCCGAACCACAGATAGATCGTGGAAAGTTATCTGTGGATCAGGAT 312
    A  L  C  P  K  P  Q  I  D  R  G  K  L  S  V  D  Q  D

313 GAATATATTGAGTCTGAAAACGTCATTGTCCAGTGTGGCTCTGGCTATGGTTTG 366
    E  Y  I  E  S  E  N  V  I  V  Q  C  G  S  G  Y  G  L

367 GTTGGTCCCAAAATTATCACTTGCACAGAAGACGGAACCTGGCACCCACGGGTG 420
    V  G  P  K  I  I  T  C  T  E  D  G  T  W  H  P  R  V

421 CCCAAGTGTGAATGGGAGTACCCCGAAGACTGTGAGCAAGTGCATGAAGGCAAA 474
    P  K  C  E  W  E  Y  P  E  D  C  E  Q  V  H  E  G  K

475 AAACTCATGCAGTGTCTCCCAAACCTGGAGGAGATAAAATTGGCCCTGGAGCTG 528
    K  L  M  Q  C  L  P  N  L  E  E  I  K  L  A  L  E  L

529 TATAAGCTGTCCCTGGAGACTAAACTACTGGAGCTTCAGATAGATAAGGAAAAG 582
    Y  K  L  S  L  E  T  K  L  L  E  L  Q  I  D  K  E  K

583 AAAGCCAAAGCGAAGTACTCAATATAGTTTTTCCAAAGAGGCGGAAAAACATGC 636
    K  A  K  A  K  Y  S  I

637 CTTGCAGCTCTCAATCCAATGTAGATCACTTGTTTACCACTTCTTCCACCTTTT 690

691 TTATACCATCATCTGCAGC 710
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FIGURE 3: Nucleotide sequence and deduced amino acid sequence from apoR cDNA. The amino-terminal 19 amino acids determined by protein sequencing are in italics. Amino acids are identified by the single-letter code. The primer used to amplify the 3′ end of the gene from spleen cDNA is underlined.

Table I: Properties of ApoR and C4BP

property	apoR	C4BP
mass (kDa)		
native	145–185	500–570[c]
reduced	23	70[c]
% carbohydrate	none	7–11[c]
p*I*	6.8 (apoR-1)[a]	6.7[c]
	5.5 (apoR-2)[a]	
SCR domains	2	8[c]
plasma concn (μg/mL)	7.8 (apoR-1)[b]	210[d]
	5.0 (apoR-2)[b]	
% on VLDL	11.5	none[d]
present on chylomicrons	yes	yes[d]
correlation with plasma cholesterol and triglyceride	none	yes[d]

[a] Rapacz et al. (1986b). [b] ApoR-1 significantly different from apoR-2, $p = 0.002$. [c] Hessing (1991). [d] Sata et al. (1976).

apoR overlaps with the carboxy-terminal third of C4BPα, including SCR repeats 7 and 8 and the carboxy-terminal tail (Figure 5). The carboxy-terminal domain of apoR has 57% and 40% identity with those of human C4BPα and C4BPβ, respectively (Figure 4). ApoR is also similar to C4BPα in that both proteins have unusually long signal peptides, 28 and 48 amino acids, respectively. However, the signal peptides are only 19% identical, and therefore are probably unrelated.

Northern Blot Hybridization. RNA preparations from 12 pig tissues were screened for apoR mRNA using a PCR-amplified insert of pR6 as a probe. A mRNA of 1.2 kb, with homology to apoR, was abundant in liver, spleen, lung, bone marrow, and lymph node (Figure 6A). ApoR mRNA was in lower abundance in muscle and adipose tissue and was essentially absent in white blood cells, heart, kidney, intestine, and brain. This distribution is consistent with apoR production in terminally differentiated macrophages. ApoR mRNA was absent in white blood cells, suggesting that blood monocytes do not express this gene.

ApoR was also detected by immunoblot in pig peritoneal exudate, which contains ~90% macrophages (Cooper, 1992). We were not able to detect apoR synthesis in cultured pig hepatocytes by immunoprecipitation (S. T. Cooper and A. D. Attie, unpublished observation). The tissue distribution of apoR mRNA and its presence in peritoneal exudate suggest that the presence of apoR in the liver cDNA library was likely due to the Küppfer cells in this tissue.

A second reactive mRNA species was present in liver, bone marrow, intestine, and adipose tissue (Figure 6A). This mRNA has the same length as human C4BPα mRNA (2.5 kb), which has 67% homology with apoR in the region of pR6. To test whether this band was C4BPα mRNA, the nylon blot was stripped and then reprobed with a radiolabeled mouse C4BPα cDNA fragment (kindly provided by Dr. Brian Tack, Scripps Research Institute). This fragment encodes the signal peptide through the fifth SCR of the corresponding C4BPα amino acid sequence, a region which is not predicted to overlap with the sequence of apoR (Figure 5). Only the band at 2.5 kb was visible upon hybridization with a C4BPα-specific probe (Figure 6B), suggesting that the upper band is indeed C4BPα and the lower band apoR. Furthermore, the 2.5-kb band observed in Figure 6A is not likely to be apoR mRNA because it is abundant in the intestine, while apoR protein synthesis was not detected in this tissue. The minor bands on the blot probably represent other members of the SCR protein family.

To determine whether there is a human homologue of apoR, RNA was isolated from human liver and lung and probed for apoR mRNA with the PCR-amplified insert of pR6. ApoR mRNA was not detected in either liver or lung, while C4BPα mRNA was observed in both tissues (Figure 7). If a human homologue of apoR exists, it must either have a different tissue distribution from the pig mRNA or be of very low abundance in humans.

Plasma Distribution and Concentration of ApoR. ApoR antisera recognized a single protein at 23 kDa in pig plasma subjected to immunoblot analysis. ApoR was detected on chylomicrons, on VLDL, and in the $d > 1.21$ g/mL infranatant of plasma with an apparent molecular mass of 23 kDa (Figure 8). As determined by competitive ELISA, the plasma concentration of apoR in apoR-1 pigs is 7.8 ± 2.8 μg/mL and in apoR-2 pigs is 5.0 ± 1.6 μg/mL (Table I). No correlation between apoR concentration and either plasma triglyceride or cholesterol concentration was observed (Cooper, 1992). Only 11.5% of apoR in plasma from a fasted pig was associated with VLDL, with the remainder in the $d > 1.21$ g/mL infranatant. ApoR accounted for 6.8% of VLDL protein, as measured by scanning densitometry of VLDL subjected to SDS–PAGE and Coomassie R-250 staining. This is similar to the percent of total VLDL protein contributed by apoE or apoB.

ApoR is not made in the intestines, yet is found on chylomicrons. This suggested that apoR could transfer onto chylomicrons in the circulation. To test this, human lipoproteins and a lipid emulsion, Intralipid, were incubated with a source of apoR (pig $d > 1.21$ g/mL infranatant). Human lipoproteins do not contain apoR (Figure 9). Thus, human lipoproteins were used as acceptor particles for measuring the transfer of apoR onto lipoproteins. ApoR transferred onto human chylomicrons and Intralipid from pig $d > 1.21$ g/mL infranatant (Figure 9). However, transfer of apoR onto human VLDL could not be detected.

Characterization of Glycosylation and Interchain Disulfide Bonds in ApoR. C4BPα has two N-linked glycosylation sites

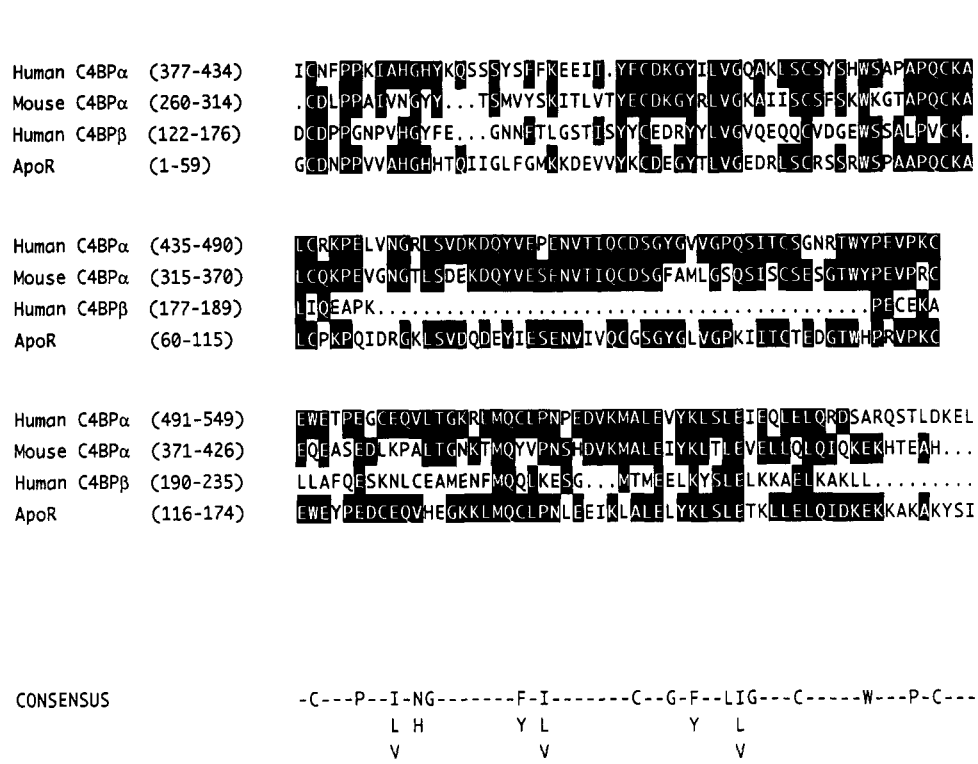

FIGURE 4: Comparison of the deduced amino acid sequences of apoR (this work), human C4BPα (Aso et al., 1991), mouse C4BPα (Kirstensen et al., 1987b), and human C4BPβ (Hillarp & Dahlbäck, 1990). Percent amino acid identity using the best alignment between paired sequences was measured. ApoR had 57% identity with human C4BPα, 49% with mouse C4BPα, and 36% with human C4BPβ. Human C4BPα had 54% identity with mouse C4BPα and 27% identity with human C4BPβ. Mouse C4BPα and human C4BPβ had 29% identity. Panel A shows the regions of each sequence that overlap with apoR. Amino acid residues present in at least three of the sequences are boxed. Panel B shows the reported SCR consensus sequence (Hessing, 1991).

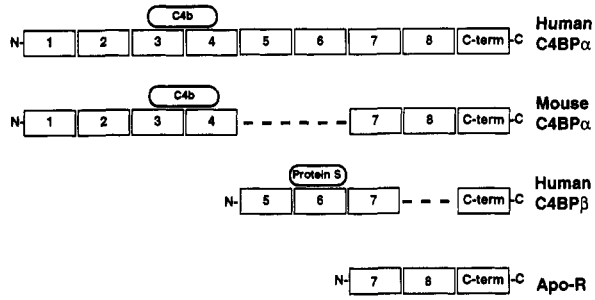

FIGURE 5: Schematic alignment between human C4BPα, mouse C4BPα, human C4BPβ, and apoR. The upper diagram represents human C4BPα, with the putative C4b-binding site indicated (Chung & Reid, 1985; Fujita et al., 1985). The second diagram represents mouse C4BPα, which is lacking SCR domains 5 and 6 from human C4BPα. The third diagram represents C4BPβ, which contains the protein S binding site in C4BP and aligns best with SCR domains 5, 6, and 7 of C4BPα. The bottom diagram represents apoR which has homology to SCR domains 7 and 8 of C4BPα. All four of the aligned protein sequences have conserved residues in their carboxy-terminal domains which do not contain the SCR consensus sequence. Alignments were performed as described in the legend of Figure 4.

in the region of overlap with apoR, Asn^{506} and Asn^{528}. Both of these amino acid residues are absent in the corresponding positions of apoR. The protein sequence of apoR contains no potential N-linked glycosylation sites. Treatment of apoR with endoglycosidase-H, an enzyme that cleaves N-linked carbohydrate, resulted in no shift in molecular weight on SDS–PAGE. Furthermore, apoR in VLDL did not react with the glycoprotein stain Alcian Blue whereas apoE, which is known to be O-glycosylated, did react with the dye (Cooper, 1992). Thus, apoR does not appear to be glycosylated.

As mentioned above, apoR migrated at 23 kDa under reducing conditions. However, under nonreducing conditions, chylomicron-associated apoR migrated at molecular masses of 145 and 185 kDa (Figure 10), implying the formation of intermolecular disulfide bonds. By two-dimensional gel electrophoresis, in which one dimension was nonreducing and the second dimension was reducing, the 185-kDa complex appeared to consist of only apoR molecules. On the basis of the molecular mass of apoR, this would represent an octamer. The 145-kDa complex appeared to be between apoR and a 45-kDa protein of unknown identity. A band at >200 kDa was also evident on this gel, which was immunoreactive with antibodies to apoR. To address the possibility that the higher molecular mass complexes might have formed during the handling of the plasma, plasma samples were treated with iodoacetic acid (5.4 mM final concentration) immediately after collection to trap free sulfhydryl groups. The samples were subjected to gel electrophoresis on reducing or nonreducing gels, electroblotted, and probed with antibodies to apoR. The >200-kDa form persisted, suggesting that the cross-links were not formed during the handling of the plasma (data not shown). The 185- and 145-kDa forms may have been formed during the isolation of the chylomicrons or may be present in such low abundance that they are only discernible upon isolation of chylomicrons.

Barium Citrate Precipitation of Pig Plasma. Treatment of plasma with barium citrate precipitates protein S, and any proteins bound to protein S. Only C4BP complexes containing C4BPβ bind protein S and are thus precipitated with barium citrate (Hillarp & Dahlbäck, 1988). ApoR does not precipitate from pig plasma in the presence of barium citrate (Figure 11). This implies that apoR did not form disulfide-linked complexes with C4BPβ. This also suggests that apoR does not bind directly to protein S alone. ApoR antisera do not cross-react with C4BPα in plasma; however, faint cross-

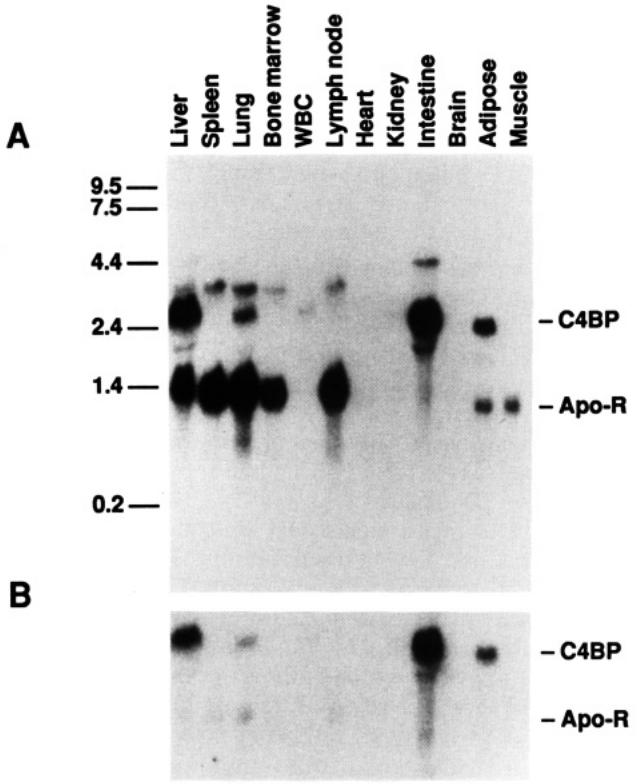

FIGURE 6: Tissue distribution and size of apoR mRNA. Twenty micrograms of total RNA from 12 pig tissues was subjected to Northern blot analysis and hybridized with a labeled PCR-amplified probe for apoR (panel A). The filter was rehybridized with a labeled fragment of mouse C4BPα encompassing the portion of the gene encoding SCR repeats 1 through 5, a region that is not predicted to overlap with apoR (panel B). ApoR and C4BPα mRNA and RNA size markers (BRL) are indicated. The tissues from which RNA was isolated are indicated at the top of panel A.

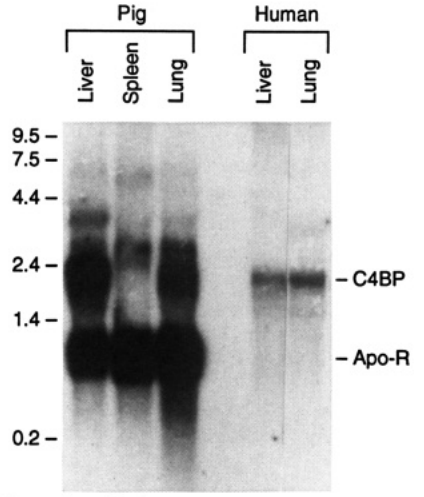

FIGURE 7: Detection of a human apoR homologue by RNA/DNA hybridization. Total RNA was isolated from human liver and lung and from pig liver, spleen, and lung. The following amounts of total RNA were loaded for each sample: pig RNAs, 20 μg of each; human liver, 10 μg; human lung, 30 μg. The filter was hybridized with a labeled PCR-amplified probe for apoR as described in the legend for Figure 5. ApoR and C4BPα mRNA and RNA size markers are indicated. The tissues from which RNA was isolated are indicated at the top.

reactivity with a 70-kDa protein in the barium citrate precipitate was apparent (Figure 11). This could be a result of concentrating C4BP in the barium citrate precipitate. The precipitate was also immunoreactive with anti-human C4BP antisera, demonstrating the presence of C4BP.

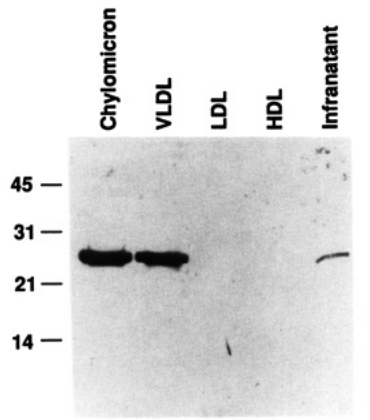

FIGURE 8: Plasma distribution of apoR. Chylomicrons were isolated from postprandial plasma 2 h after ingestion of a fatty meal (2 g of fat/kg body weight), by ultracentrifugation (30 min, 25 000 rpm) in an SW41 rotor. Lipoproteins were isolated from fasted plasma by sequential ultracentrifugation at the following densities: VLDL, $d < 1.006$ g/mL; LDL, 1.006–1.065 g/mL; HDL, 1.065–1.21 g/mL; infranatant, $d > 1.21$ g/mL. Samples were delipidated and immunoblotted with anti-apoR antisera. Thirty micrograms of each delipidated lipoprotein sample and 150 μg of infranatant were loaded in the respective lanes. Molecular mass markers in kilodaltons are indicated (Bio-Rad).

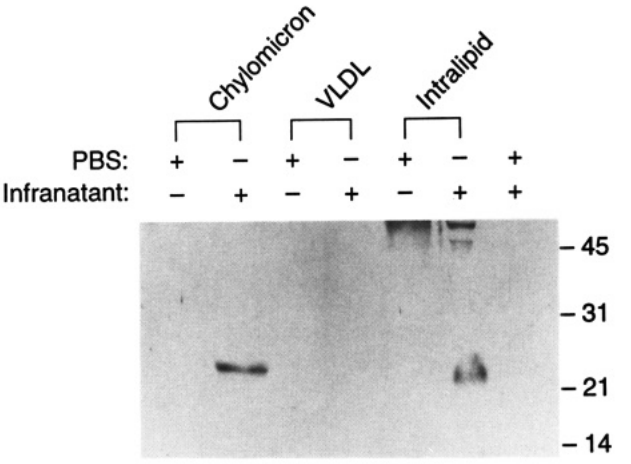

FIGURE 9: Lipid-binding properties of apoR. Pig $d > 1.21$ g/mL infranatant and PBS were incubated with either Intralipid, human chylomicrons, or human VLDL for 1 h at 25 °C. Each lipid fraction was then reisolated, delipidated, and immunoblotted with anti-apoR antisera. The incubation combinations for each lane (from left to right) are chylomicrons and PBS, chylomicrons and infranatant, VLDL and PBS, VLDL and infranatant, Intralipid and PBS, Intralipid and infranatant, and PBS and infranatant. Molecular mass markers in kilodaltons are indicated (Bio-Rad).

DISCUSSION

The most striking feature of the DNA sequence of apoR is its 67% identity with a segment of human C4BPα. The DNA sequences of human and pig apoB are only 70% identical (Maeda et al., 1988). Therefore, the homology between pig apoR and pig C4BPα may be even higher. ApoR has the same degree of identity with the 3′ end of human C4BPα, suggesting that apoR and C4BPα arose from a common ancestral protein.

Human C4BPα consists of eight tandem SCR repeats (Chung et al., 1985a). Each SCR domain is approximately 55 amino acids in length and contains 2 intradomain disulfide bonds. The SCR repeats begin at the amino terminus of C4BP. A 58 amino acid tail without an SCR motif is present at the carboxy terminus. C4BPβ has three SCR repeats which align with repeats 5, 6, and 7 of C4BPα (Hillarp & Dahlbäck,

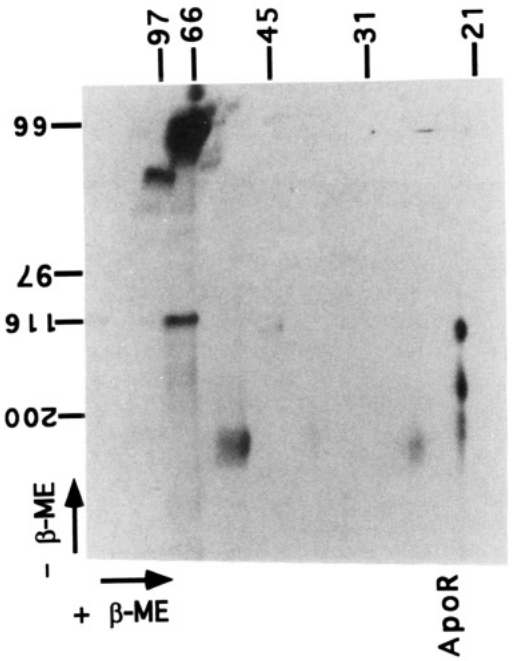

FIGURE 10: Presence of apoR in disulfide-linked complexes in plasma. Chylomicrons were collected by ultracentrifugation and passed over a Sephadex G100 column to remove any loosely-associated proteins. Delipidated chylomicrons were dissolved in SDS sample buffer without β-mercaptoethanol. Samples were subjected to SDS–PAGE; the lanes were excised and incubated with reducing sample buffer. The excised lanes were layered over a 12% gel and subjected to electrophoresis. Two-dimensional gels were stained with Coomassie R-250. Duplicate gels were immunoblotted with anti-apoR antisera to identify the bands corresponding to apoR. Molecular masses (in kilodaltons) following reducing and nonreducing SDS–PAGE are indicated to the left and at the top of the gel, respectively.

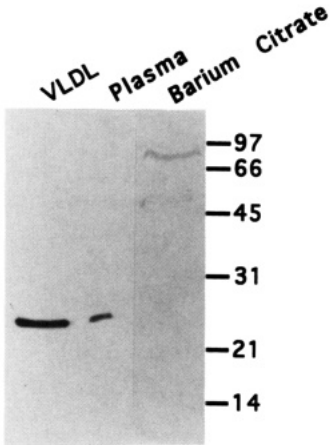

FIGURE 11: Barium citrate precipitation of pig plasma. Blood was collected in sterile 3.2% citrate, and plasma was isolated. Barium chloride was added to a final concentration of 80 mM and the barium citrate precipitate collected by centrifugation. The precipitate was washed 3 times in 0.9% saline and dissolved in 0.2 M EDTA. The solubilized pellet was dialyzed, and its protein concentration was determined. Thirty micrograms of the precipitate was subjected to SDS–PAGE and immunoblotted with anti-apoR antisera. Plasma and VLDL were included as controls. A duplicate blot was incubated with anti-human C4BPα antisera to ensure that C4BP was being precipitated from pig plasma.

1990). Mouse C4BPα has only six SCR domains, lacking domains 5 and 6 from the human C4BPα sequence (Kristensen et al., 1987b). Analysis of the apoR cDNA sequence revealed two SCR domains, aligning with SCR repeats 7 and 8 of C4BPα (Figure 5). Human C4BPα, mouse C4BPα, and human C4BPβ have considerable sequence homology in their carboxy-terminal domains (Hillarp & Dahlbäck, 1990). ApoR has high protein sequence identity with the carboxy-terminal domain of these proteins.

In C4BPα, each SCR repeat, with the exception of SCR 2, is found on an independent exon, implying exon shuffling in the formation of proteins containing SCR domains (Aso et al., 1991). Thus, it is possible that all four of the proteins described schematically in Figure 5 could have arisen by exchange of domains from a common ancestral protein.

C4BP has two binding sites, one for C4b, on the α-chain, and a second for protein S, on the β-chain (Chung & Reid, 1985; Suzuki & Nishioka, 1988). C4BPα binds free C4b, a peptide liberated in the course of the complement cascade, until it can be degraded by specific proteases (Fujita et al., 1978; Scharfstein et al., 1978). The location of the C4B-binding domain on C4BPα is at present uncertain. Electron microscopy suggests that C4b binds near the amino terminus of C4BPα (Dahlbäck et al., 1983). Fujita et al. have demonstrated that a monoclonal antibody that blocks the binding of C4BP to C4b reacts with the amino-terminal 48 kDa of C4BP, covering SCR repeats 1–6 (Fujita et al., 1985). Mouse C4BPα lacks SCR domains 5 and 6, yet binds human C4b (Kristensen et al., 1987b). These observations suggest that the C4b-binding site on C4BPα is located in SCR repeats 1–4. ApoR overlaps with SCR repeats 7 and 8, and thus it seems unlikely that apoR will have a complete C4b-binding site.

The second role of C4BP is to bind protein S (Dahlbäck & Stenflo, 1981). Protein S is a vitamin K-dependent cofactor of a serine protease, protein C, which degrades factor Va and factor VIIIa, inhibiting coagulation (Walker, 1980). Only free protein S acts as a cofactor for protein C; thus, C4BP acts as a procoagulant by binding up to 50% of the plasma protein S pool (Dahlbäck & Stenflo, 1981). Protein S can be precipitated from plasma with barium citrate; thus, proteins bound to protein S will coprecipitate. This has been employed to demonstrate that C4BP complexes that lack C4BPβ do not bind protein S (Hillarp & Dahlbäck, 1988; Hessing et al., 1990). ApoR does not appear to bind protein S, as it did not precipitate from plasma treated with barium citrate. This is consistent with apoR not forming disulfide bonds with C4BPβ.

C4BP exists in plasma as disulfide-linked complexes of C4BPα and C4BPβ molecules. Different stoichiometries of C4BPα and C4BPβ chains result in different molecular masses of the C4BP complexes. Three complexes have been proposed so far: seven α-chains with one β-chain (570 kDa); seven α-chains with no β-chain (530 kDa); and six α-chains with one β-chain (500 kDa) (Hillarp & Dahlbäck, 1988; Hillarp et al., 1989). Cys^{498} and Cys^{510}, located near the carboxy terminus of C4BPα, are proposed to form the intermolecular disulfide bonds that link the C4BPα and C4BPβ chains (Kristensen et al., 1987b). ApoR has retained cysteines in both of these proteins, and appears to form a disulfide-linked complex with other plasma proteins. The organization of the apoR molecules in the 185-kDa complex may be similar to the organization of the C4BPα molecules in heptameric C4BP. The 145-kDa complex appears to contain both apoR and a 45-kDa protein, whose identity is unknown. The 45-kDa protein is not likely to be C4BPβ, since apoR did not precipitate with barium citrate. ApoR and C4BPα complexes appear to be produced, and assembled, in different cell types. Since C4BPα and possibly C4BPβ are produced in hepatocytes, they could assemble together in disulfide-linked complexes. However, if apoR is synthesized in macrophages, it would be unlikely to be assembled intracellularly into complexes with C4BPβ.

ApoR appears to be the only member of the SCR family that is not N-glycosylated. The lack of N-glycosylation is predicted by the DNA sequence. Additionally, carbohydrate could not be detected by biochemical means. However, there is a 2-kDa discrepancy between the size of apoR predicted from the translation product of its cDNA sequence (21 kDa) and its electrophoretic mobility on SDS–PAGE (23 kDa). This could be due to a gel mobility artifact, or posttranslational modifications other than glycosylation.

The tissue distribution of apoR mRNA is consistent with a protein produced by fully differentiated macrophages. Detection of apoR by immunoblot in cells from pig peritoneal exudate supports this conclusion. The observation that apoR mRNA is not present in white blood cells indicates that apoR expression may be regulated by macrophage differentiation. In contrast, human C4BPα has been reported in hepatocytes, but not in Küppfer cells, by immunofluorescence microscopy (Kusada-Funakoshi et al., 1991). ApoR mRNA does not appear to be present in human tissues, and apoR has not been detected in human plasma by immunoblot. ApoR has also not been detected in plasma from chicken, mouse, rat, hamster, goat, and cow. Therefore, apoR may be a protein unique to pigs.

C4BPα was characterized independently as an apolipoprotein, proline-rich protein, until sequence analysis revealed that the proteins were identical (Matsuguchi et al., 1989). C4BPα is made in the liver, yet found on chylomicrons and in the $d > 1.21$ g/mL infranatant of plasma (Funakoshi et al., 1988). C4BPα has a mean plasma concentration of 210 μg/mL (Sata et al., 1976; Funakoshi et al., 1988), much higher than that of apoR. C4BPα plasma concentrations correlate with plasma triglyceride and cholesterol concentrations (Sata et al., 1976; Funakoshi et al., 1988). In contrast, the concentration of apoR does not correlate with either plasma cholesterol or triglyceride concentration (Cooper, 1992).

The plasma distributions of apoR and C4BPα are similar; a small fraction is bound to chylomicrons with the majority present in the $d > 1.21$ g/mL infranatant of plasma (Funakoshi et al., 1988). However, apoR is also present on VLDL while C4BPα has not been reported at this density range. This could be because the complexes containing apoR are much smaller than C4BPα and may be able to partition more easily onto a particle with less exposed lipid than a chylomicron. Partitioning of apoR onto human VLDL could not be demonstrated in vitro. However, if apoR is made in cells that do not produce lipoproteins, partitioning onto VLDL would have to be occurring in the bloodstream. It is also possible that differences between human and pig VLDL prevent apoR from partitioning onto human VLDL. C4BPα is also capable of exchange onto lipid emulsions, and this has been used as a purification step (Matsuguchi et al., 1989). The carboxy terminus of C4BPα can form an α-helix (Chung et al., 1985). If this helix is amphipathic, it may be responsible for the lipid affinity of C4BPα, and possibly apoR.

The presence of C4BP as a regulator of the coagulation and complement pathways on chylomicrons raises interesting questions about the interaction of lipoproteins with these two pathways. Lipid surfaces play a central role in both coagulation and complement action. Both liposomes rich in negatively charged phospholipids and large triglyceride-rich lipoproteins stimulate coagulation because they provide increased charged surface which can initiate the contact phase of coagulation (Mitropoulos et al., 1989a). An association between triglyceride-rich lipoproteins and factor VII activity, but not protein concentration, has been documented (De Sousa et al., 1988; Mitropoulos et al., 1989b).

The role of lipoproteins in complement action is more speculative. The alternative pathway can be stimulated by large foreign particles. Triglyceride-rich lipoproteins have large lipid surfaces which may be able to stimulate the complement cascade. The presence of C4BP on chylomicrons may prevent complement stimulation and membrane attack complex formation on the surface of lipoproteins, by binding C4b. Because pig C4BPα mRNA is present in intestine, C4BP may be secreted directly on nascent chylomicrons. However, the ability of C4BP to bind to lipid emulsions demonstrates some degree of lipid affinity.

The observation that apoR is present on lipoproteins and has homology with a family of proteins involved in coagulation and complement is not without precedent. There are several additional links between apolipoproteins and the coagulation and complement cascades. Clusterin (apoJ) is found on HDL particles, associated with apoAI, and functions as a complement lysis inhibitor (Jenne et al., 1991). β_2-Glycoprotein I (apoH) contains five SCR repeats and is found on chylomicrons; however, its function is unknown (Polz & Kostner, 1979; Lozier et al., 1984). Lipoprotein-associated coagulation inhibitor (LACI) is found on LDL, and disulfide-linked to apoAII on HDL (Warn-Cramer et al., 1987; Novotny et al., 1989). LACI inhibits the tissue factor/factor VIIa complex, blocking the extrinsic pathway of coagulation (Hubbard & Jennings, 1982). In addition, lipoprotein(a) particles contain a single molecule of apo(a) disulfide-linked to apoB. Apo(a) has remarkable homology with the kringle and catalytic domains of plasminogen (Loscalzo et al., 1990). An increase in plasma apo(a) concentration is a risk factor for coronary heart disease, but does not correlate with increases in plasma lipid levels (Kostner et al., 1981). This is thought to be because apo(a) can compete with plasminogen for binding sites on the surface of the endothelium, thereby inhibiting fibrinolysis (Hajjar et al., 1989; Miles et al., 1989).

ApoR appears to be a unique member of the large family of SCR-containing proteins, many of which are involved in the regulation of coagulation and the complement cascade. Because of its affinity for triglyceride-rich lipoproteins and homology with C4BPα, apoR may serve as another link between lipoprotein metabolism and the regulation of the coagulation or complement cascades.

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