High Density Lipoprotein-Binding Proteins in Liver

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(*High Density Lipoprotein*-Bindende Eiwitten in de Lever)

Proefschrift

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Marinus Petrus Gerardus de Crom
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Promotor:	Prof.Dr. H. Galjaard
Promotor:	Prof.Dr. F.G. Grosveld
Overige leden:	Prof.Dr. Th.J.C. van Berkel
	Prof.Dr. R.R. Frants
	Dr. A. van Tol
Co-promotor:	Dr. A.W.M. van der Kamp

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Gedrukt bij Offsetdrukkerij Ridderprint B.V., Ridderkerk

Nagy csukott ajtókat látok,
Hét fekete csukott ajtót!
Mért vannak az ajtók csukva?
(...)
Nyisd ki, Nyisd ki! Nekem nyisd ki!
Minden ajtó legyen nyitva!
Szél bejárjon, nap besüssön!

Ah, I see seven great shut doorways.
Seven doors all barred and bolted!
Why are all the seven bolted?
(...)
Open, open! Throw them open!
All those locks must be unfasten'd.
Wind shall scour them, light shall enter!

From Béla Bartók:
A kékszakállú herceg vára (Duke Bluebeard's Castle)
opus 11, Sz 48 (1911)
(libretto by Béla Balázs)

Voor Mirjam,
Errol en Tosca

Contents

Abbreviations

a_2-M	a_2-macroglobulin
a_2-M*	activated a_2-macroglobulin
acLDL	acetylated LDL
apo	apolipoprotein
BSA	bovine serum albumin
CHO	chinese hamster ovary
CETP	cholesteryl ester transfer protein
EGF	epidermal growth factor
ER	endoplasmic reticulum
ELAM-1	endothelial leukocyte adhesion molecule 1
FH	familial hypercholesterolemia
HB1, HB2	HDL-binding protein 1, 2
HBP	HDL-binding protein
HDL	high density lipoprotein
HMGCoA	3-hydroxy-3-methyl-glutaryl coenzyme A
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyl transferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
LRP	LDL receptor related protein
MSR	macrophage scavenger receptor
oxLDL	oxidized LDL
PA-1	plasminogen activator-1
PAGE	polyacryl amide gel electrophoresis
PAGGE	polyacryl amide gradient gel electrophoresis
RAP	receptor associated protein
SDS	sodium dodecyl sulfate
SR-A	scavenger receptor class A
SR-B	scavenger receptor class B
SRCR	scavenger receptor cysteine rich (domain)
TGFa	transforming growth factor a
tPA	tissue type plasminogen activator
uPA	urokinase type plasminogen activator
VLDL	very low density lipoprotein
WHHL-rabbits	Watanabe hereditary hyperlipidemic rabbits

Chapter I

General Introduction

1. Atherosclerosis

In one of the oldest civilizations we know, that of ancient Egypt, thoughts about the heart reflected a certain duality. On the one hand, the heart was associated with concepts like virtue, or soul. A central passage in the *Book of the Dead* of the ancient Egyptians is the description and illustration of the weighing of the soul (Fig. 1). The heart of the diseased was put on a pair of scales and balanced against the hieroglyphic symbol of virtue, the feather *maat*. If the ibis god of scribes, Thot, could register a favourable verdict, the dead man or woman was presented to the god of the dead, Osiris, and was allowed entrance into the world of the dead. If not, the heart was devoured by a horrifying beast, which event was described as the *Death of the Soul*, and meant total elimination of the individual [Rossiter, 1979]. On the other hand, the Egyptians probably were aware of the physiological function of the heart as a blood-pump and recognized a variety of heart conditions [Ghalioungui, 1973]. People suffering from them would wear a small amulet representing a heart [Howes, 1976].

In modern times, a comparable duality exists. On the one hand, the heart is associated with emotions, notably with love. On the other hand, the vital physiological function of the heart is a major concern in the

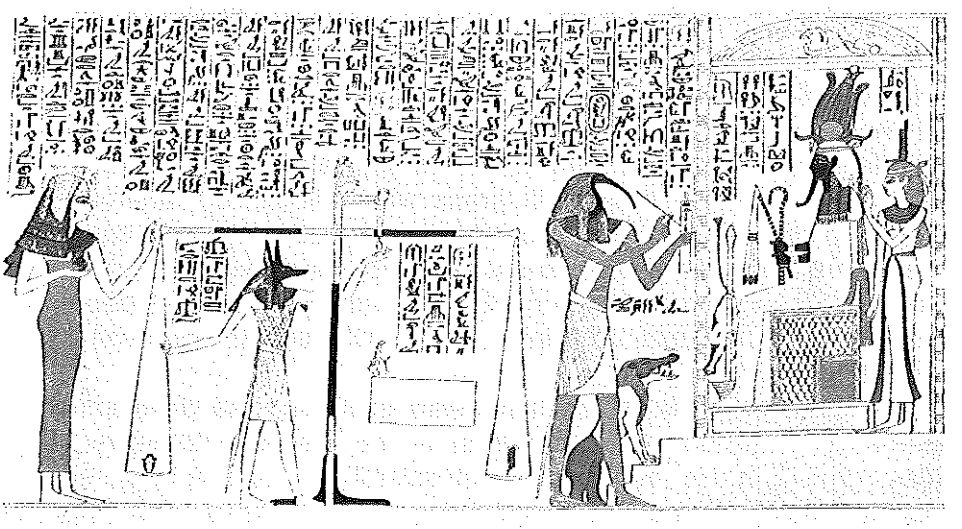

Figure 1. The judgement before Osiris. Vignette from a funeral papyrus of queen Makare of Dynasty XXI. Reproduced from G. Maspéro: *Histoire Générale de l'Art: Égypte*, Librairie Hachette et Cie, Paris, 1912.

industrialized world, since diseases of the heart are the most important cause of death and morbidity. This is almost completely attributable to the effects of atherosclerosis [Ross, 1993].

Atherosclerosis is a disorder of the arterial wall that consists of a focal accumulation of lipids and fibrous tissue in which inflammatory processes as well as proliferation and degradation of cells take place [reviewed in Ross, 1993; O'Brien and Chait, 1994]. Advanced atherosclerotic lesions can result in obstruction of the artery or haemorrhage following rupture. The ultimate result of atherosclerosis may be a heart attack, stroke or gangrene in the extremities. Atherosclerosis is considered responsible for 50% of all mortality in Europe, including The Netherlands, Japan and the U.S.A. [Ross, 1993; O'Brien and Chait, 1994; CBS, 1996].

Although the clinical manifestations of coronary artery disease usually occur in the 5th or 6th decade of life, the first pathological features have been frequently observed in autopsy specimens from young children. Thus, atherosclerosis is a long-term process.

The atherosclerotic process consists of three stages: the fatty streak, the intermediate lesion and the fibrous plaque (advanced lesion). The fatty streak is visible as a clustering of yellow spots or streaks and located mainly at branch points in the arteries. Microscopically, aggregation of macrophages and lymphocytes in the media can be observed. Fatty streaks have been frequently observed in children and young adults. Intermediate lesions are layers of smooth muscle cells and macrophages which cause a thickening of the arterial wall. Evidence from experimental animals show that they develop from fatty streaks. However, many fatty streaks will probably not develop into more advanced stages of atherosclerosis since fatty streaks are also present in areas where advanced atherosclerotic lesions are never found. Fibrous lesions are covered by a cap of connective tissue of variable size and thickness. The lesions contain smooth muscle cells, lipid-loaden macrophages called foam cells, necrotic cell debris and extracellular lipids. Lipid containing cells can also be observed in the preceeding stages.

The most widely accepted hypothesis explaining the development of atherosclerotic lesions (atherogenesis) is the response to injury theory [Ross, 1995a]. In this theory, the atherosclerotic process is triggered by a local dysfunction of the endothelial lining of the vessels. This might be caused by physical damage, metabolic changes (exposure of cell adhesion molecules or chemoattractants) or toxicological challenges (nicotinic acid, drugs, free radicals). Binding of monocytes and T-lymphocytes to the endothelial cells and migration to the underlying smooth muscle cells are

considered as manifestations of this stage. Subsequently, the monocytes differentiate into macrophages that start to accumulate lipids and become eventually foam cells. At the same time, smooth muscle cells begin to migrate and proliferate and an extracellular matrix of elastic fibers, collagen and proteoglycans is formed. A communicative network between endothelial cells, smooth muscle cells and macrophages is established by an impressive array of growth factors, cytokines and other chemicals including lipids and small molecules like nitric oxide [reviewed by Ross, 1995b]. Many of these molecules have been identified and include interleukin-1 (IL-1), tumor necrosis factor α (TNFα) interferon γ (IFNγ), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor β (TGFβ), monocyte-colony stimulating factor (M-CSF), and numerous others. The individual contribution of these factors to the atherosclerotic process is far from understood.

From epidemiological studies, a number of risk factors for the development of atherosclerosis have been established. Some of them are immutable, like increased age, male gender and postmenopausal state in women. Next to these, the most important risk factors are smoking, hypertension, diabetes mellitus, obesity and plasma cholesterol levels [Basha and Sowers, 1995; Fuster *et al.*, 1996].

The majority of plasma cholesterol is present in two classes of lipoproteins: low density lipoproteins (LDL) and high density lipoproteins (HDL) (section 2). LDL levels in plasma appeared to be positively correlated with the incidence of coronary artery disease (i.e. a high level of plasma LDL is related to a high incidence of coronary artery disease) [Brown and Goldstein, 1986]. HDL levels are negatively correlated with the incidence of coronary artery disease (section III.1) [Gordon and Rifkind, 1989; Tall, 1990]. Based on this observation, it is assumed that HDL protects in some way against the development of atherosclerosis. The mechanism of this protection is unknown, but it is widely supposed to reside in the capacity of HDL to mediate *reverse cholesterol transport*. This is the removal of excess cholesterol from extrahepatic tissues and the subsequent transfer to the liver for excretion into the bile [Pieters *et al.*, 1994; Fielding and Fielding, 1995].

Atherosclerosis is a multifactorial process, effectuated by a combination of environmental and genetic factors [Hopkins and Williams, 1989; Breslow and Dammerman, 1995]. Because cholesterol levels are an important risk factor, much research has been focussed on the elucidation of genetic factors involved in lipoprotein metabolism. LDL metabolism and the role of a specific LDL-receptor have been studied extensively and many of the

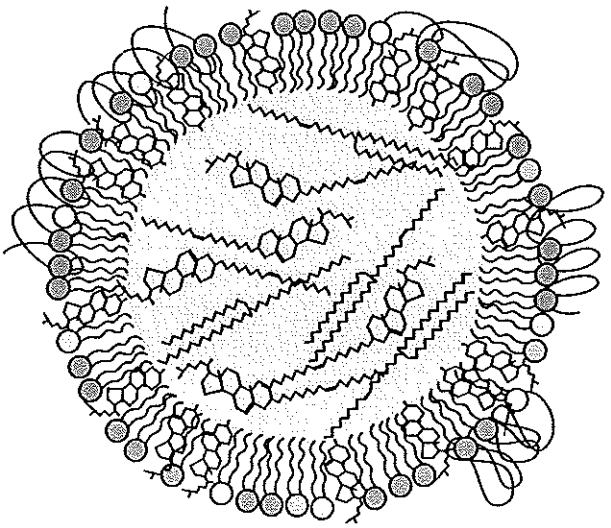

Figure 2. Structure of a lipoprotein particle.

molecular details are known (section II.1) [Goldstein *et al.,* 1986]. In contrast, many aspects of HDL metabolism remain to be elucidated.

2. Lipoproteins and apolipoproteins

At the beginning of this century, researchers realized that plasma lipids like cholesterol were emulsified by proteins [Nerking, 1901]. It was only fifty years later that lipoproteins were systematically isolated and characterized. On the analogy of the electrophorectically fractionated plasma α-, β- and γ-globulins, Oncley *et al.* [1947] separated α- and β-lipoproteins. On the other hand, Havel *et al.* [1955] used ultracentrifugal methods for the fractionation of plasma lipoproteins, resulting in a classification according to buoyant density. Those two classifications are summarized in Table I, together with some characteristics of the lipoproteins. In the past decades, the classification according to electrophoretic mobility has fallen into disuse.

Each of the lipoprotein classes is heterogeneous in size and composition. Because HDL has a striking bimodal distribution in rate zonal centrifugation, two subclasses are usually distinguished (Table I).

Lipoproteins [reviewed in Gotto *et al.*, 1986; Havel and Kane, 1995] are spherical in shape (Fig. 2). They have a hydrophobic core consisting of cholesterol esters and triglycerides, and a surface consisting of a monolayer

Table I: Lipoproteins

class	electrophoretic mobility	density (mg/dl)	diameter (nm)	molecular weight	chemical composition surface C	surface PL	surface apo's	core TG	core CE
chylomicrons	remain at origin	0.093	75-1200	50-10,000 x 10^6	2	7	2	86	3
VLDL	pre-β	0.093-1.006	30-80	10-80 x 10^6	7	18	8	55	12
IDL	slow pre-β	1.006-1.019	25-35	5-10 x 10^6	9	19	19	23	29
LDL	β	1.019-1.063	18-25	2.3 x 10^6	8	22	22	6	42
HDL_2	α	1.063-1.125	9-12	360,000	5	33	40	5	17
HDL_3	α	1.125-1.21	5-9	175,000	4	35	55	3	13

Data from: Havel and Kane [1995]. C: cholesterol; PL: phospholipids; TG: triglycerides; CE: cholesterol esters.

of phospholipids, extruding their hydrophobic fatty acid tails into the interior of the particle and exposing their hydrophyllic head groups to the exterior. Proteins associated with lipoproteins are termed apolipoproteins (discussed below). The association with the phospholipid surface of the lipoproteins occurs via amphipatic helices in the proteins. These are stretches of amino acids that are either polar or nonpolar and that are located on opposite sides of an α-helix in the protein. The hydrophobic non-polar face of the helix can associate with the lipid surface of the lipoprotein while the hydrophyllic polar face is in contact with the surrounding aqueous phase.

In the late sixties, it was proposed to designate apolipoproteins of α-lipoproteins (HDL) as apolipoprotein (apo) A and those of β-lipoproteins (LDL) as apoB. Newly discovered apolipoproteins from very low density lipoproteins (VLDL) were called apoC. Within one category, different apolipoproteins were distinguished by roman numerals [Gotto *et al.*, 1986]. Many of the apolipoproteins however, did not appear to be restricted to one category of lipoproteins. Newly discovered apolipoproteins were therefore termed apoD and apoE, which are arbitrary names that do not relate to the lipoproteins with which they are associated. The most important apolipoproteins and their properties are summarized in Table II.

The metabolism of human lipoproteins will be discussed briefly here (Fig. 3) [for reviews, see Gotto *et al.*, 1986; Kroon and Powell, 1992; Havel and Kane, 1995].

Dietary fat (also referred to as fat from exogenous sources, as opposed to fat from endogenous sources) is packaged into chylomicrons in the enterocytes. The lipid moiety of newly secreted chylomicrons consists for the greater part of triglycerides; some cholesterol is also present. Apolipoproteins are apoB-48 and apoA-I, apoA-II and apoA-IV, which are synthesized in the enterocytes and assembled into the chylomicrons. After

secretion, chylomicrons aquire apoE and apoC's from HDL in the circulation. Chylomicron triglycerides are hydrolized very rapidly by the enzyme lipoprotein lipase (LPL), that is present at the endothelial surface. LPL activity is very high in adipose tissue and skeletal muscles, where the triglycerides are stored or metabolized for energy production, respectively. Finally, a so-called chylomicron remnant remains, that has lost 80 to 90% of the triglycerides and the apoCs. Chylomicron remnants are cleared rapidly by the liver via the LDL-receptor and possibly via remnant receptors (LRP, VLDL-receptor; see section II.2). Virtually all the dietary cholesterol ends up in the hepatocytes via this pathway. This can be stored intracellularly, secreted into the bile (mostly as bile acids), or enter the blood in VLDL particles. VLDL performs the transport of liver-derived lipids (also referred to as fat from endogenous sources). They have a higher amount of cholesterol than chylomicrons, but still are rich in triglycerides. Nascent VLDL contains apoB-100 rather than apoB-48. Initially, VLDL metabolism resembles chylomicron metabolism. ApoE and apoCs are added (although nascent VLDL contains minor amounts of these apolipoproteins); triglycerides are hydrolyzed by the action of LPL. Larger VLDL remnants are taken up by hepatocytes via the LDL-receptors and remnant receptors. Smaller VLDL remnants are less rapidly cleared than the larger VLDL remnants and chylomicron remnants. The ultracentrifugally isolated intermediate density lipoprotein (IDL) fraction consists mainly of these smaller VLDL remnants. They are converted to LDL in the circulation. LDL is devoid of apoE but still can bind to the LDL-receptor via the apoB-100 moiety. LDL contains the larger part of plasma cholesterol (70%). Although the liver is the principle site of LDL catabolism, most peripheral (extra-hepatic) cells can take up LDL as well via the LDL-receptor (section II.1).

HDL metabolism has its starting point in both the liver and the intestine. Nascent HDL is discoidal and corresponds with preβ-HDL described by Fielding and coworkers [Fielding and Fielding 1995]. Discoidal HDL might be either secreted by hepatocytes or formed by the action of hepatic lipase from HDL_2. Another source of discoidal HDL is probably the redundant surface material of chylomicron remnants following triglycerides hydrolysis by LPL. Both lipid and protein components of plasma HDL are in part derived from other (remmant) lipoproteins. Spherical HDL results from the storage of cholesterol esters in the lipid core of the lipoprotein, that are formed by the action of the plasma enzyme lecithin:cholesterol acyl transferase (LCAT). HDL is believed to mediate reverse cholesterol transport (section III.3), i.e. transport of cholesterol from peripheral (extra-hepatic) tissues to the liver. HDL catabolism is a complex process.

Table II: Apolipoproteins

name	plasma conc. (mg/dl)	chromos. localiz.	amino acids (mat. prot.)	kDa	source	lipoproteins	function
A-I	130	11q23.3	243	29.0	liver, intestine	chylomicrons, HDL	activates LCAT, structural component of HDL
A-II	40	1q21-q23	77	17.4	liver, intestine	chylomicrons, HDL	unknown (activates LCAT)
A-IV	150-370	11q23-qter	376	44.5	liver, intestine	chylomicrons, HDL	unknown (activates LCAT)
B-48	<1	-	2152	241.0	intestine	chylomicrons	structural component of chylomicrons
B-100	80	2p23-p24	4536	512.7	liver	VLDL, IDL, LDL	structural component of VLDL, IDL, LDL; ligand of the LDL-receptor
C-I	6	19q13.2	57	6.6	liver	all major lipopr.	unknown
C-II	3	19q13.2	79	8.9	liver	all major lipopr.	activates LPL
C-III	12	11q23.1-q23.2	79	8.8	liver	all major lipopr.	inhibits LPL
D (A-III)	10	3q27-qter	169	19.0	many organs	HDL	unknown
E	5	19q13.2	299	34.1	liver	all major lipopr.	ligand of the LDL-receptor; possibly of specific apoE-receptor

Data from: Tenkanen and Ehnholm, 1993; Milne et al., 1993; Ginsberg, 1994; Scott et al., 1994; Havel and Kane [1995]; Human Genome Database (1997:http://gdbwww.gdb.org/).

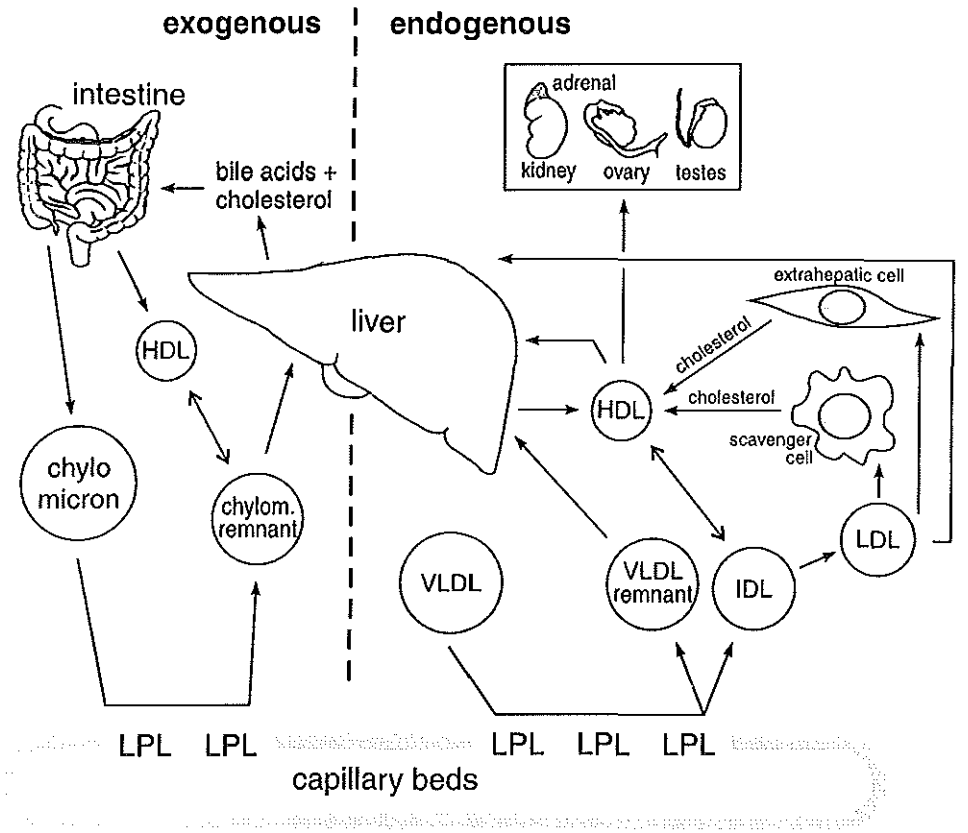

Figure 3. Metabolism of plasma lipoproteins. See text for details. Adapted from Scriver *et al.* (eds.): *The Metabolic and Molecular Bases of Inherited Disease*, New York, McGraw-Hill, 7th ed., 1995.

Quantitatively, the liver is the major site of HDL catabolism (section III.3.1.3). However, steroid producing tissues, such as the adrenal gland and ovary, have a very efficient uptake of HDL-cholesterol. In these tissues, as well as in the liver, HDL-cholesterol esters can be taken up in preference to HDL-apolipoproteins. In the kidneys, HDL apolipoproteins are preferentially catabolized. Enzymes (hepatic lipase, LCAT), and lipid transfer proteins like cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) effectuate a continuous flux of lipids between cells and lipoproteins and between lipoproteins mutually. Differences in apolipoprotein composition contribute to HDL heterogeneity (section III.2) and probably to a diversity in metabolic fate. This complexity is probably the principal cause that the details of HDL metabolism are still

far from understood and that the quantitative importance of the described processes is in most cases unknown. The identification of the most important regulatory proteins is crucial for the elucidation of the molecular details of HDL metabolism. This thesis describes the identification and isolation of HDL-binding proteins in liver that might be implicated in reverse cholesterol transport.

Chapter II

Lipoprotein Receptors

The first lipoprotein receptor that has been identified is the LDL-receptor [Brown and Goldstein, 1986]. Extensive research on this receptor has greatly increased our knowledge on LDL metabolism and on fundamental cell biological processes like receptor mediated endocytosis and the regulation of cellular cholesterol homeostasis. Although several other (candidate) lipoprotein receptors have been postulated, none of them has been unambiguously proven to be involved in lipoprotein metabolism *in vivo*.

The experimental work described in this thesis is on the identification and characterization of HDL-receptor proteins. Work that has been done on other (candidate) lipoprotein receptors can give important clues for this research, e.g. the multifunctionality of the LDL-receptor Like Protein (LRP) and of the macrophage scavenger receptors [Krieger and Herz, 1994]. One of the latter receptors, Scavenger Receptor class B, type I (SR-BI) has turned out to be a HDL-receptor in steroidogenic tissues, possibly among other functions [Landschultz *et al.*, 1996]. For this reason, this chapter will summarize what is known about the LDL-receptor (section 1), LRP (section 2.1), the VLDL-receptor (section 2.2) and the scavenger receptors (section 3). (Candidate) HDL-receptors will be discussed in chapter III.

1. Low Density Lipoprotein-Receptor (LDL-receptor)

1.1 Familial Hypercholesterolemia

Familial hypercholesterolemia (FH) is an inherited disease [reviewed in Goldstein *et al.*, 1995] that results from a mutation affecting the functioning of the LDL-receptor (see below). FH shows an autosomal dominant inheritance. Heterozygous FH occurs with a frequency of 1 in 500 individuals, classifying the disease as one of the most prevalant inborn errors of metabolism. Homozygous FH is rare; the incidence is 1 in 1.000.000 persons. Since a large number of different mutations in the LDL-receptor is known to cause FH (discussed below), homozygotes are in fact usually compound heterozygotes, having two different mutations each affecting one LDL-receptor allele. In FH patients, plasma cholesterol levels are elevated from birth, about two-fold in heterozygotes (350 to 500 mg/dl) and four to seven-fold in homozygotes (600 to 1200 mg/dl). Clinical symptoms include arcus coroneae, xanthomas and premature coronary heart disease as a consequence from atherosclerosis. Homozygous FH patients usually die from myocardial infarction between the age of 5 and 30. Heterozygous FH patients usually suffer from coronary artery disease from

their fourth decade.

It is the first genetic disorder found to cause coronary heart disease and it supplies the strongest evidence for a causal relation between elevated plasma cholesterol and atherosclerosis in man.

1.2 LDL-Receptor

Exclusively LDL-cholesterol is elevated in FH patients. LDL particles are highly increased in number, but otherwise completely normal, both in terms of lipid and protein composition per particle as well as in their metabolic characteristics. *In vitro* studies showed that binding of LDL from FH patients and controls to the LDL-receptor is similar. Radiolabeled LDL from FH patients injected into normal human volunteers behaved in the same way as LDL from controls.

Goldstein and Brown discovered that the molecular defect in FH is a lack of functional LDL-receptors [reviewed in Brown and Goldstein, 1986;

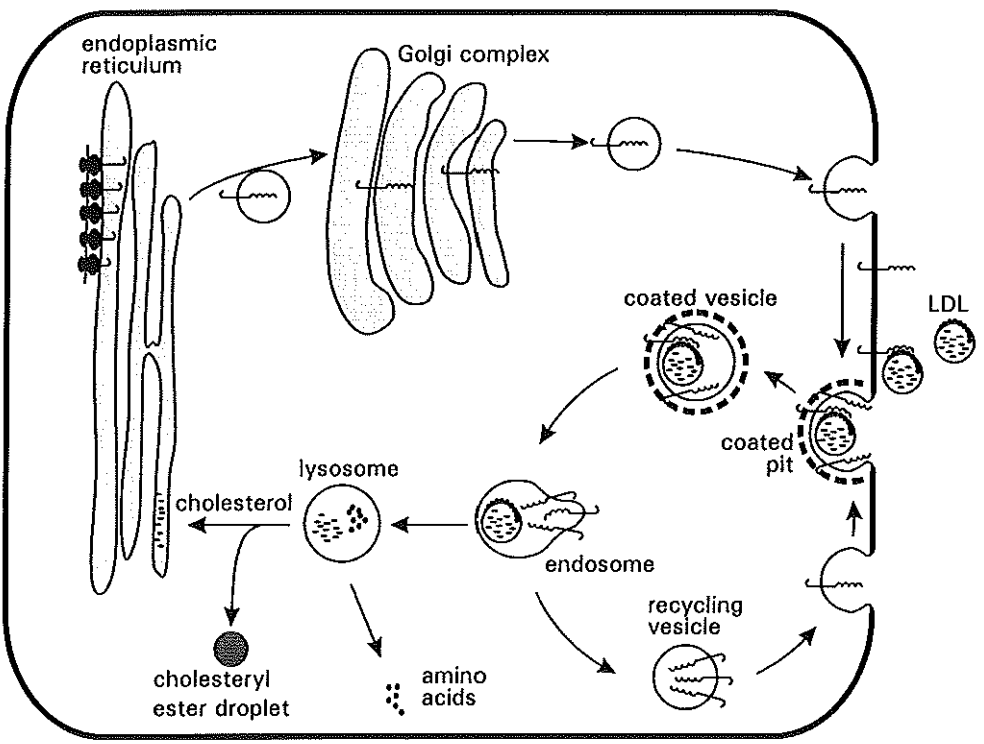

Figure 4. Receptor mediated endocytosis of LDL. See text for details. Adapted from Goldstein *et al.*, 1995.

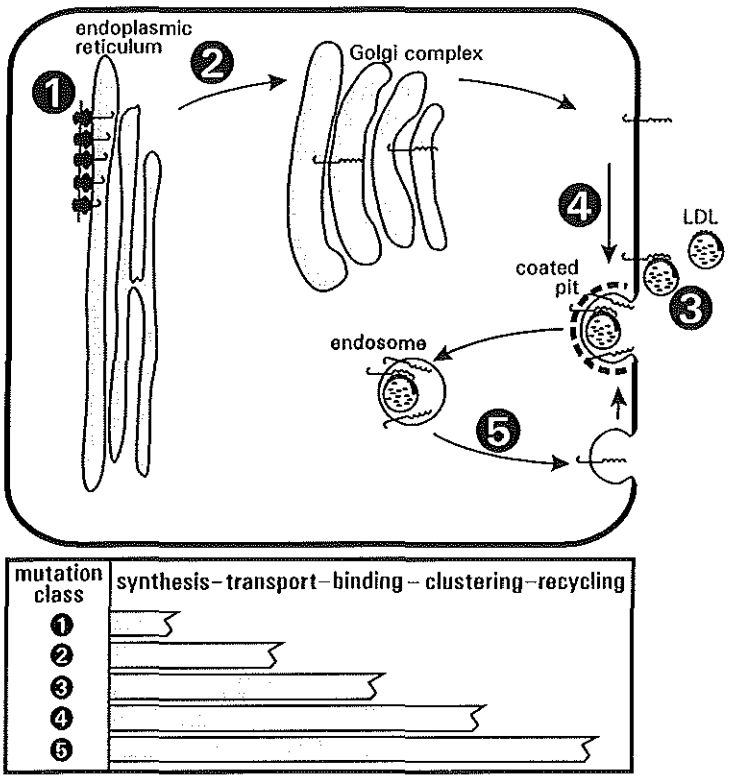

Figure 5. Classification of mutations affecting the LDL-receptor pathway. See text for details. Adapted from Hobbs *et al.*, 1990.

Goldstein *et al.*, 1995]. LDL-receptors are integral plasma membrane proteins that specifically bind LDL with high affinity. Electron microscopic studies revealed that LDL-receptors are clustered on the cell surface in small invaginations of the plasma membrane called coated pits. These have an electrondense "coat" at the intracellular side, which consists of a specific protein called clathrin. Upon further invagination these coated pits finally bud off from the plasma membrane to the interior of the cell to form a coated vesicle. Then the coat disappears and a number of vesicles fuse to form an endosome. In the endosome, the pH gradually drops as a result from the action of proton pumps in the membrane of the endosome. This causes the LDL particle to dissociate from its receptor. The receptor than locates into a part of the endosomal membrane that pinches off and fuses with the plasma membrane, by which the LDL-receptor is able to bind another LDL particle. This recycling process takes 10 minutes and occurs continuously, whether or not the receptor is occupied with LDL (Fig. 4) [Goldstein *et al.*, 1979].

Almost two hundred different mutations in the LDL-receptor gene

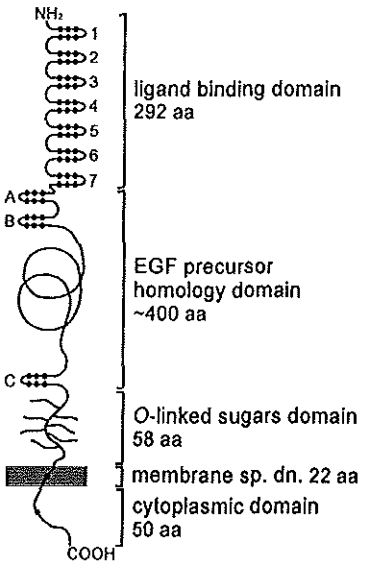

Figure 6. Domains of the mature human LDL-receptor. Cysteine residues are presented by •. The cysteine rich repeats in the ligand binding domain are designated 1–7; the growth factor repeats in the EGF precursor homology domain are designated A,B, and C. Adapted from Goldstein *et al.*, 1995.

causing FH have been described. Each of them causes a functional defect in the LDL-receptor, but at different levels. A classification of mutations has been made by Goldstein and Brown (Fig. 5) [Hobbs *et al.*, 1990; Goldstein *et al.*, 1995].

Class 1 mutations: null alleles. In cells from these patients no immunoprecipitable LDL-receptor protein is detected. In some patients, no mRNA synthesis takes place, usually as a result of a deletion including (part of) the promoter. However, in most cases there is mRNA synthesis, but a premature stop codon is caused by a nonsense mutation or by a deletion resulting in a frame shift.

Class 2 mutations: transport defective alleles. In these patients the transport of LDL-receptor protein from endoplasmic reticulum (ER) to Golgi is blocked or impaired. The reason is that the protein is improperly folded as a consequence of a missense mutation or small in frame deletion.

Class 3 mutations: binding defective alleles. In these cells cell surface LDL-receptor proteins are present, but they fail to bind LDL.

Class 4 mutations: internalization-defective alleles. In these patients, LDL-receptors are present at the cell surface and bind LDL, but fail to cluster into coated pits and are not internalized.

Class 5 mutations: recycling defective alleles. In cells from these patients, there is no uncoupling of receptor and ligand following endocytosis and subsequently no recyling of the receptor. The receptor is then finally degraded intracellularly.

The LDL-receptor is synthesized as a protein of 860 amino acids, of

which the first 21 serve as a signal sequence that is absent in the mature protein. This consists of five domains (Fig. 6). Our understanding of the function of these protein domains comes largely from the study of naturally occuring mutants (FH patients), in some cases supplemented by *in vitro* mutagenesis studies.

The first protein domain (N-terminal), the ligand binding domain, consists of seven repeats of 40 amino acids, each of which contains six cysteine residues probably all involved in disulphide bonds. This results in a very rigid structure, which explaines why many biochemical treatments like boiling in SDS do not affect the ligand binding capacity. At the C-terminus of each repeat is a triad of negatively charged amino acids present (SDE: Ser-Asp-Glu). They probably interact with domains rich in basic amino acids that are present in both ligands for the LDL-receptor, apoB-100 and apoE. These repeats are designated complement repeats, since they strongly resemble sequences found in several proteins of the complement cascade. Not surprisingly, the majority of the analyzed class 3 mutants have a mutation in the ligand binding domain. Detailed *in vitro* mutagenesis experiments were performed in order to elucidate the function of the seven 40 amino acid repeats in the ligand binding domain [Esser *et al.*, 1988; Russell *et al.*, 1989]. It was shown that deletion of repeat 1 was without effect on LDL binding activity, while deletion of any of the other repeats almost completely abolished LDL binding. Moreover, it was shown that replacement of a single conserved amino acid in any of the repeats could be equally effective, explaining how a single mutation in this region can cause FH. Binding of apoE-rich lipoproteins was only affected when repeat 5 was deleted.

The second domain is 35% identical to a portion of the epidermal growth factor (EGF) precursor. The EGF precursor is a membrane bound protein from which EGF, a peptide of 53 amino acids, is cleaved off proteolytically. The homologous part is in the non-cleaved part of the EGF precursor; there is no homology with EGF itself. It comprises three growth factor repeats, which are cysteine rich 40 amino acid sequences different from the complement repeats and a sequence containing five copies of a conserved motif YWTD (Tyr-Trp-Thr-Asp). A subset of class 3 mutations affect the EGF precursor homology domain. *In vitro* studies showed that deletions in the EGF precursor homology domain abolish LDL binding to the cellular receptor. In a ligand blot assay however, in which the protein was immobilized on nitrocellulose membranes, this mutant LDL-receptor was perfectly able to bind LDL, indicating that the EGF precursor homology domain takes care for the right spatial presentation of the ligand binding domain of the LDL-receptor on the cell surface. Other deletions gave rise to

class V mutations [Davis *et al.*, 1987], indicating that apart from its function in the LDL-receptor binding region configuration, this domain is involved in the endosomal dissociation of LDL and LDL-receptor.

The third domain is enriched in serine and threonine residues and contains two thirds of the approximately 18 O-linked sugar chains.

The fourth domain is the membrane spanning domain consisting of 22 hydrophobic amino acids.

The fifth (C-terminal) domain is the cytoplasmic tail. All known class 4 mutations have deletions affecting the cytoplasmic tail and sometimes the transmembrane region as well. In the latter case, LDL-receptors will be excreted, although they can in some cases associate with the cell surface, where they can bind LDL but do not internalize it. One class 4 mutation, the J.D. allele, has a point mutation causing a cysteine at position 807 to be substituted by a tyrosine [Davis *et al.*, 1986]. This observation led to a series of *in vitro* mutagenesis experiments, in which a tetrameric sequence, NPVY (Asn-Pro-Val-Tyr), was shown to represent the internalization signal. This sequence is completely conserved in all six species from which the LDL-receptor has been sequenced (human, rat, cow, hamster, rabbit, *Xenopus laevis*).

Mice overexpressing human LDL-receptors were among the first transgenic mouse models established [Hofmann *et al.*, 1988]. A cDNA was placed under the control of the mouse metallothionein-I promoter, Administration of cadmium led to highly increased clearance of injected radiolabeled LDL in these mice (10 times faster than in normal mice). Administration of cadmium also led to a 90% decline of the two LDL-receptor ligands, apoB-100 and apoE in plasma, while apoA-I levels were unaffected. In a later study it was shown that in mice overexpressing LDL-receptors, a high fat diet did not lead to hypercholesterolemia [Yokode *et al.*, 1990].

Mice homozygous for a targeted disruption of the LDL-receptor gene (LDL-receptor$^{-/-}$ mice) have a seven- to nine-fold increase in their plasma IDL and LDL levels [Ishibashi *et al.*, 1993]. In contrast to normal mice, low amounts of dietary cholesterol resulted in elevated plasma cholesterol levels. A high cholesterol diet induced severe atherosclerosis in these mice while control mice didn't show any sign of atherosclerosis [Ishibashi *et al.*, 1994a].

1.3 LDL-Receptor Supergene Family

In the LDL-receptor three types of protein sequences are present that have been found in other proteins as well. The proteins that share one or more of

these domains with the LDL-receptor form the LDL-receptor supergene family. The three sequences are the complement repeat, found in the ligand binding domain of the LDL-receptor, the growth factor repeats, present in the EGF precursor homology domain in the LDL-receptor, and the spacer sequences containing the YWTD motif, therefore called the YWTD region, also found in the EGF precursor homology domain in the LDL-receptor [Hobbs *et al.*, 1990].

The majority of the proteins belonging to the LDL-receptor supergene family contain exclusively growth factor repeats in one or several copies. Among them are clotting factors (Factors VII, IX, X), cell surface receptors (TGF-α precursor, ELAM-1), proteins involved in development (*Notch*, *lin-12*) and others. Some proteins share two types of sequences with the LDL-receptor (e.g. EGF-precursor, several complement factors). A limited number of proteins have all three protein domains in common. They constitute the LDL-receptor gene family [Hobbs *et al.*, 1990; Krieger and Herz, 1994]. Next to the LDL-receptor itself the two best studied proteins are the VLDL-receptor and the LDL-receptor Like Protein (LRP). They have both been implicated in lipoprotein metabolism and contain the NPXY sequence in their cytoplasmic tail that is needed for internalization via coated pits. These two proteins are described in some detail in the next paragraphs.

2. ApoE- or Remnant-Receptor

The LDL-receptor recognizes lipoproteins containing either apoB-100 (LDL) or apoE (chylomicron- or VLDL-remnants) [Herz and Willnow, 1995]. Chylomicrons, produced by the intestine, and VLDL, produced by the liver, are converted into remnant lipoproteins in the circulatory system. These have reduced levels of triglycerides, mainly caused by the action of LPL in the capillaries, and a remodelled surface, resulting in remnants in which apoE's expose their binding sites for cell surface receptors [Havel and Kane, 1995]. These remnants are quickly removed from the circulation by the liver through apoE mediated binding to the LDL-receptor and to a secondary receptor, called the remnant or apoE-receptor. This secondary receptor has been postulated because in FH patients chylomicron clearance appears to be normal [Herz *et al.*, 1988; Krieger and Herz, 1994]. Experimental evidence comes from studies in "knockout" mice deficient in the LDL-receptor, apoE or both [Ishibashi *et al.*, 1994b]. These authors showed that remnant clearance is impeded to a much higher extend in apoE$^{-/-}$ or in apoE$^{-/-}$ LDL-receptor$^{-/-}$ mice than in LDL-receptor$^{-/-}$ mice.

2.1 LDL-Receptor Related Protein (LRP)

A candidate remnant receptor was identified by Herz *et al.* [1988] who screened cDNA libraries using a probe representing the ligand binding domain of the LDL-receptor. This led to the identification of LDL-receptor Related Protein (LRP), that has a number of protein domains and motifs that are also present in the LDL-receptor.

LRP contains 4525 amino acids. After synthesis it is cleaved intracellularly between amino acids 3924 and 3925, giving rise to a large NH_2-terminal extracellular subunit, LRP515, and a smaller subunit, LRP85, containing the transmembrane region (Fig. 7). The two subunits are tightly associated in a non-covalent way [Herz *et al.*, 1990a].

Like the LDL-receptor, LRP is probably a recycling cell surface receptor because: it is a membrane bound protein with a cytoplasmic domain containing two copies of a motif that has been shown to be necessary for internalization in the LDL-receptor [Herz *et al.*, 1988]; it is present in endosomes and recycling vesicles [Lund *et al.*, 1989]; incubation of LDL-receptor deficient fibroblasts with apoE enriched VLDL (see below) in the presence of [^{14}C]-oleate resulted in intracellular accumulation of cholesteryl [^{14}C]-oleate that could be blocked by either chloroquin or an antibody directed against LRP [Kowal *et al.*, 1889]. Furthermore, studies in cultured

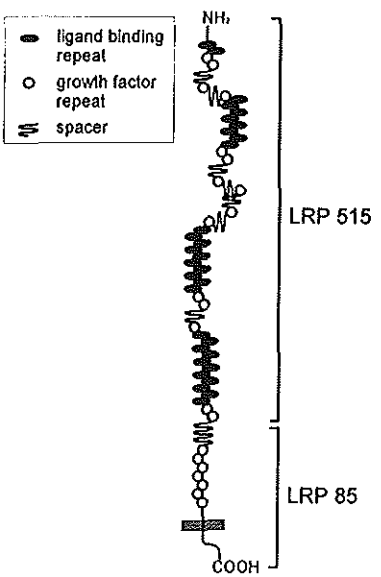

Figure 7. Domains of LRP. Adapted from Krieger and Herz, 1994.

fibroblasts showed that monoclonal antibodies directed against the LRP515 subunit, that contains the ligand binding domain, are rapidly taken up by the cells and degraded, while monoclonal antibodies directed against the LRP85 subunit are taken up and recycled [Herz *et al.*, 1990b]. This strongly suggests that the anti LRP-515 antibodies resemble ligands, that are released from the receptor in the endosomal compartment in an acid dependent way analogous to the LDL-receptor mediated cellular uptake of LDL. Injection of the monoclonal antibodies into rabbits showed a rapid uptake (half-time <10 min) by the liver [Herz *et al.*, 1990b].

LRP is believed to be a multiligand receptor [Krieger and Herz, 1994; Strickland *et al.*, 1995]. It has both lipoprotein ligands and other ligands. LRP was initially identified as a candidate apoE- or remnant-receptor. It has been shown that LRP is able to bind apoE containing lipoproteins *in vitro*, but only after artificial enrichment of the lipoproteins with apoE [Beisiegel *et al.*, 1989; Kowal *et al.*, 1989]. Interestingly, apoC is able to counteract this apoE-mediated binding [Kowal *et al.*, 1990]. An explanation for this observation has been given by the so-called secretion-recapture model (Fig. 8) [Brown *et al.*, 1991]. In this model, lipoprotein remnants become trapped into the sinusoids or, after entering the fenestrae in the liver endothelium,

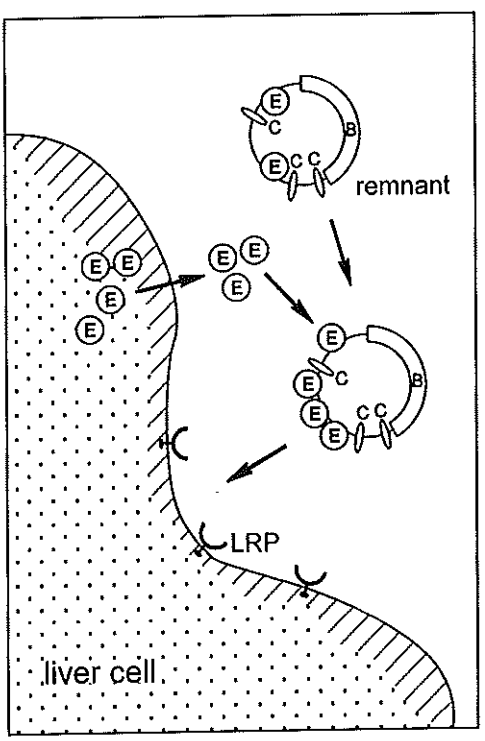

Figure 8. Secretion-recapture model for the uptake of remnant lipoproteins by hepatocytes. See text for details. Adapted from Brown *et al.*, 1991.

into the space of Disse via interactions with extracellular glycosaminoglycans. They do not interact with LRP however, because they have only low amounts of apoE and because apoC is counteracting this interaction. Hepatocytes synthesize and secrete apoE that is bound to the cell surface. This apoE can be taken up by the remnants, resulting in apoE enriched remnants, that can bind to LRP and enter the hepatocyte.

In 1990, two independent groups identified the receptor for α_2-macroglobulin (α_2-M) and concluded that this protein is identical to LRP [Kristensen *et al.*, 1990; Strickland *et al.*, 1990]. The α-macroglobulins are a group of plasma proteins that interact with a variety of proteases and control their activity by steric shielding as well as effecting rapid clearance of the proteases [reviewed in Sottrup-Jensen, 1989]. This interaction causes a conformational change that exposes the binding site for the α_2-M-receptor. This "activated" α_2-M (α_2-M*) is rapidly removed from the circulation by the liver [Sottrup-Jensen, 1989; Brown *et al.*, 1991].

A third ligand for LRP that has been identified is the plasminogen activator/inhibitor complex formed by plasminogen activator-1 (PA-1) and either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) [Andreasen *et al.*, 1994]. The plasminogen activators convert plasminogen, which is an inactive zymogen, into plasmin, a protease involved in a number of biological processes like wound healing and thrombolysis. LRP binds the PAI-1 moiety, but it can also bind non-complexed tPA [Orth *et al.*, 1994]. uPA is active on the cell-surface, where it is bound to the uPA-receptor. Inactivation of uPA by PAI-1 is followed by cellular uptake of the uPA/PAI-1 complex. This uptake is mediated by LRP [Andreasen *et al.*, 1994].

In subsequent studies, a number of other ligands of LRP were identified. Therefore, LRP is regarded as a multiligand receptor [Krieger and Herz, 1994]. Other ligands that have been described for LRP include lactoferrin [Willnow *et al.*, 1992; Meilinger *et al.*, 1995], α-1-antitrypsin-neutrophil elastase and α-1-antichymotrypsin-cathepsin G [Poller *et al.*, 1995], thrombospondin-1 [Godyna *et al.*, 1995; Mikhailenko *et al.*, 1995] hepatic lipase [Kounnas *et al.*, 1995a], secreted β-amyloid precursor protein [Kounnas *et al.*, 1995b] and exotoxin from *Pseudomonas aerigunisa* [Krieger and Herz, 1994].

In an attempt to generate LRP deficient mice by homologous recombination, it was found that LRP is essential for normal embryonal development [Herz *et al.*, 1992; 1993], since all $LRP^{-/-}$ embryos died before embryonal day 13. It is not clear to which function of LRP this observation should be attributed, but since both humans and mice deficient in apoE or LPL are viable, possible candidates are the protease-protease inhibitor

complexes that are ligands for LRP [Krieger and Herz, 1994].

At this moment, LRP remains an enigmatic protein. It binds an impressive array of ligands. Although the physiological importance of none of these interactions is known, LRP deficiency is lethal in mice, indicating that at least one of them is vital. LRP is probably able to function as a remnant receptor in the absence of LDL-receptor activity [Willnow *et al.*, 1994], but it is uncertain whether it exerts this function under normal conditions [Van Berkel *et al.*, 1994, 1995; Voorschuur *et al.*, 1994]. It is not yet clear whether LRP is involved in atherogenesis.

During the initial purification experiments of LRP, a 39 kDa protein was copurified and therefore called Receptor Associated Protein (RAP) [Kristensen *et al.*, 1990; Strickland *et al.*, 1990]. It can compete for all known ligands of LRP, which makes it a valuable experimental tool. When LRP was transiently inactivated in mice by liver expression of RAP using adenovirus vectors, a strong inhibition of α_2-M* clearance was observed while chylomicron remnant clearance was also partly impaired [Willnow *et al.*, 1994]. When LDL-receptor$^{-/-}$ mice were used, RAP expression resulted in a marked increase in chylomicron remnants. *In vitro*, RAP is also able to bind to the LDL-receptor and VLDL-receptor and compete for LDL and VLDL binding, respectively [Battey *et al.*, 1994; Medh *et al.*, 1995].

The physiological function of RAP has been elucidated recently [Bu *et al.*, 1995; Willnow *et al.*, 1995; Willnow *et al.*, 1996]. It is an intracellular protein that is mainly present in the ER. It functions as a molecular chaperone that prevents intracellular binding of receptors to their co-expressed ligands. RAP-deficient mice obtained by gene targeting have reduced levels of liver LRP. It was shown that overexpression of apoE prevents the expression of LRP, and that this effect can be counteracted by co-expression of RAP.

2.2 Very Low Density Lipoprotein-Receptor (VLDL-Receptor)

Takahashi *et al.* [1992] screened a cDNA library under low-stringency conditions using an LDL-receptor derived cDNA probe in an attempt to identify the remnant-receptor. The isolated cDNA clones were transfected into LDL-receptor deficient CHO cells which were then assayed for their ability to bind and internalize rabbit LDL, VLDL and β-VLDL. In one clone of transfected cells, the uptake of VLDL and β-VLDL, but not LDL, appeared to be highly efficient. Therefore, the protein expressed by these cells was designated the VLDL-receptor.

The VLDL-receptor is a member of the LDL-receptor gene family. Its structure more closely resembles that of the LDL-receptor than that of any

of the other identified members to date [Jingami and Yamamoto, 1995]. Both proteins consist of five domains in which the only structural difference is in the ligand binding domain: the LDL-receptor has seven, the VLDL-receptor has eight cysteine rich repeats. This may be the reason that the VLDL-receptor does not bind LDL [Takahashi *et al.*, 1992].

The tissue expression of the two proteins is completely different. The LDL-receptor is expressed in every tissue examined, but its expression in liver is the most prominent and crucial for the central role of the liver in cholesterol homeostasis. In contrast, the VLDL-receptor is hardly expressed in the liver [Takahashi *et al.*, 1992; Webb *et al.*, 1994], whereas high levels of VLDL-receptor mRNA have been found in heart, skeletal muscle and adipose tissue. Since this expression pattern resembles that of another protein involved in lipid metabolism, LPL, it has been hypothesized that the VLDL-receptor and LPL are both involved in fatty acid delivery to muscle and fat cells by lipoproteins [Yamamoto *et al.*, 1993]. However, because mRNA levels for VLDL-receptor and LPL appear to be regulated in a completely different way, this hypothesis has been seriously questioned [Jokinen *et al.*, 1994].

At present, the function of the VLDL-receptor is unknown. It has an extremely high degree of homology between species: human and rabbit VLDL-receptor proteins are 96 to 97% homologous, while the human and rabbit LDL-receptor are only 75% homologous [Sakai *et al.*, 1994; Webb *et al.*, 1994]. This suggests that its physiological function is at least as important as that of the LDL-receptor. Therefore it was quite surprising to find that mice made deficient for the VLDL-receptor by gene targeting did not show any obvious abnormalities except a slight reduction in adipose tissue mass [Frykman *et al.*, 1995]. Levels of plasma lipids and lipoproteins were not different from control mice when fed different diets. Therefore it was concluded that the VLDL-receptor does not play a significant role in the metabolism of VLDL or other lipoproteins.

In contrast to mammals, the VLDL-receptor in chickens has a well defined function [Schneider, 1995]. It mediates the uptake of large amounts of liver derived serum lipoproteins by the growing oocyte in the laying hen. It is essential for reproduction, since affected females from a chicken strain carrying a single mutation in the VLDL-receptor gene cannot lay eggs and are therefore sterile. However, VLDL-receptor deficiency in mice does not result in an apparent reduction in fertility [Frykman *et al.*, 1995].

In the 5' untranslated region of the VLDL-receptor mRNA a CGG triplet is present in 4 to 9 copies [Jingami and Yamamoto, 1995]. Trinucleotide repeats have first been described as crucial in the inheritance of the most common hereditary form of X-linked mental retardation in man [Verkerk *et*

al., 1991] and has since been found in several other monogenic diseases, including Huntington's chorea and myotonic dystrophy. During transmission from one generation to the next the number of trinucleotide repeats may increase and once a critical number is exceeded, it will affect protein synthesis or protein function [De Graaf, 1996]. Attempts to find a correlation between the CGG triplet copy number in the VLDL-receptor gene and genetic disorders in lipid metabolism have so far been unsuccesful [Jingami and Yamamoto, 1995].

3. Modified LDL and Macrophage Scavenger Receptors

3.1 Modified LDL

In atherosclerotic plaques, large amounts of LDL-derived cholesterol can be found in macrophages. However, macrophages have a low expression of LDL-receptors. Besides, in patients with FH massive depostition of LDL-derived cholesterol can be observed in macrophages, in atherosclerotic plaques and throughout the body. These observations gave rise to the postulation by Goldstein and Brown of an alternative, i.e. LDL-receptor independent pathway of LDL uptake by scavenger cells like macrophages [Goldstein *et al.*, 1979]. Since the uptake of native LDL by macrophages is low and since macrophages are known to be involved in clearance of denatured proteins, Goldstein and Brown hypothesized that uptake of LDL via the scavenger pathway was effective following modification of LDL. This might easily occur in FH patients, who have a prolonged halflife of plasma LDL. *In vitro* acetylated LDL (acLDL) was used as a model for modified LDL and it was found that macrophages have high affinity binding sites that recognize acLDL but not native LDL and that mediate uptake and degradation of acLDL followed by massive intracellular cholesterol deposition.

Steinberg and coworkers set out to identify the physiological ligand for this acLDL-receptor and came up with the hypothesis that *in vivo* oxidation of LDL, resulting in oxidized LDL (oxLDL), gives rise to such a ligand [Witztum and Steinberg, 1991]. The experimental evidence for this widely accepted hypothesis comes from the following observations: 1. Incubation of native LDL with endothelial cells or smooth muscle cells *in vitro* results in oxLDL that is rapidly taken up by macrophages; 2. Immunohistochemical studies revealed the presence of oxLDL in atherosclerotic arteries and not in normal arteries; 3. Autoantibodies recognizing oxLDL have been detected both in FH patients and in the LDL-receptor deficient rabbit strain WHHL; 4.

Antioxidants have been found to inhibit atherosclerosis in WHHL-rabbits [Witztum and Steinberg, 1991].

3.2 The Macrophage Scavenger Receptors

The binding sites on macrophages to which both acLDL and oxLDL bind have a wide specificity and are therefore called scavenger receptors [Brown and Goldstein, 1990]. The macrophage scavenger receptor (MSR) is a membrane glycoprotein that has been identified and characterized in detail by Krieger and coworkers [reviewed in Krieger and Herz, 1994]. MSR is a trimeric protein consisting of three identical subunits of 77 kDa. Two isoforms have been identified of these subunits (type I and type II) [Kodama *et al.*, 1990; Rohrer *et al.*, 1990], that later have been designated class A scavenger receptors (SR-AI and SR-AII), to distinguish them from the newly identified class B scavenger receptor (see below) [Acton *et al.*, 1994]. Type I and type II receptors are generated by alternative splicing of mRNAs that originate from a single gene. Both isoforms consist of six different protein domains (Fig. 9) [Kodama *et al.*, 1990; Rohrer *et al.*, 1990]. Domain I to V is identical in both proteins subunits, while domain VI is isoform specific [Kurihara *et al.*, 1991]. Domain V is necessary for ligand binding. Truncation of this domain abolishes the binding of acLDL [Freeman, 1994].

Domain VI in SR-AI, the Scavenger Receptor Cysteine Rich (SRCR)

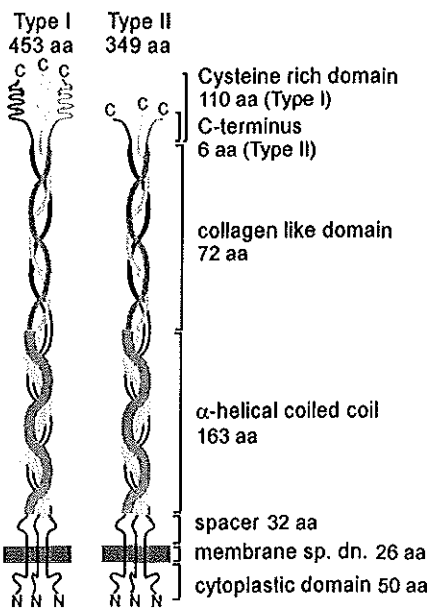

Figure 9. Domains of the scavenger receptors SR-AI and SR-AII. Adapted from Rohrer *et al.*, 1990.

domain, is present in several cell surface or secreted proteins. They form a superfamily of proteins of which many are involved in the immune system and host defence. Their SRCR-domains probably serve as binding domains for which however the ligand remains unidentified in most cases, including SR-AI [Resnick *et al.*, 1994]. Domain VI of SR-AII is only six amino acids; the SRCR is not present.

Expression of SR-A has been found exclusively in macrophages [Ylä-Herttuala *et al.*, 1991; Naito *et al.*, 1991; Krieger and Herz, 1994]. Expression of SR-A in monocyte-like HEL cells or COS cells resulted in localization of SR-A protein in the ER and Golgi apparatus, but not on the plasma membranes. Phorbol ester induded differentiation of transfected HEL cells to macrophage like cells however, resulted in localization of SR-A protein on the plasma membrane and in endosomes [Naito *et al.*, 1991], showing that the localization observed in macrophages is effected by cell type specific mechanisms. Both SR-A's can bind a number of different ligands like modified lipoproteins (acLDL, oxLDL), polyribonucleotides (e.g. polyinosinic acid), polysaccharides (e.g. dextran sulphate), anionic phospholipids (phosphatidylserine) and a number of other molecules like asbestos and bacterial toxines. Although some of these ligands are not physiologically relevant, they probably resemble structural features with the natural ligands. All these ligands are polyanionic, but a number of chemically closely related polyanions cannot serve as ligands for SR-A. Thus these scavenger receptors are multiligand receptors with a broad, but circumscribed specificity [Krieger *et al.*, 1993].

SR-A's are probably involved in host defence functions of macrophages [Krieger and Herz, 1994]. Their broad specificity is in agreement with this, as well as the observation that they are involved in macrophage recognition and clearance of certain forms of bacterial endotoxin [Hampton *et al.*, 1991]. The multiligand binding of SR-A's enable macrophages to cope with a variety of foreign and endogenous substances. For this reason SR-A has been termed "molecular flypaper" [Krieger, 1992; Krieger *et al.*, 1993].

SR-A is believed to be involved in atherogenesis. The massive accumulation of cholesterol found in macrophages in atherosclerotic plaques transforming them into foam cells, is thought to originate from oxLDL which is taken up by macrophages via SR-A. This is supported by the observation of large amounts of mRNA encoding SR-A in atherosclerotic plaques [Ylä-Herttuala *et al.*, 1991].

CD36 has been identified as an oxLDL-binding protein in expression cloning studies trying to find modified lipoprotein scavenger receptors distinct from the macrophage-specific receptors described above [Endemann *et al.*, 1993]. CD36 is expressed in a variety of tissues and is

able to bind several types of ligands, including fatty acids, thrombospondin and *Plasmodium falciparum*-infected erythrocytes. Its function is unknown [Acton *et al.*, 1994].

In a similar type of study, a novel, CD36 related, protein was shown to bind acLDL [Acton *et al.*, 1994]. This protein and CD36 itself were designated class B scavenger receptors; the newly discovered protein was termed scavenger receptor class B type I (SR-BI). Based on the DNA-sequence, this protein has a predicted length of 509 amino acids and a calculated molecular mass of 57 kDa. Later its apparent molecular weight on SDS-PAGE was shown to be ~82 kDa, the difference being attributable to glycosylation [Acton *et al.*, 1996]. It has 32% identity with CD36 along its entire length. In transfected COS cell expressing either CD36 or SR-BI it was shown that both proteins show acLDL-binding that can be inhibited by both acLDL and oxLDL, and in the case of SR-BI also by LDL. In similar experiments it was shown that SR-BI, but not CD36, can bind LDL. Northern blotting showed that SR-BI is expressed highly in fat cells and moderately in lung and liver. Expression in other tissues was very low. In 3T3 cells differentiated into adipocytes expression was high, while expression was not or hardly detectable in undifferentiated cells. It was suggested that SR-BI plays a role in lipid metabolism. The subsequent finding that SR-BI and CD36 can bind anionic phospholipids is in agreement with such a function [Rigotti *et al.*, 1995]. Recently, it was proposed that SR-BI acts as an HDL-receptor (section III.4.2) [Acton *et al.*, 1996].

Chapter III

High Density Lipoproteins

1. HDL and Atherosclerosis

The interest in HDL metabolism comes from the inverse correlation between plasma levels of HDL and the incidence of coronary artery disease in man. Although this correlation was noticed already in 1950 [Gofman *et al.*, 1950], it received little attention during the next quarter of the century. Then, Miller and Miller [1975] re-established the interest in this relation and explicitely formulated the hypothesis that HDL is an anti-atherogenic factor. This was followed by large epidemiological studies in which the inverse relationship was confirmed: The Honolulu heart study in 1976 and the Framingham and Tromsø heart studies in 1977 [Rhoads *et al.*, 1976; Gordon *et al.*, 1977; Miller *et al.*, 1977]. In the next decade, many other prospective epidemiological studies in several countries corroberated the now generally accepted view, that there is an independent, strong, inverse correlation between plasma levels of HDL and the incidence of coronary artery disease [reviewed in Gordon and Rifkind, 1989].

Several hypotheses have been proposed to explain how HDL protects against the development of atherosclerosis. By far the most widely accepted hypothesis is that HDL mediates transfer of cholesterol from extra-hepatic cells to the liver for excretion. This is called reverse cholesterol transport, as opposed to ("forward") transport from the liver to cells mediated by VLDL and LDL. Reverse cholesterol transport is discussed below (section 3).

An alternative explanation is based on the relation beween low levels of HDL and elevated plasma triglycerides. Some authors have suggested that HDL might not be a risk factor on its own, but mirror the presence or absence of another factor, i.e. high triglycerides levels [Gordon and Rifkind, 1989; Tall, 1990].

Another possible explanation for the anti-atherogenicity of HDL is the interference with the oxidation of LDL and its general protection against oxidative damage [Forte and McCall, 1994].

2. HDL Subclasses

HDL is a heterogeneous pool of lipoproteins that can be divided into several subclasses using different parameters [reviewed by Von Eckardstein *et al.*, 1994]. The most commonly used classifications are summarized in Table III and annotated below.

Density. Normally, circulating plasma HDL is present in two pools that differ in size and buoyant density, designated HDL_2 and HDL_3 [Tall, 1990;

Table III: HDL subclasses

density	ultracentrifugation	HDL_2 HDL_3	1.063 - 1.125 g/ml 1.125 - 1.21
diameter	non denaturating polyacrylamide gradient gel electrophoresis (PAGGE)	HDL_{2a} HDL_{2b} HDL_{3a} HDL_{3b} HDL_{3c}	9.7 - 12 nm 8.8 - 9.7 8.2 - 8.8 7.8 - 8.2 7.2 - 7.8
charge	agarose gel elctrophoresis	α-HDL preβ-HDL → PAGGE:	 $pre\beta_1$-HDL $pre\beta_2$-HDL $pre\beta_3$-HDL
apolipoprotein-content	sequential immuno-affinity chromatography	LpA-I LpA-I/A-II LpA-I/A-IV LpA-IV	

Havel and Kane, 1995]. They can be interconverted into one another by the action of various enzymes like LCAT, CETP and PLTP. The relation between HDL_2 and HDL_3 and the incidence of coronary artery disease has been investigated by several groups, but the results are conflicting. During the last decade, an increasing number of researchers believe that HDL_2 and HDL_3 are just physicochemically stable complexes and not subfractions with important differences in metabolic properties. [Von Eckardstein *et al.*, 1994].

Diameter. A further subclassification of HDL can be achieved by nondenaturating polyacrylamide gradient gel electrophoresis [Nichols *et al.*, 1986]. The obtained fractions do not completely match the ultracentrifugation fractions. Since the method of isolation is laborious and the functional meaning of the subfractions is unclear, this classification is rarely used.

Charge. Two-dimensional gel electrophoresis using agarose in one dimension and nondenaturating polyacrylamide gradient gel electrophoresis in the second dimension allows the separation of alpha-HDL, prebeta-1 HDL, prebeta-2 HDL and prebeta-3 HDL. These subfractions are believed to be functionally important in reverse cholesterol transport (see section 3.1.2) [Fielding and Fielding, 1995].

Apolipoprotein content. Using antibodies directed against apolipoproteins, immunoadsorption techniques allow the separation of HDL

subclasses that differ in their apolipoprotein composition. The most frequently studied subfractions are HDL particles that contain apoA-I but not apoA-II (LpA-I) and HDL particles that contain both apoA-I and apoA-II (LpA-I/A-II). In normal human plasma, 25% of circulating apoA-I is present in LpA-I particles and 65% in LpA-I/A-II particles. The majority of LpA-I is present in the HDL_2 fraction; the majority of LpA-I/A-II is present in the HDL_3 fraction [Fruchart *et al.*, 1993; Von Eckardstein *et al.*, 1994].

During the last few years, the significance of LpA-I and LpA-I/A-II subclasses in HDL metabolism and atherosclerosis has been studied by several groups. [Leroy *et al.*, 1995]. Puchois *et al.* [1987] designated LpA-I the anti-atherogenic fraction, because they observed a reduction in LpA-I but not in LpA-I/A-II in coronary artery disease patients. Although several subsequent studies showed reduced levels of LpA-I/A-II in coronary artery disease patients [Von Eckardstein *et al.*, 1994], studies in transgenic mice corroborated the initial suggestion (see section 3.2.3). While transgenic mice overexpressing human apoA-I are highly resistant against diet induced atherosclerosis, transgenic mice overexpressing both human apoA-I and apoA-II showed much smaller areas of atherosclerotic lesions following the same diet [Schultz *et al.*, 1993].

Although their significance is only partially understood, at present the LpA-I and LpA-I/A-II subfractions are generally believed to be physiologically relevant.

3. Reverse Cholesterol Transport

The most widely accepted explanation for the anti-atherogenicity of HDL is its involvement in reverse cholesterol transport [Pieters *et al.*, 1994]. This is the tranfer of cellular cholesterol by HDL to the liver, where it can be excreted directly, or following the conversion to bile acids, into the bile. Although the concept was formulated almost thirty years ago [Glomset, 1968], there is still much debate about the mechanism of the different steps in the reverse cholesterol transport pathway [Pieters *et al.*, 1994; Breslow, 1995; Fielding and Fielding, 1995].

Reverse cholesterol transport has been mostly studied *in vitro* (section 3.1), although some groups performed *in vivo* studies (section 3.2). Other *in vivo* studies, notably those on transgenic mice, that focussed on HDL metabolism and the anti-atherogenic properties of HDL rather than on reverse cholesterol transport *per se*, are discussed in this chapter as well, together with human disorders in HDL metabolism.

3.1 *In vitro* Studies

The first step is the uptake of cellular cholesterol by HDL. Several mechanisms have been proposed for this process (see section 3.1.1). The free cholesterol at the HDL surface is esterified by lecithin-cholesterol acyltransferase (LCAT), a plasma enzyme that is associated with HDL and that has apoA-I as a cofactor [Jonas, 1991; Glomset *et al.*, 1995]. The cholesterol ester is sequestered into the lipoprotein core. In this way, LCAT provides a driving force for the continuous influx of cellular free cholesterol into the HDL particle.

LCAT causes an increase in HDL particle size. Remodelling of HDL in plasma is further effectuated by the action of two lipid transfer proteins [Fielding and Fielding, 1995; Tall, 1995]. CETP (or lipid transfer protein-1) transports part of the HDL-cholesterol esters to VLDL and LDL. PLTP (or lipid transfer protein-2) has HDL-conversion activity [Jauhiainen *et al.*, 1993] and possibly enhances the efficiency of the LCAT-reaction by supplying lecithin to HDL [Tall, 1995].

The final step in the reverse cholesterol transport pathway is the uptake of HDL-cholesterol by the liver (section 3.1.3). There are three routes for this uptake. The first is receptor-mediated uptake processes of VLDL remnants and LDL by the liver, following the CETP-mediated transfer of HDL-cholesterol to VLDL and LDL. The contribution of this route to the total of cholesterol clearance by the liver is probably largely species dependent, since considerable differences in CETP-activity have been observed among species [Tall, 1995]. In rabbits, CETP-activity is high; Goldberg *et al.* [1991] estimated that liver-uptake of cholesterol occured for 70% via this route. In humans, CETP-activity is four times lower than in rabbits, while in mice and rats, CETP-activity is absent [Swenson, 1992]. The second route is direct uptake of HDL particles, the third route is selective uptake of HDL-cholesterol esters without simultaneous uptake of HDL-protein.

3.1.1 Mechanisms of Efflux

The first step in reverse cholesterol transport is the uptake of cellular cholesterol by HDL. There are three concepts explaining how this interaction takes place [Rothblat *et al.*, 1992]: passive diffusion of cholesterol from the plasma membrane to HDL, receptor mediated efflux of cellular cholesterol to HDL and retroendocytosis of HDL-particles. Each of them is based on a considerable amount of experimental evidence. However, all biochemical studies use artificial model systems, which renders the validity of the conclusions for the *in vivo* situation uncertain. It is

possible that efflux of cholesterol occurs via different pathways, maybe dependent on cell type or metabolic (cholesterol) status of the cells. Since the key factors regulating these pathways remain unidentified, it is difficult to evaluate their physiological relevance.

Efflux by passive diffusion: membrane cholesterol domains and the apoA-I-anchor model

Rothblat and coworkers extensively studied the kinetics of cholesterol efflux from cell membranes to (reconstituted) HDL and concluded that they can be best explained by passive diffusion of free cholesterol through the aquous phase between the membrane and the HDL particles [Johnson *et al.*, 1988; Johnson *et al.*, 1991]. This efflux is not affected by specific binding of HDL to the cell surface, since treatments of HDL which have been shown by others to strongly reduce binding do not reduce cholesterol efflux from cells [Karlin *et al.*, 1987; Johnson *et al.*, 1988]. However, some observations cannot be explained by the physical properties of an aqueous diffusion model. For instance, fundamental differences in efflux have been noticed dependent on the cell type used [Bernard *et al.*, 1990; Mahlberg *et al.*, 1992]. Differences have also been observed using acceptor particles with different apolipoprotein compositions [Mahlberg *et al.*, 1991]. Therefore, a speculative model has been proposed by Rothblat *et al.* [1992] that explains the complex kinetics of cholesterol efflux from cultured cells to HDL. The existence of plasma membrain domains with differences in lipid composition is a central premise in this model.

In the plasma membrane, the content and packaging of cholesterol is dependent on phospholipid composition, the phospholipid/cholesterol ratio and the presence of membrane proteins and of other lipids like sphingomyelin [Fielding and Fielding, 1995]. These factors might differ within cellular membranes, thus establishing membrane domains. Intracellular lipid transfer proteins like sterol carrier protein 2 (SCP2) and L-FABP might play a role in the maintainance of or fluctuations in the membrane domains [Jefferson *et al.*, 1991; Schroeder *et al.*, 1991]. Cholesterol efflux from cholesterol rich domains is supposed to require more energy than efflux from cholesterol-poor domains since the latter are less tightly packed and hence the association of cholesterol with the membrane is less strong. As a consequence, cholesterol is present in the plasma membrane in different kinetic pools.

Apolipoproteins associated with HDL (apoA-I, apoA-II, apoA-IV and apoC's) have amphipatic helices, which enable these proteins to associate with lipids. In apoA-I a "hinged domain" has been proposed, that could result in a part of the protein that is extending into the aqueous phase rather

than remaining associated with the surface of the HDL particle [Segrest *et al.*, 1992]. This extending part could easily associate with a cellular membrane, facilitating cholesterol efflux from the membrane to the HDL particle. In disclike HDL particles containing only apoA-I the apolipoprotein is most likely to have its "open hinge" structure [Segrest *et al.*, 1992]. These particles have been shown to be the most efficient acceptors for free cholesterol [Casto and Fielding, 1988].

Cholesterol taken up by disclike HDL, will be esterified by LCAT and generate a hydrophobic lipid core. The resulting spherical HDL particle will have its apoA-I in a reorganized conformation, resulting in a close association with the lipoprotein surface and a reduced interaction with the plasma membrane.

Receptor-mediated efflux of cellular cholesterol

Many authors have suggested that a specific HDL-receptor is involved in reverse cholesterol transport. Although several cellular HDL-binding proteins have been identified (see section 4.2), it is not clear whether any of these proteins function in HDL-mediated steps in the reverse cholesterol transport pathway or in HDL metabolism. One exception is an HDL-binding protein of 110 kDa that has been implicated in the uptake of cellular cholesterol by HDL in a series of studies by Oram and coworkers.

Graham and Oram [1987] identified an HDL-binding protein by ligand blotting of 110 kDa that is present in the membranes from a number of cultured cell types. Cholesterol loading of cells leads to an increased binding activity while binding is abolished by trypsinization of cells. Binding of HDL is not followed by cellular internalization [Oram *et al.*, 1987], but to a translocation of cholesterol from intracellular pools to the plasma membrane [Slotte *et al.*, 1987]. This translocation is mediated by a protein kinase C signalling pathway [Mendez *et al.*, 1991], suggesting that the 110 kDa HDL-binding protein might be a G-protein coupled receptor [Oram *et al.*, 1993]. This translocation model has however been questioned by others [Johnson *et al.*, 1991; Fielding and Fielding, 1995], because it is based on studies using unphysiological conditions (lipid loaded cells) and because it is unlikely that translocation of intracellular cholesterol contributes considerably to cholesterol efflux since the plasma membrane contains 80-90% of cell cholesterol.

Although a cDNA clone encoding the 110 kDa HDL-binding protein (now designated HBP) was isolated [McKnight *et al.*, 1992], conclusive evidence that this protein represents a true HDL-receptor is still lacking. HBP lacks a transmembrane domain and does not resemble any known receptor. Later it was found that HBP is identical to a protein that has been termed vigilin

and that has been isolated from chicken chondrocytes [Schmidt *et al.*, 1992; LeBoeuf *et al.*, 1994; Plenz *et al.*, 1994]. This protein is located intracellularly [Neu-Yilik *et al.*, 1993]. Its function is unknown.

Retroendocytosis of HDL particles

A decade ago, Schmitz *et al.* [1985] published an electron microscopic study showing that HDL can bind to macrophages, and is subsequently endocytosed and finally resecreted. This process is termed retroendocytosis. Rogler *et al.* [1991, 1992] also showed electron microscopic data confirming a retroendocytosis pathway for HDL in IEC-6 crypt-derived rat epithelial cells and CaCo-2 cells. Both groups used colloidal gold labeled HDL, which may give rise to scepticism about the physiological relevance of these observations since this label dramatically changes both the size and the electric charge of the particles. However, Takahishi *et al.* [1989] demonstrated a retroendocytosis pathway in rat peritoneal macrophages using HDL labeled with other tags (horseradish peroxidase and ferritin).

Additional support for a retroendocytosis pathway for HDL comes from biochemical studies showing that cultured cells are able to take up and subsequently resecrete HDL, labeled either radioactively or by a pH sensitive fluorescent probe that allows the monitoring of the microenvironmental pH of the cell-associated HDL [Takata *et al.*, 1988; DeLamatre *et al.*, 1990; Kambouris *et al.*, 1990; Rahim *et al.*, 1991]. However, other groups did not find any *in vitro* evidence for internalization in cells [Oram *et al.*, 1987; Alsat and Malassini 1991].

3.1.2 Subfractions Involved in Efflux

An intriguing question is whether certain HDL subfractions are more efficient or maybe predominant in affecting cholesterol efflux from cells. Several researchers compared cholesterol efflux to LpA-I versus LpA-I/A-II subfractions, and found them to be equally effective in most cases. However, the quantitatively minor prebeta-HDL subfraction appeared to be an extremely efficient cholesterol acceptor compared to alpha-HDL.

Prebeta- versus alpha-migrating HDL

Plasma HDL consists mainly of alpha-HDL; only 2 to 5% of the particles have prebeta (slow) electropheretic mobility [Ishida *et al.*, 1987]. Prebeta-1 HDL has also been termed lipid-poor apoA-I. The particles have a diameter of 5-6 nm; their molecular mass is about 60-70 kDa. Isolation of HDL by

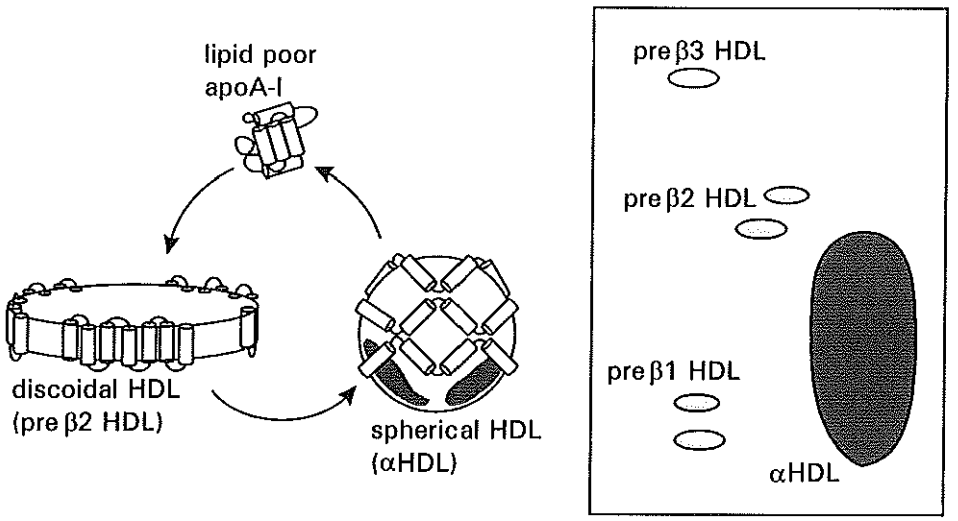

Figure 10. Structure (left) and electropheretic mobility (right) of preβ-HDL and α-HDL particles. Adapted from Pieter *et al.*, 1994; and Fielding and Fielding, 1995.

ultracentrifugation results in a complete loss of the particles. ApoA-I conformation in prebeta-HDL differs from that in other HDL (Fig. 10), as could be concluded from studies using specific proteases and the possibility to generate monoclonal antibodies that specifically recognize prebeta-1 HDL [Fielding *et al.*, 1994].

Prebeta-2 HDL or discoidal HDL is about 2 to 3% of total plasma HDL. ApoA-I is the only protein present. These particles have the shape of a disc, with the thickness of a phospholipid bilayer and a variant diameter (Fig. 10). The conformation of apoA-I in discoidal HDL as shown in Fig. 10 is deduced from computer modelling studies.

Spherical HDL (alpha-HDL) is the majority of circulating HDL. The diameter of the particles is 9-12 nm.

Castro and Fielding [1988] showed that when fibroblasts containing radioactively labeled cholesterol are briefly (1 min) incubated with normal human plasma, a relatively high amount of label can be traced in the small prebeta fraction of HDL. Subsequent incubations of 2 minutes after transfer to a dish with unlabeled cells resulted in the transfer of label to the larger prebeta-HDL fractions and to alpha-HDL. Thus cholesterol is transferred from the plasma membrane to prebeta-1 HDL, and only appears in prebeta-2 HDL later. Discoidal HDL might emerge from fused prebeta-1 particles or from the acquirement of lecitin from cell membranes by prebeta-1 particles. These processes occur mainly in lymph, which has a higher concentration

of both prebeta-1 HDL and discoidal HDL than plasma. The discoidal HDL then enters the plasma, where its cholesterol is esterified by LCAT. Discoidal HDL is a very good substrate for LCAT, probably because the conformation of apoA-I, the activator for LCAT, allows an optimal interaction between LCAT and the activating domains of apoA-I in discoidal HDL. This interaction leads to spherical HDL [Francone and Fielding, 1990]. In agreement with this concept, in LCAT deficiency discoidal HDL accumulates in the circulation that resembles prebeta-2 HDL [Glomset *et al.*, 1995]. Efflux of cholesterol from the membrane occurs via specific (60%) and nonspecific (40%) ways [Kawano *et al.*, 1993]. This has been quantified by using plasma that had been depleted of prebeta-1 HDL: this led to a 60% reduction of cholesterol efflux from cultured fibroblasts. Specific efflux is thus defined as efflux occuring ot prebeta-HDL. Intracellular lipid transfer proteins might regulate this process by controlling the cholesterol content of domains in the plasma membrane. The nonspecific part possibly involves albumin as an acceptor.

In the liver, prebeta-1 HDL can be generated from spherical HDL by the action of hepatic lipase.

LpA-I versus LpA-I/A-II

Prebeta-HDL contains apoA-I as the only apolipoprotein. Therefore, Fielding and Fielding [1995] postulate that reverse cholesterol transport occurs via LpA-I and that LpA-I/A-II HDL particles are probably not involved in it.

Other researchers compared the efficacy of LpA-I and LpA-I/A-II isolated from plasma in *in vitro* cholesterol efflux studies. Barbaras *et al.* [1986, 1987] found that LpA-I was effective in mediating cholesterol efflux from mouse fat cells Ob1771, while LpA-I/A-II was not. However, this effect seem to be restricted to the fat cells used by this group, since several other groups failed to find any difference between LpA-I and LpA-I/A-II mediated cholesterol efflux in various cell types [Johnson *et al.*, 1991; Von Hodenburg *et al.*, 1991; Oikawa *et al.*, 1993].

3.1.3 Uptake of Cholesterol by the Liver

The final step in the reverse cholesterol transport pathway is the delivery of HDL-cholesterol to the liver. The mechanism of interaction of HDL and liver cells remains ill-defined [Breslow, 1995].

HDL-binding and HDL-binding proteins

Various groups described specific binding of HDL and HDL-binding proteins in liver. However, to date a direct relation between HDL-binding and uptake

of HDL-lipids or -proteins in the liver has been demonstrated in only one study [Acton *et al.*, 1996] (see section 4).

Selective uptake of HDL cholesterol esters

In HDL decay studies, Glass *et al.* [1983] showed that the liver is quantitatively the most important organ, since it takes up 65% of an injected dose of radioactively labeled homologous HDL in rats. The adrenal gland and ovary are the most active organs per gram wet weight. In this study, the uptake of HDL-protein and HDL-cholesterol was simultaneously monitored by the use of undegradable tracers (^{125}I-tyramine cellobiose coupled to apoA-I and [^{3}H]cholesterol *ethers* [representative for the uptake of cholesterol *esters*] incorporated into the lipid core of the lipoprotein). It appeared that the uptake of the two radiolabels was disproportionate: the relative uptake of cholesterol ethers was 2-fold higher than the uptake of apoA-I in the liver, 4-fold higher in the ovary and 7-fold higher in the adrenal gland. The uptake was equal in all other tissues studied. It was concluded that HDL cholesterol esters can be taken up without parallel uptake of whole particles. This phenomenon, that was already noticed in an earlier study [Van 't Hooft *et al.*, 1981], was given the plain designation *selective uptake*, and was shown to take place in cultured cells of mouse and human origin as well [Pittman *et al.*, 1987; Rinninger and Pittman, 1988]. Selective uptake was also shown to occur in the presence of drugs inhibiting receptor recycling or endocytosis [Pittman *et al.*, 1987].

Pieters *et al.* [1991] showed that hepatocytes are responsible for the selective uptake in the liver. These cells take up HDL-cholesterol ester six times more efficiently than HDL-protein, while both liver endothelial cells and Kupffer cells show a parallel uptake of HDL-cholesterol ester and -protein. In the same study it was shown that uptake of HDL-cholesterol esters is more efficiently coupled to bile acid synthesis than the uptake of LDL-cholesterol esters.

In another study from the same laboratory [Pieters *et al.*, 1993], it was shown that although selective uptake by hepatocytes of cholesterol esters from either LpA-I or LpA-I/A-II is equally effective, the subsequent conversion into bile acids is more efficient in animals injected with LpA-I. The mechanism of this unexpected observation is unknown.

Goldberg *et al.* [1991] estimated that 10% of the uptake of HDL cholesterol esters in rabbits occured via whole particle uptake, 20% via selective uptake and 70% via transfer to LDL and VLDL. Because rabbits have a plasma cholesterol ester transfer activity that is approximately four times higher than in man, selective uptake is believed to be of major importance in HDL cholesterol ester clearance in humans.

It was initially reported that in transgenic mice expressing human apoA-I, no selective uptake of HDL cholesterol esters takes place [Chajek-Shaul *et al.*, 1991]. This conclusion was based on plasma decay studies, This issue was however recently re-evaluated by Khoo *et al.* [1995] who showed that selective uptake in liver was apparent when tissue samples were examined. It was also shown that the kidney takes up ApoA-I preferentially. This is probably the reason that selective uptake is not apparent in plasma decay studies.

Role of hepatic lipase

Hepatic lipase is an enzyme that is able to hydrolyze both triglycerides and phospholipids [Jansen and Hülsmann, 1985]. Its role in HDL metabolism is only partly understood [Bensadoun and Berryman, 1996].

Both Kadowaki *et al.* [1992] and Marques-Vidal *et al.* [1994] performed perfusion studies in rat liver using reconstituted HDL containing various radiolabels. They showed that depletion of hepatic lipase by preperfusion with heparin markedly reduced the HDL-cholesterol ester uptake. HDL reconstituted with a non-hydrolyzable phospholipid analog also resulted in a considerable reduction of HDL-cholesterol ester uptake. They concluded that hepatic lipase mediated hydrolysis of HDL-phospholipids promoted the hepatic uptake of HDL-cholesterol esters.

Overproduction of hepatic lipase in transgenic mice [Bush *et al.*, 1994] or transgenic rabbits [Fan *et al.*, 1994] results in reduced levels of plasma HDL. In hepatic lipase deficient mice generated by gene targeting levels of HDL are elevated [Homanics *et al.*, 1995].

Barrans *et al.* [1994] showed by rat liver perfusion studies that hepatic lipase activity results in the formation of preβ-1 HDL from HDL_2, and thus might play an important role in reverse cholesterol transport (section 3.1.2).

3.2 *In Vivo* Studies

3.2.1 Experimental Animals

Because it is difficult to perform analyses in whole animals and to interpret the resulting data, only a few studies have been carried out to obtain *in vivo* evidence of reverse cholesterol transport.

Miller *et al.* [1985] injected rabbits with human (native) LDL or acLDL with a dose that resulted in an almost 10-fold increase in LDL levels in plasma. Within 24 hours, LDL levels returned to the initial values. In the following days LDL levels did not change. In contrast, HDL levels progressively increased in all animals by 14 to 43%. These results suggest

that the rise of HDL levels is a regulatory stimulation of reverse cholesterol transport in these animals.

Badimon *et al.* [1989] fed rabbits an atherogenic diet for 8 weeks. Animals were given daily injections with homologous HDL or saline. In both groups of animals, the atherogenic diet resulted in fatty streak lesions in the intimal aorta. However, the area was 60% smaller in HDL-injected animals in comparison with saline-injected animals. Cholesterol deposistion in aorta and liver was also significantly lower in HDL-treated animals.

In the same laboratory, regression of atherosclerosis by HDL-injections in rabbits was studied [Badimon *et al.*, 1990]. Three groups of animals were fed an atherogenic diet for 60 days (group 1) or 90 days (group 2 and 3). During day 61 to 90, animals were given daily injections with saline (group 2) or homologous HDL (group 3). At the end of the experimental period, the area of atherosclerotic lesions was identical in group 1 and 2, but markedly reduced in group 3. This study shows that HDL can effectuate regression of pre-existing atherosclerosis. Injection of apoA-I in cholesterol fed rabbits proved to be anti-atherogenic as well [Miyazaki *et al.*, 1995].

None of these studies allowed the researchers to observe reverse cholesterol transport operating *in vivo*. Therefore, Bakkeren *et al.* [1990] used an elaborate experimental set-up in order to make direct observations of reverse cholesterol transport in rats. The animals were injected with human acLDL with [^{3}H]-labeled cholesterol esters incorporated. A permanent heart catheter allowed injection and subsequent blood sampling, showing a rapid serum decay of radioactivity. By isolating hepatocytes, endothelial cells and Kupffer cells from the liver, the authors show that cholesterol is mainly taken up by the endothelial cells (60% of the administered dose within 15 min). A subsequent release of labeled cholesterol from the endothelial cells could be monitored, that was mainly recovered in HDL. This caused a decrease in the amount of total radioactivity in the liver between 15 and 30 min after injection. In the following 1 to 2 hours, liver-associated radioactivity increased again, which was caused by uptake of cholesterol by the hepatocytes. Since rats were provided with a permanent catheter in the bile duct, continuous sampling of bile could be carried out. A gradual increase in radioactivity appearing into the bile was monitored 4 to 12 hours after injection. Radioactivity was present mainly in bile acids (90%). This study is among the most convincing evidence published showing the operation of reverse cholesterol transport, since the sequence of appearance of cholesterol label into cells, plasma HDL and bile is exactly as expected.

3.2.2 Familial Disorders of HDL Metabolism

HDL deficiencies are extremely rare. Many of them have been found in only one family. The most prevalent cause of HDL deficiency, familial LCAT-deficiency, has been described in 30 families.

Familial LCAT deficiency
Familial LCAT deficiency was discovered in Norway in 1966 [Norum and Gjone, 1967]. About 60 patients from 30 different families have been found [for a recent review, see Glomset *et al.*, 1995]. The disorder is inherited in an autosomal recessive mode. Symptoms include corneal opacities, anemia, proteinurea. Only a few patients showed signs of atherosclerosis. All lipoprotein levels are abnormal. Levels of plasma HDL are reduced to 20 to 30% of control values. A major part of circulating HDL particles are disc-shaped; spherical HDL is unusually small (~6nm). LCAT-activity in plasma is absent; LCAT protein has been detected in some patients.

Fish eye disease
From 1975 to 1995, seven patients have been reported from four different families in Sweden, Canada, Germany and The Netherlands suffering from fish eye disease. In this disorder [reviewed in Glomset *et al.*, 1995], inherited as an autosomal recessive trait, corneal opacities are the only clinical symptom. These opacities make the eyes look like those of boiled fish, hence the name of the disease. The patients have elevated levels of triglycerides and VLDL. HDL levels are 10% of normal values. HDL is mostly small and spherical; some HDL is discoidal. Cholesterol esters are absent in HDL but present in LDL and VLDL. Premature coronary artery disease is not observed in patients with fish eye disease.

LCAT-activity toward LDL and VLDL is normal, while LCAT-activity toward HDL is (nearly) absent. It has been shown that fish eye disease is caused by specific mutations in the LCAT-gene that differ from those observed in familial LCAT deficiency. Since mutations causing fish eye disease as well as mutations causing familial LCAT-deficiency both have been found in different parts of the LCAT-gene, it is impossible to attribute one of these disorders to structural defects in a functional domain of the protein.

Tangier disease
Tangier disease [reviewed in Assmann *et al.*, 1995] was originally described in two sibs from Tangier island in Virginia [Frederickson, 1961]. Today, about 55 patients are known from all over the world. They have virtually no

plasma HDL (plasma apoA-I levels < 3% of controls). Cholesterol esters are deposited in many tissues throughout the body. As a consequence, the tonsils become hyperplastic and yellowish-orange, which is a characteristic symptom of these patients. Tangier disease is inherited in an autosomal recessive mode. No mutation has been found in the apoA-I gene [Makrides *et al.*, 1988]. Symptoms of cardiovascular disease have been observed in approximately half of the Tangier patients. Autopsy data showed considerable differences in the severness of atherosclerosis, indicating heterogeneity of patients in their risk for coronary artery disease [Serfaty-Lacrosniere *et al.*; 1994]. Cultured fibroblasts from Tangier patients have an impaired efflux of cellular cholesterol to HDL or apoA-I [Walter *et al.*, 1994; Francis *et al.*, 1995]. This probably causes the rapid clearance of HDL and apoA-I particles that have been observed in Tangier patients. Although the molecular defect underlying the impaired efflux is not known, Francis *et al.* [1995] reported that high affinity binding of apoA-I to Tangier fibroblasts is strongly reduced, suggesting a defect in an HDL-binding protein that mediates the efflux of cellular cholesterol. This protein remains unidentified; levels of the candidate protein HBP (section 3.1.1) are apparently unchanged [Francis *et al.*, 1995].

ApoA-I deficiency
Deficiency of apoA-I has been described in a few individual cases [Assmann *et al.*, 1993; Breslow, 1995; Miccoli *et al.*, 1996]. In five cases, coronary artery disease was apparent. In three other patients, coronary artery disease was not apparent. However, one of them was only seven years old when the mutation was discovered [Lackner *et al.*, 1993], which could be the reason that coronary artery disease symptoms were absent. Another patient might be an atypical fish eye disease patient, since he had massive corneal opacifications and half-normal levels of LCAT-activity [Brelow, 1995]. Besides, individual heterogeneity of these patients can lead to differences in coronary artery disease susceptibility, as has been suggested for Tangier disease patients [Serfaty-Lacrosniere *et al.*, 1994].

ApoA-II deficiency
The only known case of apoA-II deficiency has been reported by Deeb *et al.* [1990]. The affected individuals are two sisters from Japan who are clinically normal and who have no signs of coronary artery disease. Their HDL-cholesterol levels are normal.

3.2.3 Transgenic Mice

In transgenic mice expressing human apoA-I the plasma levels of murine apoA-I are 85 to 95% reduced [Chajek-Shaul *et al.*, 1991; Rubin *et al.*, 1991]. The particle size distribution of HDL is also different in transgenic mice: in control mice, HDL is present in one homogeneous pool, while in the transgenic mice HDL is present in two distinct pools that resemble human HDL_2 and HDL_3 in size.

Murine apoA-II levels were reduced by 45% in human apoA-I expressing transgenic mice. As a result, HDL in these mice consist mainly of "humanlike" LpA-I.

In the study by Rubin *et al.* (1991), control mice and transgenic mice expressing human apoA-I were fed either a mildly atherogenic diet (diet A) or a highly atherogenic diet (diet B) for 14 or 18 weeks, respectively. Control mice developed atherosclerotic lesions on both diets. Diet B resulted in a 2.5-fold larger lesion area than diet A. Transgenic animals however, did not develop any lesions on diet A, and only limited atherosclerosis on diet B (lesion area 15% of controls). Thus high level expression of apoA-I protects against atherosclerosis induced by a high fat diet.

Transgenic mice expressing human apoA-II showed plasma levels of 50 – 100% of endogenous apoA-II expression [Schultz *et al.*, 1992]. As in humans, expression was liver specific, and human apoA-II was present as a homodimer in the plasma of transgenic animals. Levels of HDL-cholesterol and endogenous apoA-I and apoA-II were unchanged by expression of the transgene. HDL particle size distribution was unchanged by human apoA-II expression apart from the appearance of a separate class of small HDL particles that contain only apoA-II. The presence of these small HDL particles was more prominent in the trangenic lines expressing relatively high levels of apoA-II.

When these mice were crossed with human apoA-I expressing transgenic mice, HDL particle size distribution was markedly different from both apoA-I– or apoA-II– transgenic mice. These results show that apoA-II-expression affects the HDL particle size distribution [Schultz *et al.*, 1992].

Transgenic mice overexpressing murine apoA-II showed a 2- to 3-fold elevation of plasma levels, resulting in a 2-fold increase in HDL levels [Hedrick *et al.*, 1993]. The HDL particle size was increased.

Comparison of the results from studies of transgenic mice expressing either human or murine apoA-II shows that the effects of apoA-II expression on HDL are species dependent. Human apoA-II differs both in primary sequence as in conformation from murine apoA-II: in contrast to murine apoA-II, human apoA-II is present as a homodimer, which possibly affects

the size of apoA-II containing HDL particles [Hedrick *et al.*, 1993].

Transgenic mice overexpressing murine apoA-II are much more prone to the development of atherosclerosis than control mice [Warden *et al.*, 1993]. This led to the conclusion that not only the amount, but also the protein composition of HDL determines its anti-atherogenic properties, a conclusion confirmed by Schultz *et al.* (1993). In this study, two lines of transgenic mice were compared, one overexpressing human apoA-I and one overexpressing both human apoA-I and apoA-II. In these mice, the particle size distribution of HDL was examined. Transgenic mice expressing human apoA-I showed an HDL particle size distribution that was reminiscent to the distribution of LpA-I isolated from human plasma. In mice expressing human apoA-I and apoA-II the HDL particle size distribution resembled that of human plasma LpA-I/A-II. Mice from both transgenic lines as well as control mice were fed an atherogenic diet for 36 weeks. The two transgenic lines showed comparable lipid levels (total, HDL-cholesterol and non-HDL cholesterol), both before and after the atherogenic diet. As expected, the diet itself caused an increase in lipid levels. The extent of atheroslerosis caused by the diet was evaluated by measuring the lesion area in a defined segment of the proximal aorta. In apoA-I mice, this area proved to be 15 times smaller than in apoA-I/apoA-II mice. In non-transgenic mice, which have HDL levels half as high as the transgenic animals, the lesion area was three-fold bigger than in apoA-I/apoA-II mice. This study provides strong evidence that HDL has anti-atherogenic properties, which are effectuated by LpA-I to a much greater extent than by LpA-I/A-II.

Additional evidence for the anti-atherogenicity of apoA-I comes from the observation that crossing the human apoA-I transgene into apoE deficient mice, results in a two- to three-fold increase in HDL levels and in a drastic reduction of the atherosclerosis normally observed in apoE knock out mice [Paszty *et al.*, 1994; Plump *et al.*, 1994].

Similar observations were made in transgenic mice expressing human apo(a), that show an increased susceptiblity to diet-induced atherosclerosis [Lawn *et al.*, 1992]. Crossing these mice with human apoA-I expressing mice resulted in a 20-fold decrease in development of early atherosclerotic lesions [Liu *et al.*, 1994].

In transgenic rabbits too, overexpression of human apoA-I has been shown to inhibit diet-induced atherosclerosis [Duverger *et al.*, 1996].

Williamson *et al.* [1992] produced mice deficient in apoA-I by gene targeting. In these animals, total cholesterol and HDL-cholesterol levels are reduced by 66% and >80%, respectively. Diet-induced atherogenesis was studied in these mice [Li *et al.*, 1993]. In mice of 8 to 15 months maintained

on chow diet no signs of atherosclerosis were found, neither in apoA-I$^{-/-}$, apoA-I$^{+/-}$ nor in control animals. Surprisingly, in animals kept on a high fat diet for 12 to 32 weeks, only moderate symptoms of beginning atherosclerosis could be detected. No correlation with the apoA-I genotype of the mice could be found.

This unexpected finding might be attributed to the strain of mice used by the authors. These have a mixed 129 and C57BL6/J genetic background. In detailed studies carried out by Paigen *et al.* [1994] it was clearly established that many strains of mice are "resistant" against diet-induced atherosclerosis. This apparently includes the hybrids used by Li *et al.* [1993], since control mice only show moderate symptoms of early atherosclerosis after several months of atherogenic diet. This is in strong contrast with results obtained in C57BL6/J mice (or mice with >90% of C57BL6/J genetic background), which for this reason is the strain that is almost exclusively used in diet-induced atherogenesis studies in mice. Therefore, the final conclusions on the effect of apoA-I deficiency on diet-induced atherosclerosis have to await studies in C57BL6/J mice.

Still, lack of apoA-I might not be sufficient for a high suspectibility to atherosclerosis in mice. Possibly, a redundancy of apolipoproteins, several of which are very similar in structure, might take over the role of apoA-I. Actually, apoA-I deficient mice show a two-fold higher expression of apoE [Li *et al.*, 1993]. Overexpression of apoE in transgenic mice has been shown to result in resistance against diet-induced hypercholesterolemia [Shimano *et al.*, 1992].

In conclusion, studies in transgenic mice have provided the strongest evidence available for the anti-atherogenicity of HDL. The findings in apoA-I deficient mice are not necessarily refuting this. In humans, apoA-I deficiency in several cases is, but in other cases is not apparently associated with coronary artery disease. The factors that underlie this ambuigity are not fully appreciated.

4. HDL-Binding and HDL-Binding Proteins

4.1 HDL-Binding

Since the LDL-receptor does not bind HDL, Goldstein and Brown and coworkers proposed that cellular binding and uptake of HDL occurs via separate mechanisms [Kovanen *et al.*, 1979]. In the following decade, numerous groups studied HDL-binding to cultured cells or to plasma membranes from cultured cells or from tissues [Van Berkel *et al.*, 1980;

Tauber *et al.*, 1981; Biesbroeck *et al.*, 1983; Havekes *et al.*, 1984; Chacko, 1984; Hwang and Menon, 1985; Van Tol *et al.*, 1986; Mitchel *et al.*, 1987; Schouten *et al.*, 1988; Zsigmond *et al.*, 1988; Martin-Nizard *et al.*, 1989; Kilsdonk *et al.*, 1990]. It is generally assumed that binding of HDL is achieved by a specific receptor. A number of observations are in favour of this view: binding occurs with high affinity (K_d = 2 to 40 mg/ml); HDL-binding is competed for by unlabeled HDL, apoA-I, apoA-II but not by apoE or LDL; HDL-binding is affected by the cholesterol status of cells or hormonal stimuli [Oram *et al.*, 1983; Fidge *et al.*, 1984; Ghosh and Menon, 1986; Oppenheimer *et al.*, 1987; Talavera and Menon, 1989]; crosslinking, modification or proteolytic processing of HDL apolipoproteins abolishes HDL-binding [Chacko, 1985; Brinton *et al.*, 1986; Chacko *et al.*, 1988; Corsini *et al.*, 1988].

4.2 HDL-Binding Proteins

Several groups have identified HDL-binding proteins by ligand blotting (Table IV; see references therein). The discrepancies between these studies remain unexplained. Some of the variation in the molecular weight of the HDL-binding proteins reported can easily be attributed to the variation in the technique used to estimate this molecular weight. Proteins with reported molecular weights of 100, 108 and 110 kDa might all be the same protein. It is difficult to understand however, why Graham and Oram [1987] detect an HDL-binding protein of 80 kDa in human placenta, while Keso *et al.* [1987] find a 120 kDa apoA-I-binding protein in the same organ. Fidge [1986] detected an HDL-binding protein in rat liver of presumably 78 kDa. In a later study from the same laboratory [Tozuka and Fidge, 1989], using rat liver again, HDL-binding proteins of 100 and 120 kDa were found. Graham and Oram [1987] detected a 110 kDa HDL-binding protein in rat hepatocytes, as they did in HepG2 cells. In contrast, Kambouris *et al.* [1988] found a 80 kDa HDL-binding protein in HepG2 cells. Bond *et al.* [1991] found HDL-binding proteins of 60, 100 and 210 kDa in Hep3B cells. Another unexpected finding is the detection of a 58 kDa HDL-binding protein in luteinized ovaries of pseudopregnant rats [Ferreri and Menon, 1990] and a 108 kDa HDL-binding protein in bovine corpus luteum membranes in the same laboratory [Ferreri and Menon, 1992].

Most studies in which HDL-binding proteins have been detected have remained without follow-up. Exceptions are the studies by Oram and coworkers (discussed in section 3.1.1), by Fidge and coworkers, and the recent studies that followed the identification of SR-BI as a candidate HDL-receptor (see below).

Table IV: HDL binding proteins

kDa	source (species)	ligands	reference
78^{NR}	adrenal cortex (sheep)	HDL_3, ApoA-I, ApoA-II	Fidge et al., 1985
78^{NR}	liver, kidney (rat)	HDL_3, ApoA-I, ApoA-II	Fidge, 1986
$110^{R/NR}$	3T3 cells, J774 cells (mouse); smooth muscle cells, HepG2 cells, fibroblasts; placenta (human); endothelial cells (bovine); hepatocytes (rat)	HDL_3, ApoA-I, ApoA-II	Graham and Oram, 1987
110^{R}	J774 cells (mouse)	HDL; rec. ApoA-I-ProtA fusion protein	Monaco et al., 1987
$120^{R}/(50, 30)^{NR}$	placenta (human)	apoA-I	Keso et al., 1987
80^{NR}	HepG2 cells (human)	HDL_3	Kambouris et al., 1988
$(100, 120)^{NR}$	liver (rat; human)	HDL_3	Tozuka and Fidge, 1989
58^{R}	luteinized ovaries (rat)	HDL	Ferreri and Menon, 1990
$(80, 92)^{R}$	Ob17 cells (mouse)	apoA-I, apoA-II, apoA-IV	Barbaras et al., 1990
$(60, 100, 210)^{NR}$ 100^{R}	Hep3B cells (human)	HDL, ApoA-I-ProtA, apoA-II, apoA-IV	Bond et al., 1991
108^{R}	corpus luteum membrane (bovine)	HDL_2	Ferreri and Menon,

NR: run on SDS-PAGE under non-reducing conditions; R: run on SDS-PAGE under reducing conditions

Tozuka and Fidge [1989] described two HDL-binding proteins of 120 kDa (HB_1) and 100 kDa (HB_2) in rat liver. In human liver, HDL-binding proteins with similar molecular weights are claimed to be present. Both proteins in rat liver bind HDL_3, apoA-I and apoA-II in a ligand blot using non-reducing conditions. The proteins were purified by a combination of DEAE-chromatography and preparative SDS-PAGE. Purified proteins retained their HDL-binding activity. Upon reduction, the apparent molecular weight of the purified proteins did not change. However, HB_1 did not show HDL-binding activity under reducing condiditons, while HB_2 retained its HDL-binding activity. Antibodies were raised against the proteins. No cross-immunoreactivity was detected, suggesting that the proteins are not

structurally related. This was confirmed in amino acid analyses: differences in amino acid composition were found.

Mathai *et al.* [1990] addressed the question whether there would be changes in expression of HB_1 and HB_2 when rats were either fed a cholesterol rich diet or treated with drugs lowering the cholesterol content of the liver. Expression of the HB proteins was quantitated by excising a strip of nitrocellulose filter from the ligand blot containing HB_1 or HB_2 with ^{125}I–HDL bound to it and measuring the radioactivity in a gamma-counter. Cholesterol feeding did not seem to be effective, but some of the drug treatments resulted in a 40% and 60% reduction in HDL-binding to HB_1 and HB_2, respectively.

The use of specific antibodies recognizing either HB_1 or HB_2 allowed localization studies in rat tissues by Western blotting [Lutton and Fidge, 1994]. HB_1 and HB_2 are both present in liver, ovary and lung, but absent from or present in trace amounts in several other tissues. In spleen only HB_1 was detected. The authors suggest that the two HDL-binding proteins might therefore have different functions in HDL metabolism or in other biological processes yet to be determined.

Recently, it was reported that the scavenger receptor SR-BI (section II.3.2) probably functions as an HDL-receptor [Acton *et al.*, 1996]. Transfected cells expressing SR-BI show greatly increased binding of ^{125}I–HDL. Using fluorescently labeled HDL (DiI–HDL) the authors demonstrated that there was mainly cell-surface binding with very little cellular uptake of HDL. A considerable transfer of HDL-cholesterol esters was not accompanied by a detectable degradation of HDL-protein. Therefore, SR-BI apparently mediates cellular selective uptake of HDL-cholesterol esters (see section 3.1.3). Expression of SR-BI was investigated by Western blotting. The highest expression was found in adrenals, ovaries and the liver, which are the organs in which selective uptake of HDL-cholesterol esters occurs [Glass *et al.*, 1983]. SR-BI expression is increased in estrogen treated rats in the adrenal gland and the corpus luteal cells of the ovary [Landschulz *et al.*, 1996]. A concomitant increase of *in vivo* dil–HDL uptake in these organs was observed. In the liver, basal levels of SR-BI expression were much lower than in either the adrenal gland or in the ovary. Estrogen treatment resulted in a further decrease of SR-BI expression. Dil–HDL uptake by the liver was low in both treated and untreated rats. This study provides evidence for a function of SR-BI in selective uptake of cholesterol esters from HDL by steroidogenic organs. Further support comes from apoA-I deficient (knock-out) mice, that show increased levels of SR-BI mRNA [Wang *et al.*, 1996]. In mice adrenal steroidogenic Y1 cells, mRNA levels of

SR-BI were upgraded by ACTH treatment. Addition of HDL to the medium completely blocked this effect. Therefore, SR-BI probably functions as an HDL-receptor mediating selective uptake of HDL cholesterol esters in steroidogenic organs, using apoA-I as a ligand. This is in agreement with the observation that selective uptake of HDL cholesterol esters is blocked in apoA-I knock-out mice, but not in apoA-II or apoE knock-out mice [Plump *et al.*, 1996].

SR-BI could be a multi-functional protein. Apart from HDL, it also binds LDL (although LDL does not compete for HDL-binding), acLDL, oxLDL, maleylated BSA, and anionic phospholipids [Acton *et al.*, 1994; Rigotti *et al.*, 1995, Acton *et al.*, 1996]. The qualitative and quantitative relevance of these functions for the *in vivo* situation remains to be determined.

Finally, it should be mentioned that some authors suggested that the binding of HDL by cells occurs via non-protein interactions [Tabas and Tall, 1984]. Mendel *et al.* [1988] applied radiation inactivation to estimate the molecular mass of the HDL-binding sites on fibroblasts, which appear to be 16 kDa on average. These authors too, speculate that lipid-lipid interactions explain their data, rather than the existence of a classical receptor.

5. Conlusions

At present, HDL is generally recognized as an anti-atherogenic factor. This has been initially suggested because of the inverse relationship between plasma levels of HDL and the incidence of coronary artery disease observed in epidemiological studies. Experimental evidence came from studies in rabbits, in which injections with HDL were shown to interfere with the atherosclerotic process. Transgenic mice and rabbits that have elevated levels of HDL as a consequence of the overexpression of human apoA-I were shown to exhibit a reduced susceptibility to atherosclerosis. Besides, overexpression of apoA-I in mice was shown to counteract the effects of transgenes promoting atherosclerosis (apo(a), apoE).

On the other hand, (near) absence of HDL does not seem to inevitably lead to atherosclerosis. Some of the very rare familial disorders with (near) absence of circulating HDL have been shown to be associated with an increased risk for coronary artery disease, but for other HDL deficiencies such a relation has not been found. In apoA-I deficient mice produced by gene targeting, no increased susceptibility to atherosclerosis could be observed. Probably, HDL-deficiency leads to coronary artery disease only in

association with other risk factors or in a genetic constitution in which its effects cannot be sufficiently met.

Although alternatives have been proposed, the most widely accepted explanation for the anti-atherogenicity of HDL is its involvement in reverse cholesterol transport. There is conclusive evidence from *in vitro* experiments that HDL can perform all the functions and has all the biochemical properties needed for its role in reverse cholesterol transport. *In vivo* evidence is scarce because of the difficulty of obtaining it. Nevertheless, the evidence that is available is in agreement with the supposed role of HDL in reverse cholesterol transport.

The molecular details of the steps in the reverse cholesterol transport pathway still remain elusive. Some proposed, while others denied the involvement of HDL-binding proteins in efflux of cellular cholesterol to HDL. It is obvious, however, that HDL-binding proteins could greatly facilitate this process. The identification of such proteins is the goal of the research described in this thesis.

The contribution of distinct HDL subfractions to the uptake of cellular cholesterol is also a controversial issue. Probably, LpA-I and LpA-I/A-II have important functional differences. In transgenic mice, it has been convincingly demonstrated that LpA-I gives a better protection against the development of atherosclerosis than LpA-I/A-II. Still, most biochemical studies show that LpA-I and LpA-I/A-II are equally effective as acceptors of cellular cholesterol and most epidemiological studies show that plasma levels of LpA-I and LpA-I/A-II do not discriminate coronary artery disease patients versus controls better than plasma levels of HDL-cholesterol do.

Chapter IV

Experimental Work

1. Introduction

The goal of the work described in this thesis is to identify HDL-binding proteins in the liver. The molecular identification of the different factors (proteins) involved in hepatic HDL metabolism, offers the prospect of applying the powerful tools of molecular biology. These can be used to unravel the impact of the individual factors or combination of factors on HDL metabolism in the liver, and resolve questions concerning the validity of hypothetical pathways and their relative contribution. Much of the progress in our understanding of lipoprotein metabolism from the last decade has come from molecular biological studies, in particular studies in transgenic mice.

During the last decade, many HDL-binding proteins, hepatic and non-hepatic, have been described. Only two of them have been identified on DNA level. Oram and coworkers described a cDNA clone for HBP [McKnight *et al.*, 1992]. Conclusive evidence that this protein is involved in HDL metabolism is still lacking. Evidence by others showing that this protein is found intracellularly in chicken chondrocytes and other cells, is not in agreement with such a function [Neu-Yilik *et al.*, 1993]. The secondly identified candidate HDL-receptor is SR-BI [Acton *et al.*, 1996], which had been initially identified as a macrophage scavenger receptor. When SR-BI cDNA was tranfected to cultured cells, these cells showed an increased ^{125}I–HDL binding activity. Supporting evidence comes from a study showing that the increase of SR-BI expression in steroidogenic organs in rats is accompanied by an increase in uptake of HDL [Landschultz *et al.*, 1996]. Studies in mice made deficient in apoA-I, apoA-II or apoE by gene targeting [Plump *et al.*, 1996; Wang *et al.*, 1996] showed that SR-BI can function as an HDL-receptor with apoA-I as a ligand to mediate selective uptake of HDL-cholesterol esters by steroidogenic cells in the adrenal gland, and probably also in the ovary and in the testis. The *in vivo* role of SR-BI in hepatic HDL metabolism has not been clearly established yet.

2. Results and Discussion

In Appendix Paper 1, HDL-binding proteins in the liver were detected by ligand blotting. Two major HDL-binding proteins of 90 and 180 kDa were identified and monospecific antisera recognizing either the 90 kDa or the 180 kDa HDL-binding protein were obtained. These antisera were used to evaluate the light-microscopic immunohistochemical localization and, in an electron-microscopic study (Appendix Paper 4), the ultrastructural

localization. These are the only studies showing the histological or ultrastructural localization of HDL-binding proteins. The 180 kDa HDL-binding protein appeared to be present along the plasma membranes of the hepatocytes, the endothelial cells and the Kupffer cells lining the space of Disse and the sinuoids while the 90 kDa HDL-binding protein is localized at the apical membranes of the hepatocytes comprising the bile canaliculi and at the vesicular membranes of the endosomal/lysosomal system. This difference in ultrastructural localization suggests a functional difference of the two HDL-binding proteins. Although speculative, the localization of the 180 kDa HDL-binding protein might indicate that it interacts with plasma HDL. The 90 kDa HDL-binding protein might have a chaperonin like function in the intracellular trafficking of HDL or apoA-I. Alternatively, it could be an intracellular intermediate or catabolic product of the 180 kDa HDL-binding protein without functional HDL-binding properties *in vivo*.

Biochemical studies showed that the 90 and 180 kDa HDL-binding protein in liver and a third HDL-binding protein of 110 kDa are structurally related (Appendix Paper 2). Probably the 180 kDa HDL-binding protein is a homodimer of the 90 kDa HDL-binding protein, while the 110 kDa HDL-binding protein is a conformational variant of the monomeric protein that runs at the 110 kDa position in a polyacrylamide gel under non-reducing conditions. Additional prove for this relation is presented in Appendix Paper 3, in which it is shown that the three HDL-binding proteins have identical N-terminal amino acid sequences.

These proteins probably originate from one gene product. This gene was identified as gp96/GRP94 (Appendix Paper 3). This protein has the C-terminal tetrapeptide KDEL, which serves in many proteins as an ER retention signal [Munro and Pelham, 1987]. The function of gp96 is unknown. Several lines of evidence indicate that the protein is an ER chaperonin. Expression of the protein appears to be coordinately regulated with the expression of GRP78, which is generally recognized as an ER chaperonin. However, there is conclusive evidence that at least in some species and in some organs gp96 is localized at the plasma membrane. A number of other KDEL containing proteins are known to be able to (partially) escape from ER retention as discussed in Appendix Paper 3. In electron microscopic immunocytochemical studies in porcine liver we found that the extend of expression of the protein in the ER is negligable. However, in light microscopic immunohistochemical studies in human liver, we found a diffuse intracellular localization of the protein in isolated groups of hepatocytes next to a uniform labeling of the sinusoidal linings (representing labeling of the plasma membranes). Therefore, presumably only hepatocytes with a certain state of metabolic activity express gp96

abundantly in the ER, while plasma membrane expression is constitutive. Therefore we hypothesize that hepatic gp96 is a protein with a dual subcellular localization, in the ER and at the plasma membrane, that probably reflects bifunctionality.

The bifunctionality probably troubles our expression studies. It seems not unlikely that the ER function of the protein is strictly regulated and maybe vital. All the expression systems that we studied, transiently transfected COS cells (Appendix Paper 3), stably transfected cell lines (Appendix Paper 5), micro-injected *Xenopus* oocytes (data not shown) and several lines of transgenic mice (Appendix Paper 6) appeared to be highly resistant against high level expression of gp96.

A more technical explanation for the lack of overproduction of gp96 in most of the expression systems we used is that we invariably included gp96 cDNA in the expression constructs. Although genomic sequences from the human β-globin gene were added in most cases in order to obtain higher mRNA stability an a more efficient translation, the incorporation of large fragments of cDNA is a notorious impediment in expression studies in eukaryotic cells or transgenic mice. Therefore, we will generate transgenic mice with a cosmid containing the gp96 gene (see below).

In order to focus on the function of plasma membrane expressed gp96, DNA constructs were generated with a deletion of the KDEL sequence (ΔKDEL). Since the mechanism by which some KDEL-containing proteins, including gp96, escape from ER retention is unknown, we decided to introduce a single mutation that changes the leucine of the KDEL sequence in a stop codon. COS cells transiently tansfected with a $gp96^{\Delta KDEL}$ cDNA containing construct, showed plasma membrane expression, albeit in a very low percentage of the cells (section V.5). This experiment showed that the ΔKDEL construct rendered the desired result. A Δ.KDEL construct also induced plasma membrane expression in MEL cells, and in one experiment an increased HDL-binding was found. Unfortunately attempts to isolate a cell line stably expressing the protein were unsuccesful, which impeded a reproduction of these results (section V.8). Trangenic mice generated with a $gp96^{\Delta KDEL}$ construct did not show appreciable expression.

In conclusion, a fairly high level of plasma membrane expressed gp96, that would allow the evaluation of its HDL-binding capacities, could not be achieved, although a number of expression systems have been tested. The dual localization of the protein and its probable bifunctionality linked to this, complicates the studies on the functionality of the plasma membrane localized form. To date, the question whether gp96 is involved in HDL metabolism cannot be answered definitely.

As mentioned before, the use of cDNA constructs might have seriously

hampered protein expression in the systems that have been used. Therefore, transgenic mice will be generated with a cosmid encompassing the complete human gp96 gene flanked by extended 5' and 3' sequences.

In this cosmid, we will engineer a ΔKDEL mutation analogous to the ΔKDEL mutation that has been introduced in gp96 cDNA. This will allow us to test the effect of plasma membrane expression in transgenic mice or in transfected cells. Next to this we will make another mutation changing the cysteine residue at position 138 that is involved in dimerization [Qu *et al.*, 1994]. This will allow us to investigate the function of the monomeric versus the homodimeric protein.

Obviously, the generation of mice deficient in gp96 by gene targeting could be very informative. However, in such a mouse model the supposed bifunctionality might highly complicate the results. The opportunity to breed these mice with different lines of transgenic mice (expressing the wild type, the ΔKDEL protein or the gp96 with a mutated Cys_{138}) may be of considerable help.

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Summary

Cholesterol is a molecule with a Janus face: although it is essential for human life, it is also considered as a plague of the industrialized world, since it is one of the key factors in the prevailing cause of death and morbidity: cardiovascular diseases. High levels of plasma cholesterol are associated with an increased risk for cardiovascular diseases. However, cholesterol appears as "bad" cholesterol and "good" cholesterol, which is present in low density lipoproteins (LDL) and high density lipoproteins (HDL), respectively. These are complexes of proteins and lipids that transport cholesterol in plasma. The quantitatively minor fraction of HDL apparently protects against the development of atherosclerosis, since relatively high levels of HDL are coupled to a low risk of atherosclerosis. The most widely accepted explanation for the anti-atherogenicity of HDL is that it mediates "reverse cholesterol transport". This is the transfer of cholesterol from extrahepatic cells to the liver, where cholesterol can be excreted from the body via the bile.

The molecular details of reverse cholesterol transport are still far from understood. It is not known how HDL interacts with either extrahepatic cells, where HDL has to function as a cholesterol acceptor, or with liver cells, where HDL has to function as a cholesterol donor. Obviously, cellular HDL-binding proteins could be effective mediators of these HDL–cell contacts. However, to date the functional activity in HDL metabolism of none of the reported HDL-binding proteins has been demonstrated.

In this thesis, it is demonstrated that HDL-binding proteins could be detected in porcine and human liver by ligand blotting (Appendix Paper 1). Two major HDL-binding proteins of 90 and 180 kDa were identified that have a strikingly different localization in porcine liver as evident from immunohistochemical studies: the 90 kDa protein in localized within the hepatocellular plates, while the 180 kDa protein is exclusively present along the lining of the sinusoids. In Appendix Paper 2 biochemical evidence is presented showing that the 90, 180 kDa and a third minor HDL-binding protein of 110 kDa are structurally related, which is confirmed by the finding that they have identical N-terminal amino acid compositions (Appendix Paper 3). Subsequently, this protein was identified as gp96, also known as GRP94. Gp96 contains the endoplasmic reticulum (ER) retention signal KDEL (a C-terminal tetrapeptide) but several lines of evidence indicate that the protein can at least in part escape ER retention and localize at the plasma membrane. We showed in electron microscopic immuno-cytochemical studies (Appendix Paper 4) that the homodimeric 180 kDa protein actually is localized at the plasma membrane in liver. The

monomeric 90 kDa form is found sporadically at the plasma membrane, notably in coated pits, indicating that it is involved in receptor mediated endocytosis. Intracellularly, the 90 kDa protein is present at the membranes of the vesicles of the endosomal/lysosomal system and at the membranes of the bile canaliculi, but hardly in the ER.

In order to test whether gp96 is able to function as an HDL-binding protein in a physiological context, we used several expression systems. These studies were complicated by the inability to induce a stable, high level of protein expression and by the dual localization/function of the protein.

In transiently expressed COS cells (Appendix Paper 3), expression was found in a very low percentage of the cells and appeared to be restricted to the ER. Plasma membrane localization was observed in immunocytochemistry experiments in COS cells transfected with a construct in which the KDEL sequence was deleted ($gp96^{\Delta KDEL}$). However, the percentage of cells expressing the protein was extremely low.

Although various cell lines and different DNA constructs were tested, a cell line stably expressing gp96 to appreciable levels could not be obtained (Appendix Paper 5). Invariably, clones of cells expressing gp96 proved to be unstable. Still, one unstable clone of MEL cells expressing $gp96^{\Delta KDEL}$ showed an increased binding of ^{125}I–HDL.

Lines of transgenic mice were generated (Appendix Paper 6) with gp96 containing DNA constructs anticipated to effectuate either liver-specific (ABH), T-cell specific (CDH) or generalized expression of the transgene. Two lines of ABH mice (having the transgene under control of the mouse albumin enhancer and promoter) and two lines of CDH mice (having the transgene under control of the human CD2 locus control region and promoter) showed appreciable expression of gp96. Still, HDL-binding to either liver- or thymus-derived plasma membrane preparations was not increased (comparisons were made with control litter mates). It could be demonstrated that the level of gp96-expression in T-cells from CDH mice was increased about twofold. However, this expression proved to be exclusively intracellular, presumably localized in the ER. Therefore, transgenic mice were generated using a construct containing $gp96^{\Delta KDEL}$ in order to induce plasma membrane expression, and the mouse major histocompatibility class I promoter $H2K^b$ in order to induce generalized expression. However, in these mice expression of the transgene could not be detected in any of the tissues tested.

In conclusion, both the level of protein expression as well as the localization of the expressed protein are complicating factors in expression studies of gp96. Future experiments will concentrate on the function of

(plasma membrane localized) gp96 and will specifically address the involvement of gp96 in HDL metabolism. For this purpose, transgenic mice can be generated with genomic sequences encoding gp96 or $gp96^{\Delta KDEL}$, as well as mice carrying a cysteine mutation that will prevent dimer formation. Gp96 deficient mice will be generated by gene targeting.

Samenvatting

Cholesterol is een molecuul met een Janus-gezicht: enerzijds is het essentieel voor menselijk leven, anderzijds is het levensbedreigend. Een verhoogd niveau van cholesterol in het bloed is namelijk één van de belangrijkste risico-factoren voor het verkrijgen van atherosclerose. Cholesterol komt in het bloed voor in complexen van vetten en eiwitten, die LDL (*low density lipoprotein*) en HDL (*high density lipoprotein*) worden genoemd. Vooral LDL blijkt verantwoordelijk te zijn voor het ongunstige effect van een verhoogd niveau van cholesterol. Het in kwantitatief opzicht veel minder prominente HDL lijkt juist te beschermen tegen de ontwikkeling van atherosclerose. De oorzaak hiervan wordt gezocht in de rol die HDL waarschijnlijk speelt in het "omgekeerde cholesterol transport". Daarmee wordt het cholesterol-transport bedoeld van lichaamscellen naar de lever, waar cholesterol het lichaam kan verlaten via de gal.

Op moleculair niveau zijn de details van het omgekeerde cholesterol transport nog grotendeels onbegrepen. Het is niet bekend hoe de interactie plaats vindt van HDL met lichaamscellen, waar HDL als cholesterol-acceptor moet optreden, of van HDL met levercellen, waar HDL als cholesterol-donor moet fungeren. Een cellulair HDL-bindend eiwit zou in deze processen een sleutelrol kunnen vervullen. Hoewel inmiddels verscheidene HDL-bindende eiwitten zijn beschreven, is men er tot nu toe niet in geslaagd om aan één van deze een duidelijke fysiologische betekenis toe te kennen.

In dit proefschrift wordt beschreven dat HDL-bindende eiwitten kunnen worden aangetoond door middel van *ligand blotting* in zowel varkens- als humane lever (Appendix Paper 1). Deze eiwitten hebben molecuul-gewichten van 90 en 180 kDa en blijken een verschillende cellulaire localisatie te hebben in de lever: het 180 kDa eiwit is vooral aanwezig op de begrenzingen van de sinusoïden, terwijl het 90 kDa eiwit wordt aangetroffen in de hepatocellulaire platen. In Appendix Paper 2 wordt met behulp van biochemische analyses aangetoond dat het 90 en 180 kDa HDL-bindend eiwit, alsmede een derde, minder prominent aanwezig HDL-bindend eiwit van 110 kDa, een onderlinge structurele verwantschap vertonen. In Appendix Paper 3 wordt aangetoond dat deze eiwitten bovendien een gelijke N-terminale aminozuur volgorde hebben. Waarschijnlijk is het 180 kDa eiwit een homodimeer van het 90 kDa eiwit, terwijl het 110 kDa eiwit een structurele variant van de monomeer is. Dit eiwit is vervolgens geïdentificeerd als gp96, ook wel bekend als GRP94. Gp96 bevat het C-terminale tetrapeptide KDEL dat een bekend endoplasmatisch reticulum (ER) retentie-signaal is. Er zijn echter diverse aanwijzingen dat dit eiwit (ten

dele) aan de ER retentie kan ontsnappen en dan tot expressie komt op de plasma membraan. In Appendix Paper 4 presenteren wij electronen-microscopische immunocytochemische localisatie studies, die aantonen dat het 180 kDa eiwit tot expressie komt op de membraan van de levercellen. Het 90 kDa eiwit wordt slechts sporadisch aangetroffen op de plasma membraan, maar blijkt wel aanwezig te zijn in *coated pits*, hetgeen impliceert dat het betrokken is bij receptor gemedieerde endocytose. Verder wordt het 90 kDa eiwit vooral intracellulair gevonden, met name aan de membranen van de blaasjes van het endosomale/lysosomale systeem, en aan de plasma membranen die de galcanaliculi vormen. Wij vonden nauwelijks ER labeling.

Om de fysiologische betekenis van dit HDL-bindend eiwit te onderzoeken, hebben wij diverse expressie systemen gebruikt. Deze studies werden echter ernstig bemoeilijkt door het feit dat het onmogelijk bleek te zijn een hoge en stabiele eiwit-expressie te verkrijgen en door de tweeledige localisatie van het eiwit.

In COS cellen (Appendix Paper 3) bleek expressie van het eiwit na transfectie slechts in een zeer klein percentage van de cellen aantoonbaar te zijn. Dit eiwit was voornamelijk in het ER gelocaliseerd. Wanneer COS cellen worden getransfecteerd met een construct waarin de KDEL sequentie is gedeleteerd ($gp96^{\Delta KDEL}$), blijkt de expressie van het eiwit wel gelocaliseerd te zijn op de plasma membraan, maar in een uitzonderlijk laag percentage van de getransfecteerde cellen.

In Appendix Paper 5 zijn experimenten beschreven die zijn uitgevoerd ten einde een cellijn met stabiele expressie van gp96 te isoleren. Het bleek nagenoeg onmogelijk te zijn een dergelijke cellijn te verkrijgen. Niettemin kon in een (onstabiele) MEL cellijn die getransfecteerd was met $gp96^{\Delta KDEL}$ eiwit-expressie op de plasma membraan worden aangetoond alsmede verhoogde binding van ^{125}I–HDL.

Verscheidene lijnen van transgene muizen zijn gegenereerd met verschillende gp96 bevattende constructen (Appendix Paper 6), die werden geacht lever-specifieke ofwel T-cel specifieke expressie te bewerkstelligen. In eerstgenoemde muizenlijnen bleek de eiwit-expressie op zijn best vergeljkbaar met de endogene expressie; in de laatstgenoemde was weliswaar een ongeveer tweevoudig verhoogde expressie aantoonbaar, maar deze bleek geheel intracellulair te zijn. In geen van beide soorten muizenlijnen kon verhoogde HDL-binding aan lever- c.q. T-cel preparaten worden aangetoond. In muizen met een $gp96^{\Delta KDEL}$ construct onder controle van de MHC klasse I $H2K^b$ promoter bleek geen expressie van het transgen te kunnen worden aangetoond.

Concluderend, zowel het niveau van eiwitexpressie als ook de

localisatie van het gp96 leveren problemen op bij de bestudering van de functie van het eiwit. Volgende experimenten zullen erop gericht zijn een antwoord te vinden op de vraag of gp96 een rol speelt in het HDL metabolisme. Daarvoor kunnen transgene muizen worden gegenereerd met genomische (in plaats van cDNA-) sequenties coderend voor gp96 en $gp96^{\Delta KDEL}$, alsmede transgene muizen met een mutatie van de cysteïne die verantwoordelijk is voor de dimerisatie van gp96. Gp96 deficiënte muizen zullen worden gegenereerd door *gene targeting*.

Curriculum vitæ

Marinus Petrus Gerardus (Rini) de Crom

6 maart 1962:
geboren te Bergen op Zoom

29 mei 1980:
diploma gymnasium β (Mollerlyceum, Bergen op Zoom)

17 oktober 1983:
kandidaatsexamen Biologie (B1; Rijksuniversiteit Utrecht)

26 mei 1986:
doctoraal examen Biologie (cum laude; Rijksuniversiteit Utrecht)

Hoofdvak: Moleculaire Neurobiologie; Dr. D.H.G. Versteeg en Prof. Dr. W.H. Gispen, Rudolf Magnus Instituut voor Farmacologie, Faculteit der Geneeskunde RUU (hoofd: Prof. Dr. D. de Wied) en Prof. Dr. A.H. Mulder, Laboratorium voor Farmacologie, Faculteit der Geneeskunde, Vrije Universiteit Amsterdam (hoofd: Prof. Dr. P.G. Smelik).
Bijvak 1: Vergelijkende Endocrinologie; Dr. M.M. Bevers en Prof. Dr. A.H. Willemse, Kliniek voor Verloskunde, Gynaecologie en Kunstmatige Inseminatie, Faculteit der Diergeneeskunde, RUU (hoofd: Prof. Dr. Dr.h.c. C.H.W. De Bois en Prof. Dr. A.H. Willemse)
Bijvak 2: Biochemie; Prof. Dr. K.W.A. Wirtz, Biochemisch Laboratorium, Subfaculteit Scheikunde, RUU (hoofd: Prof. Dr. L.L.M. van Deenen)

1 juni 1986 - heden:
wetenschappelijk onderzoeker, M.G.C.-Afd. Celbiologie Genetica, Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam

Dankwoord

De laatste woorden die ik ga opschrijven (typen, printen, drukken) zijn misschien wel de belangrijkste. Op de eerste plaats omdat ze gaan over de mensen die een essentiële bijdrage hebben geleverd aan het werk en aan de werksfeer. In de tweede plaats omdat deze tekst de hoofdmoot vormt van het gedeelte van het proefschrift dat ook gelezen zal worden. Ik ga het *niet* kort houden; daarvoor zijn er teveel mensen die het verdienen om hier, letterlijk en figuurlijk, met name genoemd te worden. Daar gaan we dan.

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Lieve Mirjam, Errol en Tosca ("Papa, ga je nou weer al *comploeteren*?"), het is af. Zien jullie die hartjes op de voorkant?

Appendix Paper 1

Isolation and Histological Localization of High Density Lipoprotein-Binding Proteins in Porcine Liver

Rini P.G. de Crom,
Rien van Haperen,
Rob Willemsen,
Arthur W.M. van der Kamp

MGC-Department of Cell Biology and Genetics,
Erasmus University Rotterdam,
3000 DR Rotterdam,
The Netherlands

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The anti-atherogenic properties of HDL are thought to reside in their involvement in the reverse cholesterol transport pathway. Specific HDL-binding proteins could play a key role in this process. Two HDL-binding proteins of approximately 90 and 180 kDa were identified in porcine liver by ligand blotting and purified to apparent homogeneity by a combination of protein extraction, DEAE-cellulose chromatography, ConA-Sepharose chromatography, and preparative SDS-PAGE. Binding of ^{125}I–HDL by these proteins could be actively competed for by unlabeled HDL but not by LDL. Polyclonal antisera have been raised against these two proteins. Each antiserum recognized only one of the HDL-binding proteins, indicating that they are not immunologically related. Moreover, striking differences in localization were observed in immunohistochemical studies. The 90 kDa protein is located within the hepato-cellular plates, while the 180 kDa protein is present along the lining of the sinusoids. These results suggest functional differences between the two HDL-binding proteins described.

High density lipoproteins (HDL) are widely believed to play a protective role in the development of coronary heart disease (CHD) (1). This hypothesis is based on epidemiological data, which show a strong inverse correlation between plasma HDL-cholesterol level and the prevalence of CHD (2-4), and on intervention studies, which indicate that elevation of HDL-cholesterol level is effective in the primary prevention of CHD (5). The anti-atherogenic properties of HDL are probably based on the ability of HDL to promote efflux of cholesterol from peripheral cells and to deliver it to the liver for excretion, a concept termed reverse cholesterol transport (6-9).

The binding characteristics of HDL to cultured cells and to purified plasma membrane fractions strongly suggest the existence of a specific, high-affinity HDL receptor (10-13). Because such a receptor could have a key position in the process of reverse cholesterol transport, several groups have sought to identify HDL-binding proteins by using ligand blot studies. Their results differ in the molecular masses of HDL-binding proteins found (14-19), which may be attributable to species and/or tissue specificity.

It is conceivable that in the reverse cholesterol transport pathway, the tissues involved in cholesterol efflux (peripheral tissues) have a different HDL metabolism than the liver, which is involved in cholesterol clearance. A peripheral HDL-binding protein has been described and characterized by Oram and coworkers (17, 20, 21). We investigated HDL-binding proteins in the liver. In the present study, two HDL-binding proteins in porcine liver are

described. Their histological localization is notably different, presumably reflecting their involvement in different steps in the reverse cholesterol transport pathway.

Experimental Procedures

Lipoproteins
LDL (d = 1.019 – 1.063 g/ml) and HDL (d = 1.063 – 1.21 g/ml) were isolated from human plasma of healthy volunteers by sequential ultracentrifugation (22). The same procedure was followed for the isolation of porcine lipoproteins. HDL was subjected to heparin-Sepharose affinity chromatography to remove apoB/E containing particles (23). Lipoproteins were iodinated by the iodine monochloride method (24). Specific activities were about 300 cpm/ng protein. All studies were performed with human lipoproteins, unless indicated otherwise.

Ligand blot assay
Samples of protein fractions were dialysed against 5 mM NH_4HCO_3, 1 mM EDTA and were concentrated by lyophilization. Protein fractions were electrophorized on 7% polyacrylamide slab gels in the presence of sodium dodecyl sulfate (SDS-PAGE) under non-reducing conditions (25) and electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell, 0.45 μm) (26). Nitrocellulose strips were incubated for 2 hours with blocking buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$, 1% [wt/vol] bovine serum albumin, 1% [wt/vol] low fat milk powder [a gift from Nutricia B.V., Zoetermeer, The Netherlands]) containing 50 μg/ml LDL, followed by a 2 hour incubation with blocking buffer containing 125 μg/ml LDL and 25 μg/ml HDL. Subsequently, strips were washed with blocking buffer followed by a short wash in 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$ (buffer A), and incubated for 10 min with buffer A containing 0.1% glutardialdehyde. HDL was visualized using rabbit anti-human HDL antiserum and peroxidase-conjugated, swine anti-rabbit immunoglobulins (Dako, Denmark) as the secondary antibody. Alternatively, blots were incubated with blocking buffer as described, followed by an incubation with blocking buffer containing 10 μg/ml ^{125}I–HDL. Blots were washed extensively in blocking buffer. After drying, protein bands were visualized by autoradiography.

Solubilization of liver proteins
All operations were carried out at 0 – 4 °C. Porcine livers obtained from the Department of Experimental Cardiology, Erasmus University Rotterdam, were collected on ice immediately after death of the animal, cut into small pieces and stored at –70 °C. For protein solubilization, 100 g liver tissue was minced using surgical blades and homogenized in 200 ml 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (buffer B), using a Ultra-Turrax TP 18-10. Tissue debris was removed by centrifugation (10 min, 1350 g). The supernatant was centrifuged (60 min, 100 000 g) and the pellet resuspended in 120 ml buffer B containing

1% (vol/vol) Triton X–100 by aspiration with a 22-gauge needle. The suspension was stirred for 10 min and slowly diluted 10 times in 10 mM Tris–HCl pH 8.0, 2 mM $CaCl_2$, 1% (vol/vol) Triton X–100. After removal of particulate material by centrifugation (30 min, 20 000 *g*) a clear supernatant was obtained.

DEAE-cellulose chromatography
The solubilized fraction was applied to a DEAE-cellulose (DE-52; Whatman) column (2.4 × 10 cm) at a flow rate of 30 ml/hr. The column was washed with 10 mM Tris–HCl pH 8.0, 2 mM $CaCl_2$, 15 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (buffer C). Elution was performed with buffer C containing increasing concentrations of NaCl.

ConA-Sepharose chromatography
DEAE-cellulose fractions containing HDL-binding activity were pooled and dialysed against 20 mM sodiumphosphate pH 6.8, 0.25 mM $CaCl_2$, 0.25 mM $MnSO_4$ and 10 mM NaCl (buffer D) and applied to a ConA-Sepharose (Pharmacia) column (2.4 × 7 cm). The column was washed with buffer D containing 0.5 M NaCl and eluted with buffer D containing 1 M NaCl and 1 M methyl-α-D-glucopyranoside.

Preparative SDS-PAGE
Proteins eluted from ConA-Sepharose were subjected to SDS-PAGE. A small vertical strip of the gel was analyzed in the ligand blot assay. Using this as a guide, HDL-binding proteins were cut out and extracted by electro-elution. These proteins were subjected to another round of preparative SDS-PAGE until they appeared to be homogeneous on silver-stained gels. After transfer onto nitrocellulose membranes, a small horizontal strip containing the HDL-binding activity was cut out. After solubilizing the nitrocellulose with dimethyl sulfoxide, this fraction was used to immunize the rabbits.

Antisera
Antibodies to HDL or HDL-binding proteins were prepared by standard techniques. In brief, 50 µg protein was added to 1 ml of phosphate buffered saline and 1 ml incomplete Freund's adjuvant. A rabbit (New Zealand White) was injected subcutaneously with this mixture. Two boosters with the same mixture were given 15 days and 25 days after the first injection. Eight days later the blood was collected and serum was obtained by centrifugation at 400 *g* after clotting. All animals were cared for in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Erasmus University Rotterdam (prot.nr. 128.89.55) and with the European Committee Standards on the care and use of laboratory animals (Ministry of Welfare, Health and Cultural Affairs, The Netherlands).

Immunoblotting
Protein samples were subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. These were incubated with blocking buffer as described for the ligand blot assay procedure followed by a 2 hour incubation with blocking buffer containing 0.05% (vol/vol) Tween-20 and anti-HDL-binding protein antiserum (dilution

1:1000). Peroxidase-conjugated, swine anti-rabbit immunoglobulins (Dako, Denmark) were used as the secondary antibody.

Immunoprecipitation
ConA-Sepharose eluate was dialyzed against 20 mM sodiumphosphate buffer pH 7.2, containing 150 mM NaCl. To 400 µg ConA-Sepharose eluate, various amounts of anti-HDL-binding protein antiserum were added as indicated in the legend of Figure 6 and incubated for 16 hours at 4 °C. 150 ml of a 1:1 suspension of proteinA-Sepharose (Pharmacia) was added, and the mixture was incubated for 2 hours at room temperature. After centrifugation (5 min, 400 *g*), the supernatant was separated from the immunoprecipitate. Both were boiled in SDS-PAGE sample buffer and analyzed in the ligand blot assay.

Immunohistochemistry
Porcine liver was fixed and prepared for ultracryotomy as described before (28). Semithin cryosections (0.5 – 1.0 µm) were incubated with polyclonal antibodies against HDL-binding proteins. Antigen-antibody complexes were visualized with FITC-conjugated goat anti-rabbit immunoglobulins (Nordic, Tilburg, The Netherlands) and studied by fluorescence microscopy.

Results

Porcine liver proteins were solubilized in Triton X–100. The yield was about 2600 mg of solubilized material from 100 g liver tissue (wet weight). This fraction was subjected to ion-exchange chromatography on DEAE-cellulose. Triton X–100 was then replaced by CHAPS to prevent interference in the ligand blot assay (29). Proteins were eluted with buffer containing CHAPS and NaCl. Samples of these fractions were analyzed for the presence of HDL-binding proteins in the ligand blot assay. A discontinuous NaCl gradient was used for elution. Two HDL-binding proteins of approximately 90 and 180 kDa molecular mass could be detected (Figure 1), eluting preferentially with NaCl concentrations between 50 and 125 mM NaCl. A third protein band of approximately 100 kDa was disregarded, as it was also observed in the ligand blot assay under control conditions (Figure 1, lanes 4a – 6a).

The 50 – 125 mM NaCl eluate was pooled and subjected to ConA-Sepharose affinity chromatography. HDL-binding activity proved to be present in the retained fraction only (Figure 2), indicating that these HDL-binding proteins are glycoproteins. Apart from the 90 and 180 kDa proteins, two minor bands of approximately 110 and 130 kDa were observed. The presence of these bands was dependent on the preparation used. In some preparations, both were absent; in other preparations, only a 110 kDa HDL-

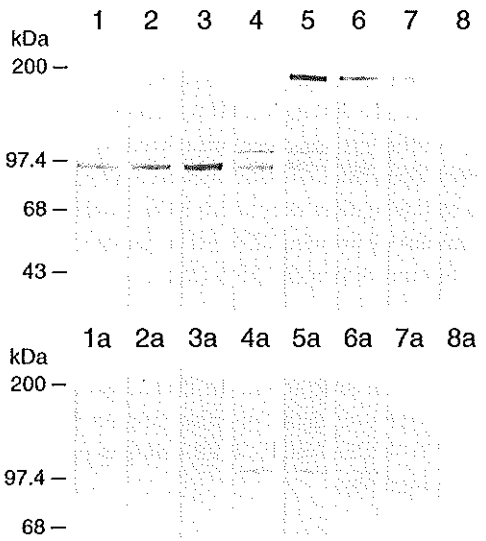

Figure 1. DEAE-cellulose chromatography. Proteins were solubilized from porcine liver and applied to DEAE-cellulose. Fractions were eluted with the following concentrations of NaCl: 50 mM (lanes 1,2); 75 mM (lanes 3,4); 100 mM (lanes 5,6); 125 mM (lanes 7,8). Samples of these fractions were subjected to the HDL ligand blot assay. In lanes 1a – 8a, corresponding to lanes 1 – 8, the HDL-incubation was omitted from the ligand blotting procedure. The unretained fraction, which was collected as a single pool, did not contain HDL-binding activity (not shown).

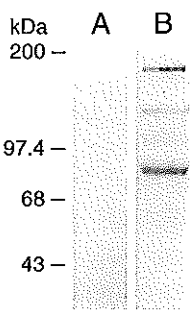

Figure 2. ConA-Sepharose chromatography. DEAE-fractions containing HDL-binding activity were applied to ConA-Sepharose. Two fractions were obtained: an unretained fraction (lane A) and a retained fraction (lane B), which eluted with methyl-α-D-glucopyranoside. Samples of both fractions were analyzed in the HDL ligand blot assay.

binding protein was observed. In all cases the 90 and 180 kDa proteins were by far the most prominent bands.

The retained fraction was analyzed in ligand blots using ^{125}I–HDL in absence or presence of a 20 fold excess on a mol/mol basis of unlabeled HDL or LDL (Figure 3). Again, two HDL-binding proteins of approximately 90 and 180 kDa were visible (Figure 3, lane A). The 110 and 130 kDa proteins were present as minor bands. Binding was completely inhibited when unlabeled HDL was present in the incubation medium (Figure 3, lane B), while LDL had little effect (Figure 3, lane C).

The use of either porcine or human HDL in the ligand blot assay

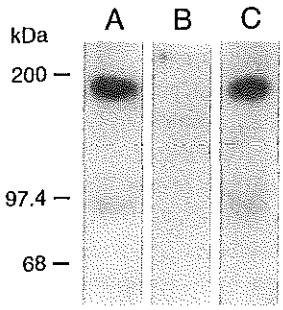

Figure 3. ^{125}I–HDL ligand blotting. ConA-Sepharose purified proteins were analyzed in the HDL ligand blot assay. Blots were incubated with 10 μg/ml ^{125}I–HDL in the absence (lane A) or presence of 20 fold excess on a mol/mol basis of unlabeled lipoprotein (lane B: 200 μg/ml HDL; lane C: 1100 μg/ml LDL). HDL-binding proteins were visualized by autoradiography. For calculations of mol/ mol based excesses of lipoproteins, the following data were used: LDL: molecular weight, 2.3×10^6; amount of protein, 22%; HDL: molecular weight, 1.75×10^5; amount of protein, 55% (30).

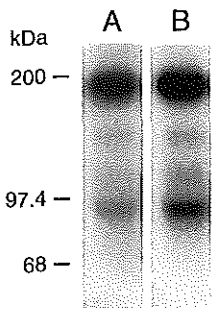

Figure 4. Comparison between porcine and human HDL. ConA-Sepharose purified proteins were analyzed in the HDL-ligand blot assay. Blots were incubated with 10 μg/ml of either porcine ^{125}I–HDL (lane A) or human ^{125}I–HDL (lane B). HDL-binding proteins were visualized by autoradiography.

resulted in an identical pattern of HDL-binding proteins in this fraction (Figure 4).

Further purification was obtained by repetitive preparative SDS-PAGE. The ConA-Sepharose retained fraction was subjected to SDS-PAGE. Slices containing proteins of 90 and 180 kDa molecular mass, respectively, were cut out from gels (Figure 5, left panel). Proteins were electro-eluted from these gel slices and subjected to SDS-PAGE again. After two rounds of SDS-PAGE, gel slicing and electro-elution, the proteins were purified to homogeneity as judged from silver-stained gels. In a typical preparation, the yield from 2600 mg solubilized protein was 480 μg and 260 μg of 90 and 180 kDa protein, respectively. After protein transfer onto nitrocellulose, a small strip was subjected to the ligand blot assay. At the position of the HDL-binding proteins, a horizontal strip of the nitrocellulose sheet was cut out. Proteins were eluted from this strip with dimethyl sulfoxide and used to immunize the rabbits. Two antisera were obtained, recognizing proteins of approximately 90 and 180 kDa respectively (Figure 5, right panel).

In immunoprecipitation studies with the antiserum raised against the 180 kDa HDL-binding protein, it was shown that with increasing amounts of

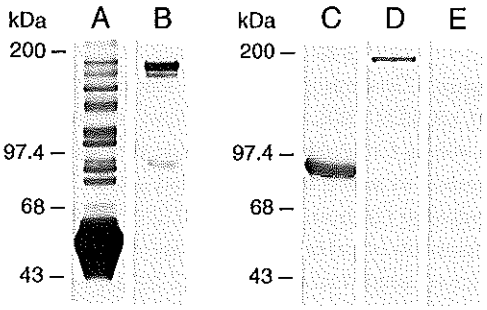

Figure 5. Preparative SDS-PAGE and immunoblotting of porcine liver proteins. *Left,* lane A: SDS-PAGE gels of ConA-Sepharose purified proteins. Slices containing proteins of 90 and 180 kDa were cut out from the gels for preparative SDS-PAGE as indicated in the text; lane B: HDL ligand blot assay on ConA-Sepharose proteins. *Right,* immunoblotting with antisera against purified proteins. Solubilized porcine liver proteins were subjected to SDS-PAGE, tranferred onto nitrocellulose membranes, and incubated with polyclonal antisera raised against the 90 kDa (lane C) and 180 kDa (lane D) HDL-binding proteins or with preimmune serum (lane E), followed by an incubation with peroxidase conjugated anti-rabbit IgG.

antiserum, HDL-binding activity to a 180 kDa protein intensifies in the precipitate (Figure 6, top), while it concomitantly attenuates in the non-precipitated fraction (Figure 6, bottom). No coprecipitation of the 90 kDa HDL-binding protein was observed in these experiments. Thus, it can be concluded that the antiserum obtained recognizes an HDL-binding protein of 180 kDa. Attempts to obtain similar results using the antiserum raised against the 90 kDa HDL-binding protein were unsuccesful. This was due to the fact that no immunoprecipitation could be achieved with this antiserum, as revealed by immunoblotting: no protein could be detected in the "precipitated" fraction, while the immunoreactivity in the non-precipitated fraction remained unchanged (results not shown).Immunohistochemistry was performed with these antibodies on semi-thin frozen sections of porcine liver. The localization of the two HDL-binding proteins appeared to be (14-19). Some groups detected two HDL-binding proteins within one tissue type (18, 19). This diversity may in part be attributable to species and/or tissue specificity. In rat luteal cells for instance, where HDL-cholesterol serves as a precursor for steroidogenesis, an HDL-binding protein of 58 kDa has been reported (31). Recently, an 80 kDa protein has been described in mouse adipose cells, which serve as a cholesterol depot (16). In many peripheral cells, a 110 kDa HDL-binding protein is present (17), mediating cholesterol efflux from these cells by a non-endocytotic mechanism (20, 21). On loading the cells with cholesterol, the amount of markedly different (Figure

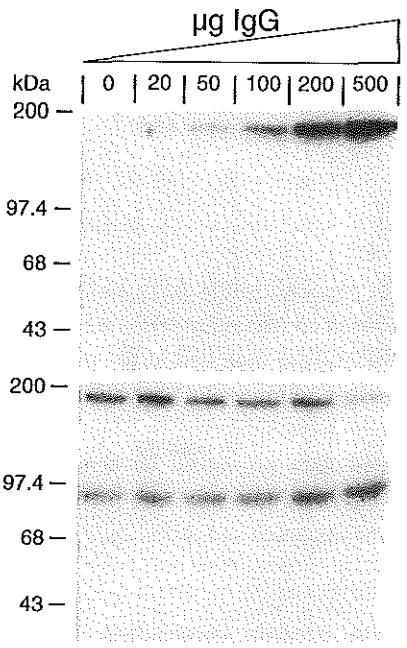

Figure 6. Immunoprecipitation studies using the anti-180 kDa HDL-binding protein antiserum. To 400 µg ConA-Sepharose eluate the indicated amounts of antiserum were added. After incubation (16 hours, 4 °C), 150 µl of a 1:1 suspension of proteinA-Sepharose was added. After an additional incubation (2 hours, room temperature), the supernatant was separated from the immunoprecipitate by centrifugation (5 min, 400 *g*). Both were boiled in SDS-PAGE sample buffer and analyzed in the ligand blot assay. *Top*: immunoprecipitate; *bottom*: non-precipitated fraction.

7). While the 90 kDa HDL-binding protein is located within the hepatocellular plates, the 180 kDa HDL-binding protein is present along the lining of the sinusoids.

Discussion

HDL is presumed to act as a vehicle for cholesterol in the reverse cholesterol transport pathway. In this pathway, one or more HDL-binding proteins could be involved (8, 9). A variety of HDL-binding proteins has been described by several research groups using ligand blotting studies this 110 kDa protein was shown to increase, suggesting a regulative mechanism for increased cholesterol efflux (17).

Several groups have reported the presence of HDL-binding proteins in liver. However, their data are not consistent. In rat liver, HDL-binding proteins of 78 kDa (14) and 100 and 120 kDa (19) have been described, while in the rat hepatoma cell line Fao, a 110 kDa HDL-binding protein has been detected (15). In the human hepatoma cell line HepG2, both an 80 kDa (32) and a 110 kDa (17) HDL-binding protein have been reported. There are no reports on the functional differences between these proteins.

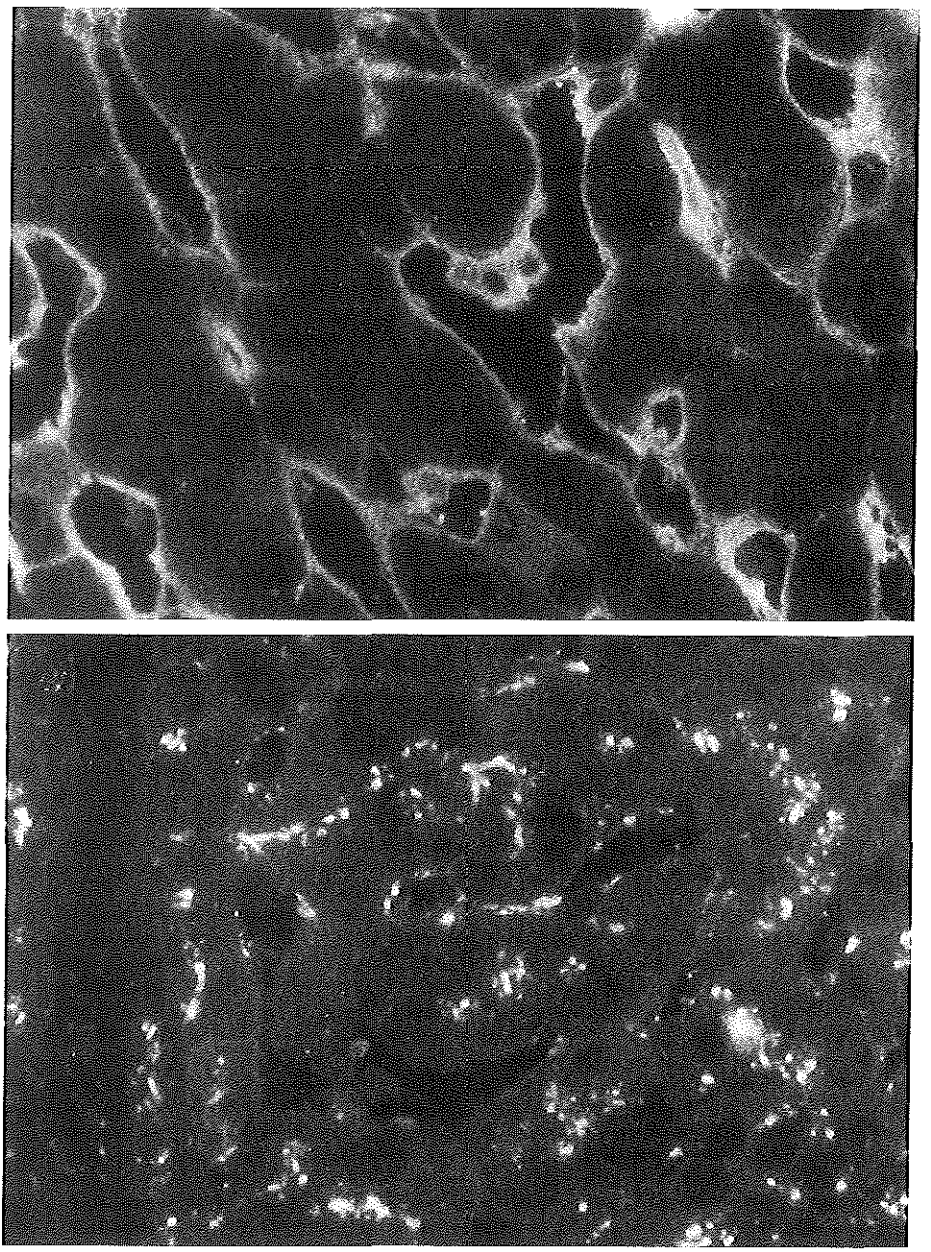

Figure 7. Immunohistochemistry of porcine liver sections. Semithin (0.5 – 1.0 μm) cryosections of porcine liver were incubated with polyclonal antibodies raised against the 90 kDa (*top*) and the 180 kDa HDL-binding proteins (*bottom*). Antigen-antibody complexes were visualized with FITC-conjugated goat anti-rabbit immunoglobulins.

In this study, we show that in porcine liver two major HDL-binding proteins of approximately 90 and 180 kDa are present. These proteins were purified to apparent homogeneity and used to raise polyclonal antisera. Each of the antisera obtained recognized only one of the HDL-binding proteins, indicating that these proteins are unrelated, at least immunologically. Moreover, striking differences were observed in histochemical localization studies on porcine liver sections using these antisera. The distribution of the 90 kDa HDL-binding protein within the hepatocellular plates is suggestive for a localization at or near the bile canaliculi, while the 180 kDa HDL-binding protein is present along the lining of the sinusoids. These results suggest a functional difference between the two HDL-binding proteins described.

In the reverse cholesterol transport pathway, HDL delivers cholesterol to the liver for excretion into the bile. Recent evidence for this pathway is produced by perfusion studies with monkey and rat liver (33, 34). Therefore, a liver specific HDL metabolism is obligatory. We describe two proteins in porcine liver which bind HDL in a ligand blot assay carried out on nitrocellulose filters. Their function in HDL-metabolism remains to be elucidated. However, some speculations can be made regarding the differences in localization. Assuming that the 180 kDa protein is a cell-surface protein, it could bind plasma HDL. In this respect, it could be equivalent to the peripheral HDL-binding protein of 110 kDa that mediates cholesterol efflux (17, 20, 21). However, on binding to the 110 kDa protein, no cellular uptake of HDL occurs (20). In contrast, HDL is taken up by liver cells (35-39). In hepatocytes, HDL-cholesterol can be converted to bile acids for excretion into the bile. However, a considerable part enters the bile as free cholesterol (33). The 90 kDa HDL-binding protein, if indeed located near the bile canaliculi, is possibly interacting in this process. The 90 kDa protein could also be a mediator for excretion of apolipoproteins A-I or A-II into the bile (40), where these apolipoproteins probably serve as cholesterol crystal formation inhibitors (41, 42).

HDL particles can also be reexcreted or transported to lysosomes (35-37, 39, 43). In these routes, other still undefined HDL-binding proteins could be involved. Indeed, we observed two minor HDL-binding protein bands after ConA-Sepharose chromatography in several preparations. One of these proteins has a molecular mass of 110 kDa and might be identical to the 110 kDa protein in HepG2 cells described by Graham and Oram (14). Further purification and characterization of these proteins is in progress.

Acknowledgments

We thank Frans W. Verheijen, and Marina A.W. Umans-Eckenhausen for critically reading the manuscript; Ruud Koppenol, Mirko Kuit and Tom de Vries Lentsch for preparation of the figures; and Jeannette Lokker for secretarial assistence.

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Appendix Paper 2

Structural Relation Between High Density Lipoprotein-Binding Proteins in Porcine Liver

Rini P.G. de Crom,
Rien van Haperen,
Pim Visser,
Rob Willemsen,
Arthur W.M. van der Kamp

MGC-Department of Cell Biology and Genetics,
Erasmus University Rotterdam,
3000 DR Rotterdam,
The Netherlands

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We have found strong evidence for a relation between three high density lipoprotein (HDL)-binding proteins of 90, 110 and 180 kDa present in porcine liver that were detected by ligand blotting. Because HDL-binding proteins with identical molecular masses were detected in human liver, all subsequent experiments were performed with porcine liver proteins. An antiserum raised against a highly purified preparation of the 90 kDa HDL-binding protein, designated 90-PC, showed cross-immunoreactivity with the 110 and 180 kDa HDL-binding proteins. Purified protein preparations of the 90, 110 and 180 kDa HDL-binding proteins were obtained and analyzed by polyacrylamide gel electrophoresis with sodium dodecyl sulfate. Under nonreducing conditions these preparations showed protein bands with the expected molecular masses. Reduction of these preparations resulted in protein bands of 90 kDa. Ligand blotting experiments with ^{125}I–HDL showed protein bands of 90, 110 and 180 kDa under nonreducing conditions, and a 90 kDa protein band in all three preparations under reducing conditions. Immunoblotting experiments with the 90-PC antiserum resulted in a similar pattern. The three protein preparations were then subjected to cyanogen bromide cleavage and the resulting peptides separated on gel. Immunoblotting with the 90-PC antibody revealed a pattern of protein bands that was remarkably similar in the three protein preparations. Immunohistochemical localization studies with the 90-PC antibody showed that the HDL-binding proteins were present both at the borders of the sinusoids as well as within the hepatocellular plates. We conclude that the 180 kDa is a homodimer of a monomeric HDL-binding protein present in two conformation variants of 90 and 110 kDa.

Epidemiological studies show an inverse correlation between the plasma levels of high density lipoprotein (HDL) and the incidence of atherosclerosis. Therefore, HDL is believed to possess anti-atherogenic properties [1-2]. This concept is supported by studies with laboratory animals, in which the administration of HDL was shown to have anti-atherogenic effects [3,4]. Very strong evidence comes from observations of transgenic mice that overexpress human apoA-I. These animals have higher levels of HDL in their plasma, and the development of atherosclerosis induced by a hypercholesterolemic diet is inhibited in comparison to control animals [5]. The anti-atherogenicity of HDL probably resides in its capability to transfer cholesterol from peripheral tissues to the liver for excretion, a process called reverse cholesterol transport [6,7]. Detailed knowledge about the functioning

of the reverse cholesterol transport pathway and about factors that regulate and affect it, is still largely lacking.

There are two processes in this pathway in which HDL-binding proteins may be involved. These are the efflux of cholesterol from peripheral cells to HDL and the uptake of HDL-particles or HDL-cholesterol by the liver.

Controversy exists about the mechanism by which HDL is able to take up cholesterol from cells in peripheral tissues [8]. Some authors do not find positive evidence for a relation between HDL-binding and cholesterol efflux from cells to HDL particles [9,10]. Oram and coworkers however, describe an HDL-binding protein of 110 kDa that has been shown to be involved in cholesterol efflux [11,12]. Recently, a cDNA clone that probably encodes this protein has been isolated [13].

In the liver, HDL-binding proteins have also been described. Tozuka and Fidge [14] have found two HDL-binding proteins of 100 and 120 kDa in rat liver. In the human hepatoma cell line Hep3B, HDL-binding proteins of 60, 100 and 210 kDa have been described [15]. We have characterized two HDL-binding proteins of 90 and 180 kDa in porcine liver. We have also found evidence for two HDL-binding proteins of 110 and 130 kDa in porcine liver, which appear to be present in minor quantities [16]. In this article we describe a structural relation between the 90, 110 and 180 kDa proteins.

Experimental Procedures

Lipoproteins

Plasma from healthy human volunteers was used for the isolation of LDL (d = 1.019 – 1.063 g/ml) and HDL (d = 1.063 – 1.21 g/ml) by sequential ultracentrifugation [17]. HDL was subjected to heparin-Sepharose affinity chromatography to remove apoB/E containing particles [18]. Analysis of lipoprotein preparations was performed by SDS-PAGE [19]. In the HDL preparations, apoA-I, apoA-II and apoC's could be detected; no apoE was present. In the LDL preparations, apoB-100 appeared to be the only protein constituent. HDL was radiolabeled with ^{125}I by the iodine monochloride method [20]. 96 – 98% of the radioactivity was protein bound, 1.5 – 3% was present in phospholipids, and 1% was free. The protein-bound radioactivity was present in apoA-I (60 – 65%), in apoA-II (23 – 25%), and in apoC's (very small amounts). Specific activities were about 300 cpm/ng protein.

HDL-binding assay

HDL-binding proteins were studied in a ligand blot assay as described [16]. In brief, protein fractions were subjected to SDS-PAGE and electrophoretically transferred onto

nitrocellulose membranes (0.45 μm; Schleicher & Schuell) [21]. These membranes were incubated with blocking buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$, 1% [wt/vol] bovine serum albumin, 1% [wt/vol] low fat milk powder [a gift from Nutricia B.V., Zoetermeer, The Netherlands], 50 μg protein/ml LDL), and subsequently with 5 ml of blocking buffer containing 10 μg protein/ml ^{125}I–HDL in tubes under continuous agitation. The HDL bound to the HDL-binding proteins on the nitrocellulose membranes was visualized by exposure to Kodak X-Omat AR films in cassettes containing two regular intensifying screens (Eastman Kodak Company, Rochester, New York) (exposure time 3 – 10 days).

Solubilization of liver proteins

All operations were carried out at 0 – 4 °C. Porcine livers obtained from the Department of Experimental Cardiology, Erasmus University Rotterdam, were collected on ice immediately after death of the animal, cut into small pieces, and stored at –70 °C. The pigs weighed ± 60 kg and were 3 – 4 months old. For protein solubilization, a plasma membrane enriched fraction was prepared essentially according to Schneider *et al.* [22]. Liver tissue (100 g) was minced using surgical blades and homogenized with an Ultra-Turrax TP 18-10 in 200 ml 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride,Tissue debris was removed by centrifugation (10 min, 1350 *g*). The supernatant was centrifuged (60 min, 100 000 *g*) and the pellet resuspended in 120 ml homogenization buffer containing 1% (vol/vol) Triton X–100 by aspiration with a 22-gauge needle. The suspension was stirred for 10 min and slowly diluted 10 times in 10 mM Tris–HCl pH 8.0, 2 mM $CaCl_2$, 1% (vol/vol) Triton X–100. After removal of particulate material by centrifugation (30 min, 20 000 *g*) a clear supernatant was obtained.

DEAE-cellulose chromatography

The solubilized fraction was applied to a DEAE-cellulose (DE-52; Whatman) column (2.4 × 10 cm) at a flow rate of 30 ml/hour. The column was washed with 10 mM Tris–HCl pH 8.0, 2 mM $CaCl_2$, 15 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate). Elution was performed with washing buffer containing increasing concentrations of NaCl.

Concanavalin A (ConA) chromatography

DEAE-cellulose fractions containing HDL-binding activity were pooled and dialyzed against 20 mM sodium phosphate pH 6.8, 0.25 mM $CaCl_2$, 0.25 mM $MnSO_4$ and 10 mM NaCl (ConA-buffer). ConA-Sepharose 4B (Sigma) was added to these fractions. The reaction was allowed to proceed overnight at 4 °C under gentle agitation. The gel was transferred to a column and washed with ConA-buffer containing 1 M NaCl and eluted with ConA-buffer containing 0.5 M NaCl and 1 M methyl-α-D-glucopyranoside.

Preparative continuous elution electrophoresis

Protein samples were run on a Bio-Rad Model 491 Prep Cell apparatus, following the manufacturer's instructions. Typically, in the first run approximately 15 mg protein was applied on a 5.5% polyacrylamide gel in the presence of SDS. Fractions of 10 ml were collected. These fractions were lyophilized and tested in the HDL-binding assay. Fractions containing one of the HDL-binding proteins were pooled and subjected to a second round of continuous elution electrophoresis. In this second round, approximately 1 mg of protein was applied to the gel. The composition of the gel was varied according to the molecular mass of the protein of interest (7% for the 90 kDa protein). To achieve optimal separation, subsequent runs with small variations in percent acrylamide were sometimes required if proteins of approximately the same molecular masses were present in the preparation.

CNBr fragmentation

Chemical cleavage using CNBr was performed essentially according to Matsudaira [23]. Protein samples for CNBr fragmentation were dialyzed against 10 mM NH_4HCO_3 and lyophilized. Aliquots (200 ml) were added from a 80 mg/ml CNBr solution in 70% formic acid and incubated at room temperature in the dark under nitrogen gas. The sample was diluted 10 × with water, and lyophilized. Sample buffer was added and fragments were run on a 16% Tricine (N-[Tris(hydroxy-methyl)-methyl] glycine)-SDS-PAGE gel according to Schägger and Von Jagow [24].

Antisera

Antibodies to HDL or HDL-binding proteins were prepared by standard techniques [25]. In brief, 50 mg protein was applied to an SDS-PAGE gel and electrophoretically transferred onto nitrocellulose membranes. Small strips of these membranes with the protein of interest were cut out and dissolved in dimethyl sulphoxide. 1 ml incomplete Freund's adjuvant was added. A rabbit (New Zealand White) was injected subcutaneously with this mixture. Two boosters with the same mixture were given at intervals of 28 days. Ten days after the last injection the blood was collected and the serum was obtained by centrifugation at 1300 *g* after clotting. All animals were cared for in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Erasmus University Rotterdam (prot.nr. 132.91.52) and with the European Committee Standards on the care and use of laboratory animals (Ministry of Welfare, Health and Cultural Affairs, The Netherlands).

Immunoblotting

Protein samples were subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes, which were incubated with blocking buffer (without LDL) containing 0.05% (vol/vol) Tween-20 as described for the ligand blot assay procedure,

followed by a 2 hour incubation with blocking buffer containing 0.05% (vol/vol) Tween-20 and anti-HDL-binding protein antiserum (dilution 1:1000). Alkaline phosphatase-conjugated, goat $F(ab')_2$ anti-rabbit immunoglobulins (Tago, Inc, Burlingame, CA, USA) were used as the secondary antibody. Reactive proteins were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Control incubations were performed using either pre-immune serum or omitting the first antibody. Virtually no protein bands were visible in these control experiments, unless indicated otherwise.

Elution of specific antibodies from nitrocellulose membranes

Protein samples were subjected to SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. These membranes were subjected to the immunoblotting procedure. For the incubation with the secondary antibody and subsequent visualization, a vertical strip cut from each nitrocellulose membrane was used. With this strip as a reference, horizontal strips were cut from the unstained nitrocellulose membrane containing immunoreactive proteins of 90 and 180 kDa. The antibodies were eluted by a procedure adapted from Smith and Fisher [26]. Membranes were washed with 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$, 01% (vol/vol) Tween-20 and eluted with a 30 second wash with 0.1 M glycine-HCl pH 2.3, 1 M NaCl, 10% (vol/vol) 1,4-dioxane. Samples were neutralized by the addition of 1 M Tris to pH 7.4. Bovine serum albumin was added to a concentration of 100 mg/ml.

Immunohistochemistry

The porcine liver was fixed and prepared for ultracryotome sectioning as described [27]. Semithin cryosections (0.5 – 1.0 μm) were incubated with a Protein A Sepharose CL-4B (Pharmacia) purified IgG fraction of polyclonal antibodies against HDL-binding proteins (dilution 1:10) for 1 hour at room temperature in a humid incubator. Antigen-antibody complexes were visualized with FITC goat anti-rabbit immunoglobulins (Nordic, Tilburg, The Netherlands) (diluted 1:80; incubated at room temperature for 1 hour in a humid incubator) and studied by fluorescence microscopy.

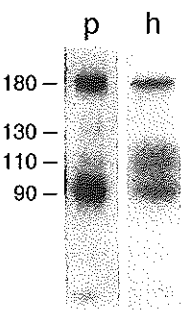

Figure 1. HDL-binding proteins in porcine and human liver. ConA-fractions from either porcine (p) or human (h) liver were analyzed in the HDL-binding assay as described in "Experimental Procedures". Proteins were run on a 6.5% SDS-PAGE gel under nonreducing conditions and blotted onto nitrocellulose membranes. In each lane, 100 μg of protein is applied. The filters were incubated with ^{125}I–HDL (10 μg protein/ml) followed by autoradiography. The molecular mass of the HDL-binding proteins is indicated.

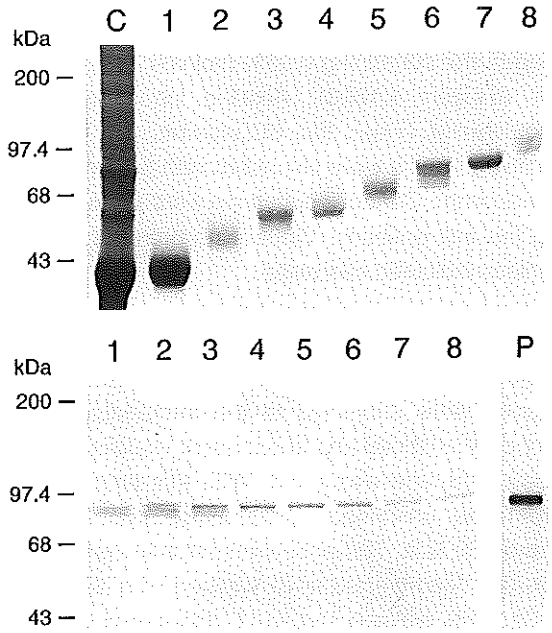

Figure 2. Purification of 90 kDa HDL-binding protein by preparative continuous elution electrophoresis. Proteins from the ConA-fraction of porcine liver were subjected to preparative continuous elution electrophoresis. In the first run, 15 mg protein was applied without reducing agents to a 5.5% SDS-polyacrylamide gel. Eluted proteins were collected in 10 ml fractions. Small samples of consecutive fractions were applied to a 5.5% SDS-polyacrylamide slab gel, run under nonreducing conditions, and proteins were stained with Coomassie Brilliant Blue (*top*: lane C, starting material; lanes 1 – 8: eight consecutive fractions). Fractions containing proteins of approximately 90 kDa (including the 90 kDa HDL-binding protein as tested in the HDL-binding assay) were pooled and run on a 7% SDS-polyacrylamide gel under nonreducing conditions by preparative continuous elution electrophoresis. Eluted proteins were collected in consecutive 2 ml fractions, of which 20 µl samples were run on a 7% SDS-polyacrylamide slab gel under nonreducing conditions, and protein stained with Coomassie Brilliant Blue (*bottom*). Lanes 1 – 8 show eight consecutive fractions, including those containing the 90 kDa HDL-binding protein. Fractions were tested for the presence of the 90 kDa HDL-binding protein in the HDL-binding assay (results not shown). Those fractions in which the 90 kDa HDL-binding protein was present in an apparently homogenous form (lanes 5 – 7) were pooled and concentrated by lyophilization.. The lane on the right (P) is a 5 µg sample from this preparation run on a 7% SDS-polyacrylamide slab gel under nonreducing conditions; protein were stained with Coomassie Brilliant Blue.

Results

Membrane proteins from porcine liver were applied to DEAE-cellulose and eluted with a discontinuous salt gradient (50, 250, 1000 mM NaCl) as described[16]. Fractions containing HDL-binding proteins (the 250 mM NaCl eluate), as established in the HDL-binding assay, were pooled and dialyzed against ConA-buffer as described in "Experimental Procedures" and subjected to ConA-Sepharose chromatography. Retained proteins were eluted by methyl-α-D-glucopyranoside. Protein recoveries were approximately 12% after DEAE-cellulose and 3% after ConA-Sepharose

chromatography. This protein preparation, which is referred to as the ConA-fraction, appeared to contain two major HDL-binding proteins of 90 and 180 kDa an two less prominent ones of 110 and 130 kDa (Fig. 1). For comparison, a ConA-fraction from human liver was prepared in exactly the same way. This fraction showed HDL-binding proteins of 90, 110 and 180 kDa in approximately equal amounts (Fig. 1). Because of the easy availability, we performed all subsequent studies with porcine liver. The ConA-fraction was subjected to preparative continuous elution electrophoresis on a Bio-Rad Model 491 Prep Cell system. During the first run, fractions of 10 ml were collected. Fig. 2, top panel, shows samples of consecutive fractions containing proteins of increasing molecular mass ranging from approximately 60-110 kDa. These fractions were tested for HDL-binding activity in the HDL-binding assay. Those fractions containing the 90 kDa HDL-binding protein were pooled and subjected to another run of continuous elution electrophoresis while the composition of the gel was varied to obtain optimal separation in the range of molecular masses of proteins still present in the applied preparation. Those fractions in which the 90 kDa HDL-binding protein was present in an apparently homogeneous form were pooled and used for further study (Fig. 2, lower panel, right lane). From 100 grams of liver tissue, approximately 0.5 mg purified 90 kDa HDL-binding protein was isolated.

This purified fraction was used to develop a high titer polyclonal antiserum in rabbits. As shown in Fig. 3, this antiserum, designated 90-PC, reacted with the purified 90 kDa HDL-binding protein on an immunoblot. It also recognized a protein band of 90 kDa in the ConA-fraction. In addition, an unambiguous cross-reaction with protein bands of 110 and 180 kDa was observed in this fraction. Both 110 and 180 kDa are molecular masses of HDL-binding proteins. This cross-reactivity of an antiserum raised against a purified preparation of the 90 kDa HDL-binding protein suggests a structural relation between these three proteins. In order to evaluate this cross-reactivity, nitrocellulose membranes on which Con-A fraction proteins were blotted were incubated with 90-PC antiserum followed by elution of

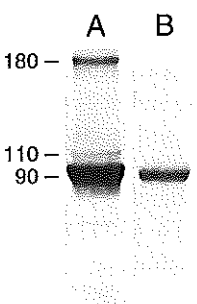

Figure 3. Polyclonal antiserum 90-PC raised against purified 90 kDa HDL-binding protein preparation from porcine liver, was tested by immunoblotting for reactivity. Gels were run under nonreducing conditions. Lane A: porcine ConA-fraction (100 μg protein); Lane B: purified 90 kDa HDL-binding protein preparation (5μg protein).

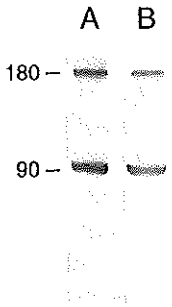

Figure 4. 90-PC antibodies eluted from proteins on nitrocellulose membranes. Horizontal strips of nitrocellulose were cut at the 90 kDa and 180 kDa position, and antibodies were eluted from these strips as described in "Experimental Procedures". These antibody preparations were used in subsequent immunoblotting procedures with porcine ConA-fraction proteins. Lane A: antibody preparation eluted from 90 kDa protein; Lane B: antibody preparation eluted from 180 kDa protein. In control incubations with only the secondary antibody, a minor protein band of 150 kDa was sometimes observed.

antibodies from strips of the nitrocellulose membranes cut out at the 90 and 180 kDa postitions respectively. These antibody eluates were used in immunoblotting experiments using Con-A fraction proteins (Fig. 4). These experiments showed that antibodies eluted from the 90 kDa position, as well as those eluted from the 180 kDa position showed cross-reactivity with proteins of 90 and 180 kDa. No protein band was visible at 110 kDa, probably because the amount of 110 kDa protein is below the level of detection.

To investigate the possible relation between the 90, 110 and 180 kDa HDL-binding proteins, we prepared purified protein fractions of the 110 and 180 kDa proteins from the ConA-fraction, using preparative continuous elution electrophoresis as described above for the purification of the 90 kDa protein. However, compared with the 90 kDa HDL-binding protein, the yield of these proteins was much lower (approximately 10 – 25 ×).

The purified protein preparations were examined on a Coomassie Brilliant Blue stained SDS-PAGE gel, run under either nonreducing or reducing conditions (Fig. 5). For the 180 kDa protein, a purified preparation of apparant homogeneity was obtained. For the 110 kDa protein however, only partial purification was achieved. The protein band of 110 kDa on a

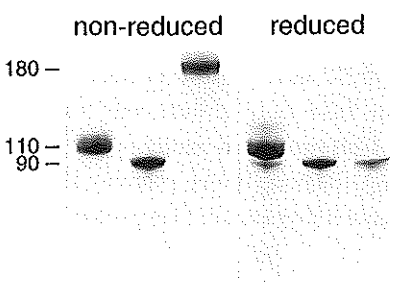

Figure 5. Fractions of HDL-binding proteins from porcine liver were purified as described in the text and examined on Coomassie Brilliant Blue stained SDS-polyacrylamide gels, under both nonreducing and under reducing conditions. Lanes 1 and 4: 110 kDa HDL-binding protein preparation, 10 µg; lanes 2 and 5: 90 kDa HDL-binding protein preparation, 5 µg; lanes 3 and 6: 180 kDa HDL-binding protein preparation, µg.

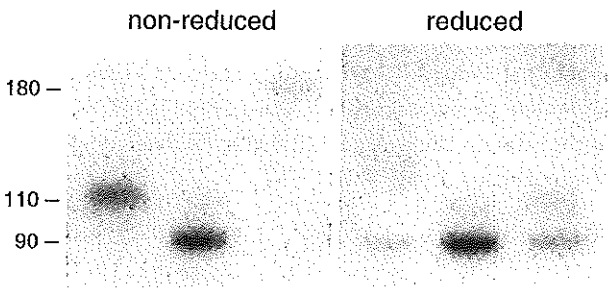

Figure 6. Purified fractions of HDL-binding proteins from porcine liver, examined in the HDL-binding assay as described in "Experimental Procedures". Lanes 1 and 4: 110 kDa HDL-binding protein preparation, 10 µg; lanes 2 and 5: 90 kDa HDL-binding protein preparation, 5 µg; lanes 3 and 6: 180 kDa HDL-binding protein preparation, 2 µg.

nonreducing SDS-PAGE gel consists of three proteins, as became apparent when a sample was run under reducing conditions. Surprisingly, under reducing conditions the 90 kDa as well as the 180 kDa preparation showed a single protein band with an apparent molecular mass of 90 kDa, whereas a protein band of 90 kDa was also present in the 110 kDa preparation. The 90 kDa band in the 110 kDa protein preparation on reducing SDS-PAGE gels may originate from a protein consisting of two subunits with a molecular mass of 90 and 20 kDa, respectively. Therefore, a sample of the 110 kDa preparation was run on a gradient gel under reducing conditions to focus on the possible presence of small protein fragments. No protein bands smaller than 90 kDa could be detected (results not shown).

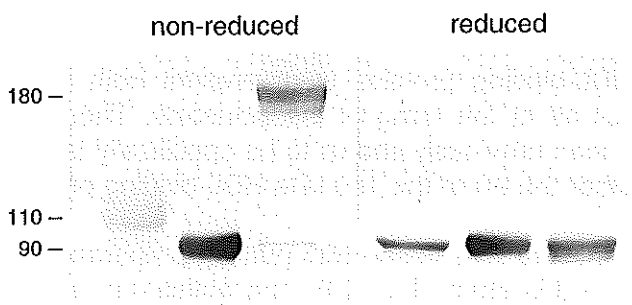

Figure 7. Immunoreactivity of 90-PC antiserum with purified fractions of HDL-binding proteins from porcine liver, which were examined by immunoblotting as described in "Experimental Procedures". Lanes 1 and 4: 110 kDa HDL-binding protein preparation, 10 µg; lanes 2 and 5: 90 kDa HDL-binding protein preparation, 5 µg; lanes 3 and 6: 180 kDa HDL-binding protein preparation, 2 µg.

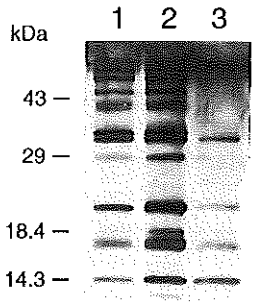

Figure 8. CNBr-fragment maps of HDL-binding proteins from porcine liver run on a Tricine-SDS-PAGE gel, electrophoretically transferred onto nitrocellulose, and immunoblotted with 90-PC antiserum. Lane 1: 110 kDa HDL-binding protein preparation, 50 μg; lane 2: 90 kDa HDL-binding protein preparation, 25 μg; lane 3: 180 kDa HDL-binding protein preparation, 10 μg.

HDL-binding capacities of the purified protein fractions (90, 110, and 180 kDa) were examined. As shown in Fig. 6, under nonreducing conditions each protein preparation showed HDL-binding activity. Under reducing conditions, in all three preparations HDL-binding activity was present at the 90 kDa position only.

In Fig. 7, reaction of the three size fractions with 90-PC antiserum is shown under either nonreducing or reducing conditions. Protein bands of 90, 110 and 180 kDa were visible under nonreducing conditions, and only a 90 kDa band was visible in each preparation under reducing conditions.

We subjected the three protein fractions to CNBr cleavage. The samples were run on a Tricine-SDS-PAGE gel and blotted onto nitrocellulose. With the 90-PC antiserum, immunoblots of the 90, 110 and 180 kDa protein fractions were produced (Fig. 8). This resulted in specific patterns of protein bands. These patterns show striking similarities, strongly suggesting that the three preparations consist of the same protein in different conformations.

The newly developed 90-PC antiserum was also applied in an immunohistochemical localization study with porcine liver sections (Fig 9). This study showed that HDL-binding proteins were present both in the hepatocellular plates as well as at the lining of the sinusoids. These are localization sites that have been previously shown to be specifically labeled with antisera recognizing either the 90 or the 180 kDa HDL-binding proteins respectively [16].

The reactivity of these antisera was tested with purified preparations of HDL-binding proteins used in this study (Fig. 10). The antiserum directed against the 90 kDa HDL-binding protein reacted with the purified 90 kDa protein preparation under nonreducing conditions, but hardly reacted under reducing conditions. The antiserum directed against the 180 kDa HDL-binding protein reacted with purified 180 kDa protein preparation under nonreducing conditions but not under reducing conditions.

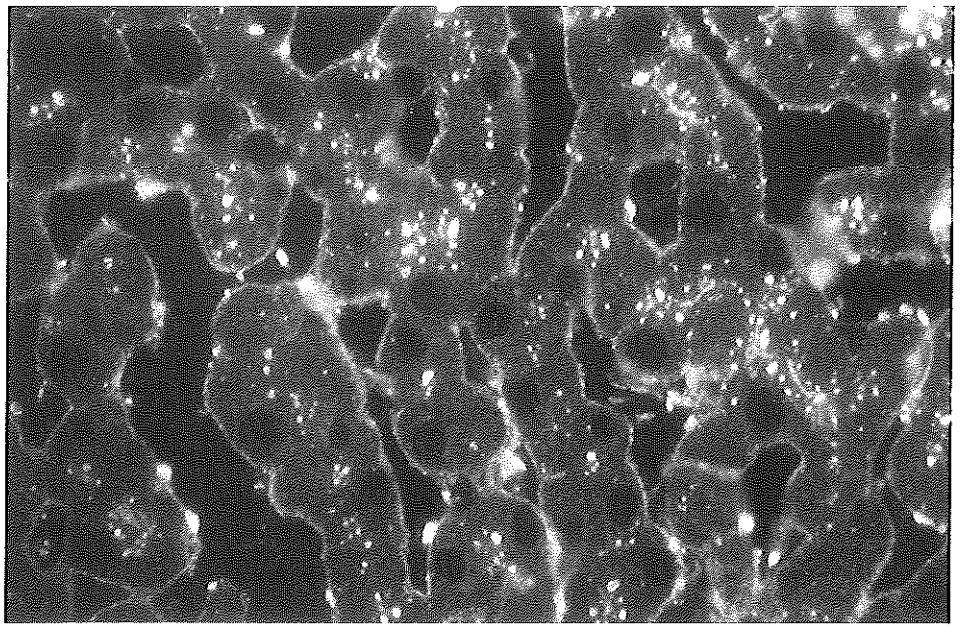

Figure 9. Immunohistochemistry of porcine liver sections. Semithin (0.5 – 1.0 μm) cryosections of porcine liver incubated with the 90-PC antiserum. Antigen-antibody complexes were visualized with FITC-conjugated goat anti-rabbit immunoglobulins. (original magnification: × 450).

Discussion

We have found strong evidence for a relation between three HDL-binding proteins in porcine liver. These proteins have apparent molecular masses of 90, 110 and 180 kDa as determined by SDS-PAGE under nonreducing conditions.

The 180 kDa protein can be reduced, resulting in a single protein band of 90 kDa that still shows HDL-binding activity. Both the unreduced 180 kDa protein as well as the reduced 90 kDa protein resulting from reduction of the 180 kDa protein are recognized by the 90-PC antiserum that was raised against the purified 90 kDa HDL-binding protein. Antibodies from the 90-PC antiserum that eluted from the 180 kDa protein on nitrocellulose membranes after immunoblotting cross-reacted with a 90 kDa protein. Also, antibodies eluted from the 90 kDa protein cross-reacted with a 180 kDa protein. Therefore, 90-PC antiserum is not a mixture of antibodies reacting with either the 90 or the 180 kDa protein and the 90-PC antibodies have genuine

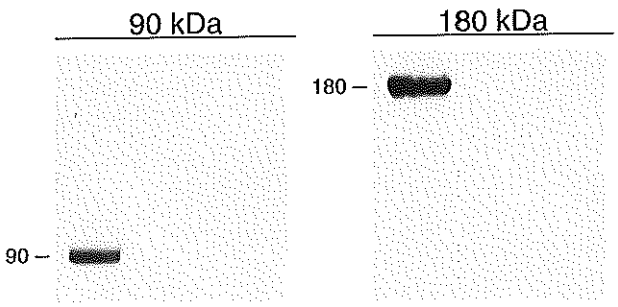

Figure 10. Immunoreactivity of purified HDL-binding proteins with antisera directed against the 90 kDa or 180 kDa HDL-binding proteins. Purified preparations of the 90 kDa (*left*) or 180 kDa (*right*) HDL-binding protein were run under either nonreducing (nr) or reducing (r) conditions, and tested by immunoblotting for reactivity with antisera directed against the 90 kDa HDL-binding protein or (*left*) the 180 kDa HDL-binding protein (*right*)as reported [16].

cross-reactivity with these two proteins. These results suggest that the 180 kDa protein is a dimer of the 90 kDa protein. We produced CNBr-cleavage maps with antibody 90-PC (Fig. 8). Protein fragment bands appearing in both samples show a strikingly similar pattern, suggesting that the 180 kDa protein consists of two identical 90 kDa subunits; i.e., that the 180 kDa form is a homodimer of the 90 kDa HDL-binding protein. In the purification procedure, the yield of 90 kDa HDL-binding protein is higher than that of the 180 kDa HDL-binding protein. Therefore, we could use a larger amount of the 90 kDa protein preparation than of the 180 kDa protein preparation. As a consequence, the faintest bands in the 90 kDa sample (Fig. 8, lane 1) are not visible in the 180 kDa sample (Fig. 8, lane 3). However, no protein fragment bands were detected in the 180 kDa sample that were absent in the 90 kDa sample. This would be highly unlikely if these two preparations had come from two different proteins or if one preparation had contained an additional protein.

A partially purified preparation of the 110 kDa HDL-binding protein also showed a 90 kDa protein band after reduction followed by SDS-PAGE, which was shown to bind HDL and to cross-react with 90-PC antiserum. In a protein-stained gel, no protein bands smaller than 90 kDa were observed under reducing conditions. Thus, the 110 kDa form apparently does not consist of two subunits of 90 and 20 kDa. The 110 kDa protein is probably a protein conformation variant of the 90 kDa protein that has a running pattern in an SDS-PAGE system under nonreducing conditions that leads to a

calculated mass of 110 kDa. This may be caused by an internal disulfide bond. Reduction allows the protein to run at a 90 kDa position. A CNBr map of the 110 kDa HDL-binding protein appears to have given a pattern identical to that of the 90 kDa HDL-binding protein, leading to the conclusion that the 110 kDa protein is an alternative conformation of the 90 kDa HDL-binding protein.

The newly developed 90-PC antiserum that was raised against the 90 kDa HDL-binding protein and that cross-reacted with 110 and 180 kDa HDL-binding proteins was tested in a histochemical study. We have previously shown that the 90 kDa HDL-binding protein is localized within the hepatocellular plates, whereas the 180 kDa HDL-binding protein is situated at the lining of the sinusoids [16]. The cross-reactivity of our newly developed 90-PC antiserum should result in a combination of these localization sites. This anticipation proved to be correct, as shown in Fig. 7. We cannot readily attribute an extra signal to the cross-reactivity with the 110 kDa HDL-binding protein. Its specific localization is unknown, since we do not have an antiserum that specifically recognizes the 110 kDa HDL-binding protein. Moreover, localization of the 110 kDa protein may (partially) overlap that of one or both of the other HDL-binding proteins, whereas subtle differences in localization might be overlooked because of limitations in the resolution of the technique.

In a previous study, we described monospecific antisera raised against the 90 and 180 kDa HDL-binding proteins [16]. This led us to assume that there was no simple relation between the 90 and 180 kDa proteins. We tested these antisera with purified HDL-binding protein preparations as described in immunoblotting experiments in this study (Fig. 10). On the basis of the results, we can make the following conclusions.

The monospecific 180 kDa antiserum apparently recognizes a structural determinant that is present only in the dimeric form for the following reasons: 1. The antiserum shows no cross-immunoreactivity with the purified 90 kDa or 110 kDa HDL-binding protein preparations under either nonreducing or under reducing conditions; 2. The antiserum reacts with the purified 180 kDa HDL-binding protein under nonreducing conditions but not with the 90 kDa protein band, that results of reduction of the purified 180 kDa HDL-binding protein. Thus, the antiserum recognizes the dimeric but not the monomeric form of the protein, which implies that the structural determinant present on the dimeric form has induced a dominant immunogenic response.

The monospecificity of the previously reported antiserum directed against the 90 kDa HDL-binding protein [16] is puzzling. This antiserum recognizes purified 90 kDa HDL-binding protein preparations under nonreducing conditions but very poorly under reducing conditions. Purified

110 kDa or 180 kDa protein preparations are not recognized by the antiserum under either nonreducing or under reducing conditions (results not shown). An explanation could be that this antiserum reacts with a unique determinant of the nonreduced 90 kDa HDL-binding protein on nitrocellulose membranes. This hypothesis is supported by the following observations. During preparation of this antiserum, we found that the protein is not very immunogenic. When preparations of electro-eluted protein in solution were used to produce antisera, no immunogenic response was evoked in the rabbits. Only when we used preparations obtained by separating proteins on SDS-PAGE, transferring them to nitrocellulose membranes and solubilizing the nitrocellulose in dimethyl sulfoxide, did we succeed in raising an antiserum. The protein probably has a defined conformation as a result of these procedures, which exposes the a structural domain that evokes an immune response. As a result, the antiserum reacts preferentially with this structural domain. This might also explain why our attempts to immunoprecipitate protein from solution with this antiserum were unsuccesful, in contrast to similar experiments using the monospecific antiserum directed against the 180 kDa HDL-binding protein [16]. In the latter case, the amount of immunoprecipitated protein was high enough to show that it had HDL-binding activity. For the monospecific antiserum to the 90 kDa protein however, we have no actual proof that it is indeed directed against an HDL-binding protein, and therefore, we cannot exclude the possibility that it recognizes a contaminating protein in the purified 90 kDa protein fraction. Although the localization pattern in the hepatocellular plates in the immunohistochemical studies with this antiserum resembles part of the pattern for the newly developed 90-PC antiserum, it does not give conclusive evidence that the two antisera recognize the same protein. This evidence will eventually come from isolation and expression of cDNA clones encoding the HDL-binding protein followed by immunochemical studies.

It is surprising that two antisera raised against purified preparations of the same protein have different characteristics. The 90-PC antiserum described in this study can also be raised against proteins eluted from nitrocellulose. However, the immunization procedure differs at two steps from the procedure used in our previous study [16]. First, the amount of protein used for immunization was probably much higher for the 90-PC antiserum than for the antiserum directed against 90 kDa HDL-binding protein in our previous study. In the latter case, we probably overestimated the amount of protein, since we now know that the electroelution procedure in the final purification step results in a very low recovery compared with the preparative continuous elution electrophoresis step we used in the present study. Second, the immunization scheme is different. Two boosters were

given with intervals of 28 days, whereas in the previous study boosters were given at 15 and 25 days after the first injection. This timing may be crucial, since in the test with ConA-fraction proteins the cross-reactivity of 90-PC antiserum after the first booster with the 180 kDa HDL-binding protein was rather weak, whereas cross-reactivity with the 110 kDa HDL-binding protein became apparent only after the second booster. This disparity cannot be attributed to differences in protein preparation, since the same preparation was used for the immunization and subsequent boosts.

Several attempts have been done to achieve immunoprecipitations with the 90-PC antiserum. The amount of immunoprecipitated protein appeared to be too low to allow detection of HDL-binding activity in the HDL-binding assay. However, in cultured porcine hepatocytes labeled with [^{3}H]-leucine, proteins of 90, 110 and 180 kDa have been immunoprecipitated using the 90-PC antiserum (unpublished results, T.I. Kerpel, R.P.G. de Crom, A.W.M. van der Kamp, Department of Cell Biology & Genetics, Rotterdam).

The three related HDL-binding proteins from porcine liver that we describe in this study are also present in human liver (Fig. 1). In Hep3B cells, a human hepatoma cell line, HDL-binding proteins of 60, 100 and 210 kDa have been reported [15]. When gels are run under reducing conditions, the 210 kDa HDL-binding signal disappears while the 100 kDa HDL-binding signal intensifies. Therefore, a monomer-dimer relationship between the 100 and 210 kDa HDL-binding proteins has been suggested. These proteins probably resemble the 90 or 110 and 180 kDa HDL-binding proteins we describe in this study.

Oram and coworkers have described an HDL-binding protein of 110 kDa in a number of cultured cells [11]. Recently, they have isolated a cDNA clone that probably encodes this protein [13]. Although we have found a 110 kDa HDL-binding protein in both human and porcine liver, these two proteins are probably not identical. Whereas Graham and Oram have found a 110 kDa HDL-binding protein in a solubilized membrane protein preparation, we have detected HDL-binding proteins of 90 and 180 kDa in a preparation that is obtained in virtually the same way. The 110 kDa HDL-binding proteins in our studies is detectable only after further purification. In contrast, Oram and coworkers have never reported the detection of 90 or 180 kDa HDL-binding proteins. Moreover, Graham and Oram have found no change in relative migration of the 110 kDa protein on SDS-PAGE after reduction [11], whereas in this study we show that the 110 kDa HDL-binding protein from liver migrates at the 90 kDa position under reducing conditions. Probably, the 110 kDa protein that has been studied by Oram and coworkers, described mainly in peripheral cells like bovine aortic endothelial cells and mouse macrophages (J774), is involved in cholesterol efflux from cells to

HDL, as indeed is strongly suggested by a number of studies from this group [28]. The proteins we describe in liver are more likely to be involved in the uptake of HDL cholesterol by the liver. Therefore, there is probably also a functional difference between these proteins.

Tozuka and Fidge found HDL-binding proteins of 100 and 120 kDa in rat liver [14]. Because estimations of molecular mass by SDS-PAGE are related to protein standards, deviations can occur between different laboratories. Therefore, the proteins found by Tozuka and Fidge could be homologues of the 90 and 110 kDa HDL-binding proteins in this study. A comparison between nonreducing and reducing conditions has revealed that the 100 kDa protein is not affected by reduction, which agrees with the results on the 90 kDa HDL-binding protein in our study. In contrast, the 120 kDa protein shows a strongly diminished HDL-binding activity but no change in mobility on SDS-PAGE after reduction, which contrasts with our observations on the 110 kDa protein. At this moment, it is not clear wether there is a relation between these proteins.

Antisera raised against porcine HDL-binding proteins do not crossreact with human proteins. This was tested both in immunoblotting experiments and in immunohistochemical studies (results not shown), which greatly impairs the possibility of performing physiological studies in hepatoma cell culture systems, since a system of porcine origin is not available. These studies are nevertheless essential for evaluation of the physiological function of the HDL-binding proteins described. At the moment, we are preparing experiments with primary hepatocytes derived from pigs.

Some speculation with regard to the functional differences between the HDL-binding proteins described can be made on the basis of their immunohistochemically determined localizations. We found HDL-binding proteins localized both at the lining of the sinusoids and in the hepatocellular plates. The proteins localized at the sinusoids are possibly involved in cellular uptake of HDL. The proteins localized within the hepatocellular plates may be involved in retroendocytosis or metabolism of HDL. Another possiblity is that the 180 kDa HDL-binding protein present at the sinusoidal borders binds HDL to facilitate cholesterol influx to liver cells, and/or interaction with hepatic lipase, which has the same localization [29]. The 90 kDa HDL-binding protein may be an intracellular metabolic intermediate. Because no specific localization of the 110 kDa HDL-binding protein has been determined, speculation on its functional role seems inappropriate. Confirmation of these speculative models, as well as establishment of the role of individual HDL-binding proteins in hepatic HDL metabolism, clearly awaits further research.

Acknowledgments

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Appendix Paper 3

Identification of Structurally Related HDL-Binding Proteins in Liver as gp96/GRP94

Rini P.G. de Crom,
Rien van Haperen,
Rick Janssens,
Pim Visser,
Arthur W.M. van der Kamp

MGC-Department of Cell Biology and Genetics,
Erasmus University Rotterdam,
3000 DR Rotterdam,
The Netherlands

submitted

Proteins that have the ability to bind high density lipoproteins (HDL) *in vitro* are present in both porcine and human liver. We have shown in a previous study that three HDL-binding proteins in porcine liver, of 90, 110 and 180 kDa, are stucturally related. Probably the 180 kDa protein is a homodimer of the 90 kDa protein, while the minor 110 kDa protein is a conformational variant of the monomer. In this study these proteins are identified as porcine gp96/GRP94. This protein is known to occur as a homodimer. Its function is unknown. It is supposed to have a dual subcellular localization: it is both an endoplasmic reticulum resident protein and a plasma membrane protein, possibly in a cell type specific manner. N-terminal amino acid sequences of the 90, 110 and 180 kDa proteins as well as amino acid sequences derived from CNBr-fragments of the 90 kDa protein show a near 100% homology with gp96/GRP94 from different species. Full length cDNA clones encoding the porcine or human protein were obtained by screening cDNA libraries from porcine or human liver. The human cDNA was used to obtain a bacterially expressed protein fragment, and a polyclonal antiserum was raised in rabbits. The antiserum reacted with protein bands of 90 and 180 kDa in human liver in immunoblots. Immunohistochemistry in human liver sections using this antiserum revealed the same localization pattern as has been previously observed in porcine liver using antisera raised against purified proteins from porcine liver. Either porcine or human gp96/GRP94 was transiently expressed in COS-1 cells. However, from immunocytochemistry studies it was apparent that the expressed protein was exclusively localized in the endoplasmic reticulum. Transfection of COS-1 cells with constructs in which the C-terminal KDEL sequence had been mutated, resulted in plasma membrane localized expression of protein, but only in an extremely low percentage of cells.

Plasma levels of HDL are among the best indicators of the risk of coronary artery disease [Lacko, 1994; Barter and Rye, 1996a]. Although this notion has led to intensive research on HDL metabolism, many aspects are still under debate [Lacko, 1994; Breslow, 1995]. One of these aspects is the role of HDL-binding proteins in the liver [Acton *et al.*, 1996].

We identified HDL-binding proteins of 90, 110 and 180 kDa in porcine liver by ligand blotting (molecular masses estimated by sodium dodecylsulfate-polyacrylamide gel electrophoresis [SDS-PAGE] under non-reducing conditions) [De Crom *et al.*, 1992]. In a later study, we showed that

these three proteins are structurally related [De Crom *et al.*, 1994]. The principle arguments for this conclusion are: a polyclonal antibody raised against the purified 90 kDa HDL-binding protein shows cross-reactivity with the 110 and 180 kDa proteins on an immunoblot; under reducing conditions, purified protein preparations of 90, 110 and 180 kDa all migrate at the 90 kDa position in SDS-PAGE, and still show immunoreactivity with the antiserum as well as HDL-binding activity in a ligand blot assay; CNBr-fragment maps of the three proteins are highly similar.

In this paper we show that the 90, 110 and 180 kDa protein have the same N-terminal amino acid sequence. Amino acid sequences of several CNBr-fragments of the 90 kDa protein show an almost 100% identity with a protein known under various names, like mouse endoplasmin or ERp99, and human tra1, gp96 or GRP94 [Mazzarella and Green, 1987]. cDNA clones, encoding both the porcine and human homologue of gp96 were isolated and tested in expression studies using transiently transfected COS-1 cells.

Experimental Procedures

N-terminal sequencing.
Purification of HDL-binding proteins and CNBr-cleavage of purified protein preparations were performed as described before [De Crom *et al.*, 1994]. The protein preparations or CNBr-fragments were subjected to SDS-PAGE and electrophoretically transferred to Pro-Blott™membranes (Applied Biosystems) [Towbin *et al.*, 1979]. These membranes were stained with Coomassie Briliant Blue and protein bands of interest were carefully cut out. These pieces of membrane were directly applied to a 473A Applied Biosystems protein sequencer for N-terminal amino acid sequence determination.

Synthesis of a cDNA probe.
Based on the amino acid sequences of purified proteins and CNBr-fragments, degenerated oligonucleotides were chosen. Non-degenerated oligonucleotides were based on the published sequence of mouse ERp99 [Mazzarella and Green, 1987]. The oligonucleotides were synthesized using an Applied Biosystems oligonucleotide synthesizer. From RNA isolated from either porcine or murine liver, first strand cDNA was synthesized with reverse transcriptase (RT). This was used as a template for polymerase chain reaction (PCR), performed in a Perkin Elmer Cetus PCR apparatus. The PCR reactions in which degenerated oligonucleotides and porcine cDNA were used were not succesful. However, PCR products representing mouse cDNA fragments were obtained and cloned into the EcoRI-site of pTZ19 via the restriction sites incorporated in the synthetic oligonucleotide primers of the PCR reaction.

Screening of cDNA libraries.
λgt11 cDNA libraries from porcine liver (CloneTech PL1001b) and human liver (CloneTech HL1115b) were screened with cDNA probes labeled with [α-^{32}P]ATP. I clones with the largest inserts were subcloned into pTZ19. Partial cDNA clones were digested with restriction enzymes and ligated in order to obtain full length cDNA inserts by standard techniques [Sambrook *et al.* 1989].

DNA sequencing.
The DNA sequence was determined on both strands by the dideoxy-nucleotide chain-termination method (^{T7}Sequencing kit™, Pharmacia), using a series of synthetic oligonucleotides.

Computer analyses.
Comparisons for homology were performed by FASTA using the data bases of GenBank/EMBL, and Swiss-Prot. Predictions of protein domains were performed by the PSORT program (http://psort.nibb.ac.jp/).

Protein expression in E. coli and generation of antisera.
cDNA sequences were subcloned into the vector pGEX (Pharmacia) and transfected to BL21 *E. coli* cells by standard techniques [Sambrook *et al.* 1989]. Induction of protein expression and isolation of bacterially expressed protein was performed according to Smith and Corcoran [1994]. Bacterially expressed protein was purified by preparative SDS-PAGE using a Bio-Rad Prep Cell. Polyclonal antisera were raised in rabbits as described [De Crom *et al.*, 1992]. Antibodies were prepared by standard techniques [Harlow and Lane, 1988]. Briefly, 50 mg protein was applied to a SDS-PAGE gel and electrophoretically transferred onto nitrocellulose membranes. Small strips of these membranes with the protein of interest were cut out and dissolved in dimethyl sulphoxide. 0.5 ml complete Freund's adjuvant was added. A rabbit (New Zealand White) was injected intracutaneously with this mixture. Two boosters with the same mixture containing 0.5 ml incomplete Freund's adjuvant were given with intervals of 28 days. Ten days after the last injection the blood was collected and serum was obtained by centrifugation at 1300 *g* after clotting. All animals were cared for in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Erasmus University Rotterdam (prot.nr. 132.94.01) and with the European Committee Standards on the care and use of laboratory animals (Ministry of Welfare, Health and Cultural Affairs, The Netherlands).

Protein expression in COS-1 cells.
Porcine or human cDNA, either full length (gp96) or carrying a mutation that gives rise to a protein that lacks the C-terminal KDEL tetrapeptide (gp96$^{\Delta KDEL}$), was inserted into the eukaryotic expression vector pCD-X [Okayama and Berg, 1983]. ΔKDEL cDNA was constructed by inserting a linker oligonucleotide (5'–CTGAATAAGATGAACTGTAAATT-ATACTCTCACCATTTG–3') by ligation between the PvuII and BamHI restriction sites that are present both in porcine and human cDNA (PvuII site at positions 2395 and 2497, respectively; BamHI site at positions 2434 and 2536, respectively), which results in a

K_{801}→STOP mutation, completely eliminating the KDEL sequence. COS-1 cells were grown in 98 mm diameter dishes and transfected with 10 μg of plasmid DNA by the DEAE-dextran method [Sambrook *et al.*, 1989]. The transfected cells were analyzed at 48–72 hr post transfection. For immunocytochemistry experiments, cells were grown and transfected in 24 well plates on 15 mm diameter cover slips. Alternative transfection protocols that were tested in order to achieve a higher percentage of expressing cells used lipofectin™ (Gibco BRL), lipofectamin™ (Gibco BRL), or Transfectam© (Promega), all applied according to the manufacturers instructions.

Immunoblotting, immunocytochemistry and immunohistochemistry.
Immunoblotting was performed exactly as described before [De Crom *et al.*, 1994]. Immunocytochemistry was performed on cells grown on coverslips as described by Van Dongen *et al.* [1984].

For immunocytochemistry, porcine liver was fixed and prepared for ultracryotomy as described before [Willemsen *et al.*, 1988]. Semithin cryosections (0.5-1.0 μm) were incubated with a Protein A Sepharose CL-4B (Pharmacia) purified IgG fraction of polyclonal antibodies against HDL-binding proteins for one hour at room temperature in a humid incubator. Antigen-antibody complexes were visualized with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins (Nordic, Tilburg, The Netherlands) (diluted 1:80; incubated at room temperature for one hour in a humid incubator) and studied by fluorescence microscopy.

Results

The N-terminal amino acid sequence of purified 90 kDa, 110 kDa and 180 kDa HDL-binding protein and of four different CNBr-fragments from the 90 kDa protein were determined. As shown in Fig.1, the 90, 110 and 180 kDa HDL-binding protein have identical N-termini. The variation at the 9th amino acid is due to occasional difficulties in the interpretation of the output of the sequencer at that particular position. The 32 kDa CNBr-fragment has the same N-terminus, indicating that this is the N-terminal fragment of the protein. Data-base searches revealed that all the determined amino acid sequences have a very high homology with three known proteins from different species: mouse ERp99, human gp96 and chicken hsp108 (all porcine sequences are presented in bold).

We synthesized both degenerated oligonucleotides based on the amino acid sequences obtained and non-degenerated oligonucleotides based on the mouse ERp99 sequence. We performed RT-PCR using these oligonucleotides and RNA isolated from either porcine or murine liver. The combination of the degenerated oligonucleotides with any of the two RNA preparations failed to yield PCR-products. Therefore we used the non-

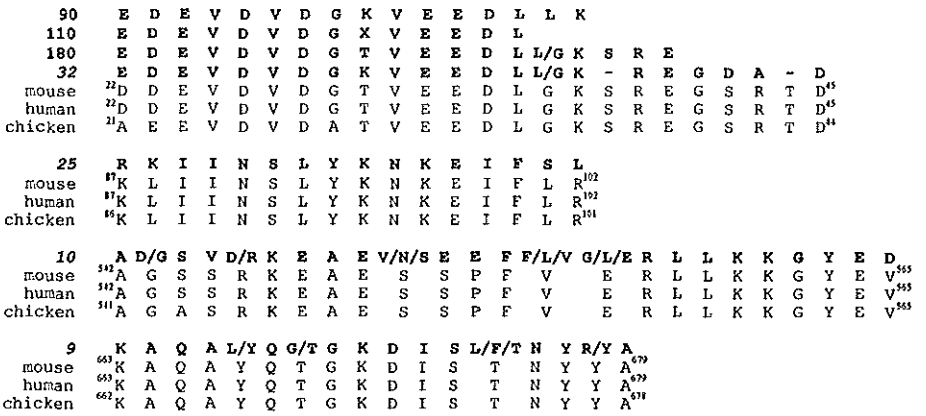


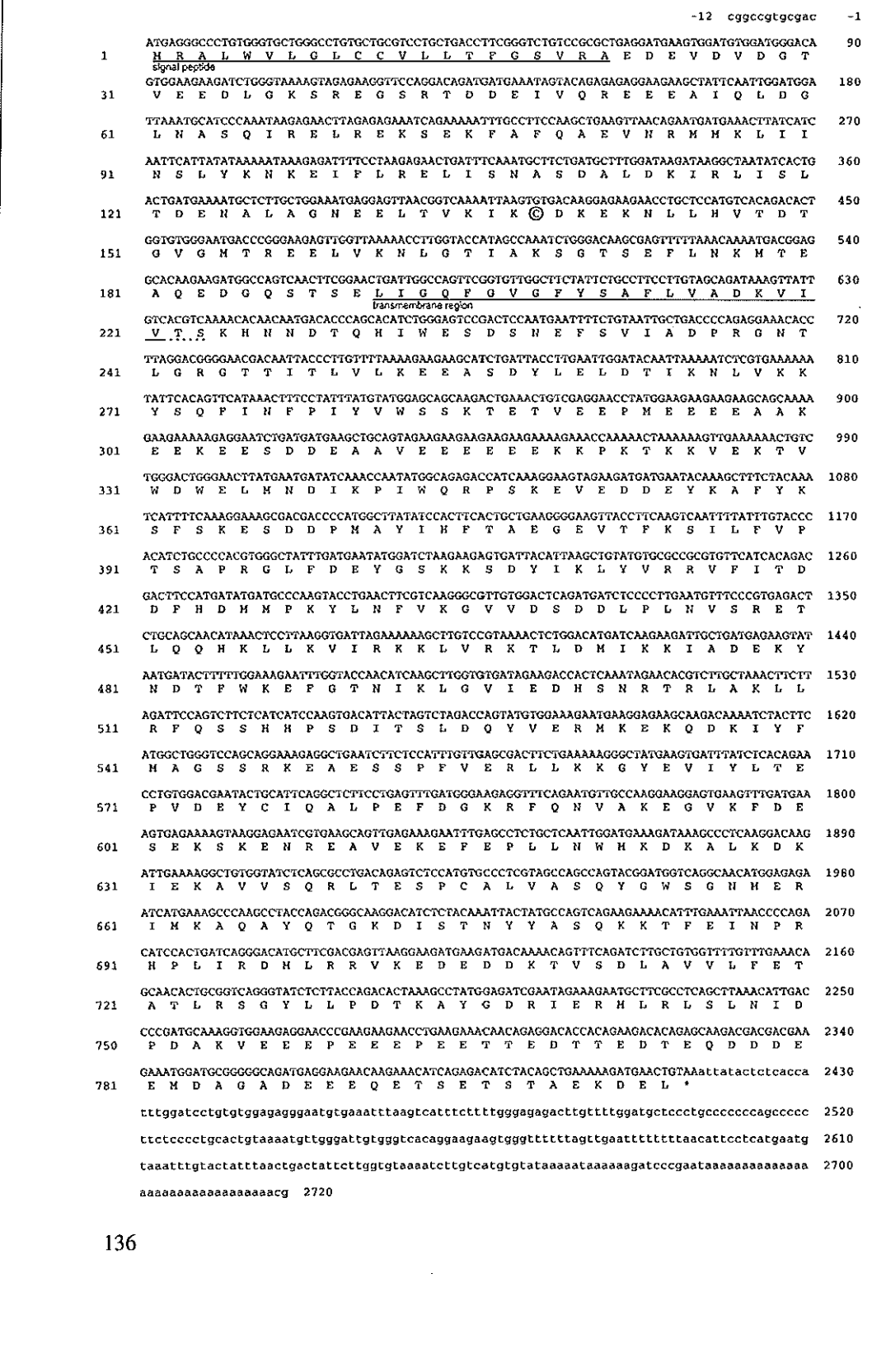
Figure 1. N-terminal amino acid sequences of purified porcine protein preparations of the 90, 110 and 180 kDa HDL-binding proteins and of 9, 10, 25 and 32 kDa CNBr-fragments of the porcine 90 kDa protein are presented in bold. Uncertainties and variations within the porcine sequences are due to occasional difficulties in the interpretations of the output of the sequencer. Comparisons with data-base sequences of mouse, human and chicken homologues are presented (accession numbers J02735, X15187, M14772 respectively).

degenerated oligonucleotides to isolate two cDNA fragments representing the 5' and the 3' part of the complete mouse cDNA sequence respectively.

Using those cDNA fragments as heterologous probes, we screened a porcine liver λgt11 cDNA library. In several screenings only clones with partial cDNA inserts were found. Therefore, a cDNA clone containing the full length cDNA was assembled from two individual clones.

From this clone, the complete cDNA sequence of porcine gp96 (p-gp96) was determined (Fig. 2). The deduced protein sequence contains both an N-terminal signal sequence, which is no longer present in the mature protein (Fig. 1), and a C-terminal KDEL-sequence, which in many proteins serves as an endoplasmic reticulum (ER) retention signal. The 21-23 amino acids beginning at Leu-191 that possibly comprise a membrane spanning region [Mazzarella and Green, 1987] is completely conserved in the porcine sequence (Fig. 2).

Figure 2. Nucleotide and deduced amino acid sequences of porcine gp96 cDNA. The putative signal sequence is underlined. The putative transmembrane region, homologous to the putative transmembrane region in mouse Erp99 [Mazzarella and Green, 1987], is underlined as well. The cysteine residue that is involved in dimerization [Qu *et al.*, 1994] is circled.


-12 cggccgtgcgac -1
ATGAGGGCCCTGTGGGTGCTGGGCCTGTGCTGCGTCCTGCTGACCTTCGGGTCTGTCCGCGCTGAGGATGAAGTGGATGTGGATGGGACA 90
M R A L W V L G L C C V L L T F G S V R A E D E V D V D G T
signal peptide
GTGGAAGAAGATCTGGGTAAAAGTAGAGAAGGTTCCAGGACAGATGATGAAATAGTACAGAGAGAGGAAGAAGCTATTCAATTGGATGGA 180
V E E D L G K S R E G S R T D D E I V Q R E E E A I Q L D G
TTAAATGCATCCCAAATAAGAGAACTTAGAGAGAAATCAGAAAAATTTGCCTTCCAAGCTGAAGTTAACAGAATGATGAAACTTATCATC 270
L N A S Q I R E L R E K S E K F A F Q A E V N R M M K L I I
AATTCATTATATAAAAATAAAGAGATTTTCCTAAGAGAACTGATTTCAAATGCTTCTGATGCTTTGGATAAGATAAGGCTAATATCACTG 360
N S L Y K N K E I F L R E L I S N A S D A L D K I R L I S L
ACTGATGAAAATGCTCTTGCTGGAAATGAGGAGTTAACGGTCAAAATTAAGTGTGACAAGGAGAAGAACCTGCTCCATGTCACAGACACT 450
T D E N A L A G N E E L T V K I K © D K E K N L L H V T D T
GGTGTGGGAATGACCCGGGAAGAGTTGGTTAAAAACCTTGGTACCATAGCCAAATCTGGGACAAGCGAGTTTTTAAACAAAATGACGGAG 540
G V G M T R E E L V K N L G T I A K S G T S E F L N K M T E
GCACAAGAAGATGGCCAGTCAACTTCGGAACTGATTGGCCAGTTCGGTGTTGGCTTCTATTCTGCCTTCCTTGTAGCAGATAAAGTTATT 630
A Q E D G Q S T S E L I G Q F G V G F Y S A F L V A D K V I
transmembrane region
GTCACGTCAAAACACAACAATGACACCCAGCACATCTGGGAGTCCGACTCCAATGAATTTTCTGTAATTGCTGACCCCAGAGGAAACACC 720
V T S K H N N D T Q H I W E S D S N E F S V I A D P R G N T
TTAGGACGGGGAACGACAATTACCCTTGTTTTAAAAGAAGAAGCATCTGATTACCTTGAATTGGATACAATTAAAAATCTCGTGAAAAAA 810
L G R G T T I T L V L K E E A S D Y L E L D T I K N L V K K
TATTCACAGTTCATAAACTTTCCTATTTATGTATGGAGCAGCAAGACTGAAACTGTCGAGGAACCTATGGAAGAAGAAGAAGCAGCAAAA 900
Y S Q F I N F P I Y V W S S K T E T V E E P M E E E E A A K
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E E K E E S D D E A A V E E E E E E K K P K T K K V E K T V
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W D W E L M N D I K P I W Q R P S K E V E D D E Y K A F Y K
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S F S K E S D D P M A Y I H F T A E G E V T F K S I L F V P
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D F H D M M P K Y L N F V K G V V D S D D L P L N V S R E T
CTGCAGCAACATAAACTCCTTAAGGTGATTAGAAAAAAGCTTGTCCGTAAAACTCTGGACATGATCAAGAAGATTGCTGATGAGAAGTAT 1440
L Q Q H K L L K V I R K K L V R K T L D M I K K I A D E K Y
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R F Q S S H H P S D I T S L D Q Y V E R M K E K Q D K I Y F
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M A G S S R K E A E S S P F V E R L L K K G Y E V I Y L T E
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P V D E Y C I Q A L P E F D G K R F Q N V A K E G V K F D E
AGTGAGAAAAGTAAGGAGAATCGTGAAGCAGTTGAGAAAGAATTTGAGCCTCTGCTCAATTGGATGAAAGATAAAGCCCTCAAGGACAAG 1890
S E K S K E N R E A V E K E F E P L L N W M K D K A L K D K
ATTGAAAAGGCTGTGGTATCTCAGCGCCTGACAGAGTCTCCATGTGCCCTCGTAGCCAGCCAGTACGGATGGTCAGGCAACATGGAGAGA 1980
I E K A V V S Q R L T E S P C A L V A S Q Y G W S G N M E R
ATCATGAAAGCCCAAGCCTACCAGACGGGCAAGGACATCTCTACAAATTACTATGCCAGTCAGAAGAAAACATTTGAAATTAACCCCAGA 2070
I M K A Q A Y Q T G K D I S T N Y Y A S Q K K T F E I N P R
CATCCACTGATCAGGGACATGCTTCGACGAGTTAAGGAAGATGAAGATGACAAAACAGTTTCAGATCTTGCTGTGGTTTTGTTTGAAACA 2160
H P L I R D M L R R V K E D E D D K T V S D L A V V L F E T
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A T L R S G Y L L P D T K A Y G D R I E R M L R L S L N I D
CCCGATGCAAAGGTGGAAGAGGAACCCGAAGAAGAACCTGAAGAAACAACAGAGGACACCACAGAAGACACAGAGCAAGACGACGACGAA 2340
P D A K V E E E P E E E P E E T T E D T T E D T E Q D D D E
GAAATGGATGCGGGGGCAGATGAGGAAGAACAAGAAACATCAGAGACATCTACAGCTGAAAAAGATGAACTGTAAattatactctcacca 2430
E M D A G A D E E E Q E T S E T S T A E K D E L *
tttggatcctgtgtggagagggaatgtgaaatttaagtcatttctttttgggagagacttgttttggatgctccctgccccccccagccccc 2520
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aaaaaaaaaaaaaaaaacg 2720

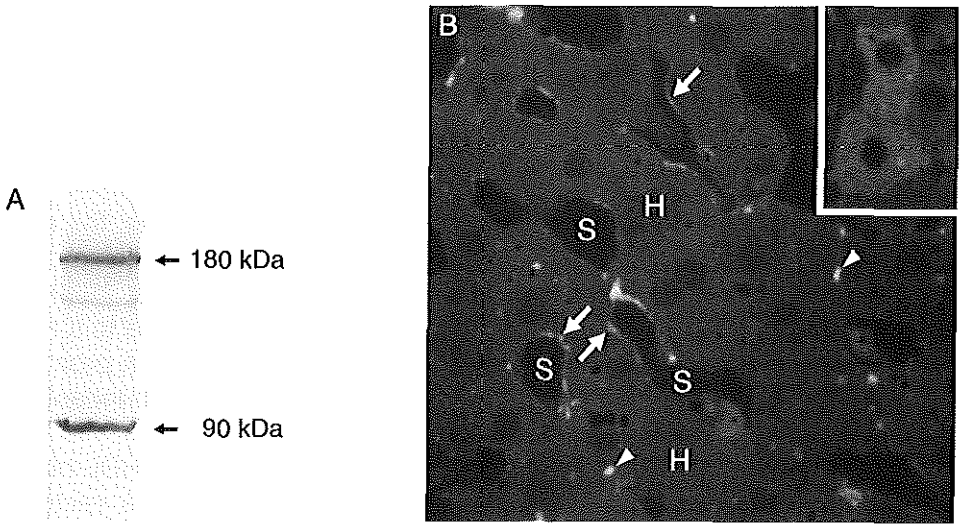


Figure 3. A. Immunoblot of anti–(h-gp96) with a protein homogenate of human liver. Protein bands of 90 and 180 kDa are indicated. B. Immunohistochemistry of human liver. Semithin cryosections of human liver were incubated with anti–(h-gp96) and fluorescein isothiocyanate-conjugated goat anti-(rabbit IgG) as a secondary antibody as described in "Experimental Procedures". Expression of gp96 was detected at the borders of the hepatocellular plates (H) and sinusoids (S) (arrows) and within the hepatocellular plates (arrowheads). *Insert:* Occassionally, hepatocytes were found with a strong intracellular fluorescence, probably representing localization in the ER.

We wanted to extend our analyses to the human protein (h-gp96). Since none of the antibodies we raised against the porcine proteins cross-reacts with sections of human liver in immunohistochemistry studies, we raised antibodies recognizing h-gp96. Therefore, we used a protein fragment expressed in *E. coli* transfected with human cDNA. With the porcine cDNA as a probe, we screened a human liver λgt11 cDNA library in order to isolate a cDNA clone encoding the human cDNA. A clone containing the full length cDNA was obtained by ligation of the inserts of two partial overlapping clones. The cDNA was completely sequenced; it perfectly matched the published sequence of human gp96 (GenBank accession number X15187; not shown).

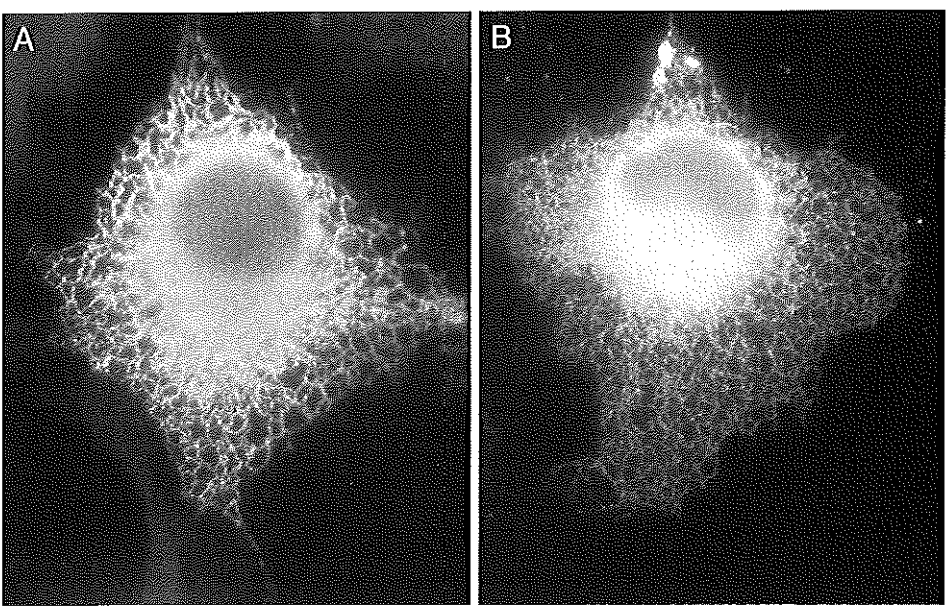
A cDNA fragment, comprising the C-terminal part of the protein (amino acids 677 to 801) was cloned into pGEX for expression in *E. coli*. Produced protein was purified by preparative SDS-PAGE. Rabbits were injected with this protein preparation and an antiserum was obtained.

In immunoblotting studies, this anti–(h-gp96) recognizes protein bands of 90 and 180 kDa in human liver (Fig. 3a). A protein band of 110 kDa was not found. However, in porcine liver this is a minor band that is usually hardly visible in immunoblots of crude material [De Crom *et al.*, 1994]. Therefore it is probably below detection level in the human liver. It might also be degraded, since the liver used in this experiment comes from autopsy.

In immunohistochemistry studies, using human liver biopsies, the newly developed antiserum showed a localization pattern along the lining of the sinusoids as found before in porcine liver [De Crom *et al.*, 1992, 1994]; the localization within the hepatocellular plates was less obvious due to a rather high background (Fig. 3b). Occasionally, cells were found with a prominent intracellular fluorescence pattern, that might represent abundant ER localization (Fig. 3b, insert).

To determine if the isolated cDNA clones encode an HDL-binding protein, both the human and porcine cDNAs were cloned in a vector for expression in COS-1 cells. Cells were analyzed at two or three days

Figure 4. Immunocytochemistry of COS cells transiently transfected with p-gp96 (*A*) or h-gp96 (*B*). Transfection of COS cells and immunocytochemistry were performed as described in "Experimental Procedures". The observed localization pattern is indicative for expression in the ER.



following transfection.

In immunoblotting experiments it was found that non-transfected COS-1 cells express gp96 while in COS-1 cells transfected with either porcine or human cDNA there is no significant increase in gp96-expression (not shown). Immunocytochemistry studies however, that permit the inspection of individual cells, revealed that a very low number of cells did express the protein, and that this protein is located intracellularly, showing a distribution highly suggestive for ER localization (Fig. 4). In immunohistochemistry studies in porcine liver it has been shown that the protein is localized predominantly at the lining of the sinusoids, while there is no evidence for an appreciable ER localization [De Crom *et al.*, 1992, 1994], while in human liver the same localization pattern was found along with a putative ER localization present only in a minority of the hepatocytes (Fig. 3b).

In order to circumvent this ER localization in transfected COS-1 cells, vectors were generated containing porcine gp96 cDNA with a deletion of the C-terminal KDEL-sequence (p-gp96$^{\Delta KDEL}$). COS-1 cells were transfected and analyzed after two to three days. Immunocytochemistry revealed, that only a very small minority (< 1%) of the cells expressed gp96. The protein was localized at the perifery of the cell indicating plasma membrane localization (Fig. 5). Several attempts to increase the percentage of expressing cells by using other or modified transfection protocols proved to be unsuccesful.

Discussion

In this paper, we identify the HDL-binding proteins detected in porcine liver [De Crom *et al.*, 1992, 1994] as the porcine homologue of gp96. This protein is known from several species and have been given various names: mouse ERp99 [Lewis *et al.*, 1985; Mazzarella and Green, 1987] and endoplasmin [Koch *et al.*, 1986], human GRP94 [Lee *et al.*, 1984], tra1 and gp96 [Maki *et al.*, 1990; 1993], and chicken hsp108 [Kleinsek *et al.*, 1986]. Gp96 has 50% identity with the cytosolic hsp90 proteins [Maki *et al.*, 1990] and is therefore included in the stress-90 protein family [Gething and Sambrook, 1992]. It is quite an exceptional member of this family, though, since it contains an N-terminal signal sequence for ER translocation and the C-terminal KDEL-sequence for ER-retrieval [Munro and Pelham, 1987]. The protein is induced by glucose starvation and by disruption of the intracellular calcium stores, but not by heat [Chang *et al.*, 1989; Melnick *et al.*, 1992]. Its specific function is unknown [Gething and Sambrook, 1992], although several groups suggested that the protein is a chaperonin like GRP78 (BiP). This hypothesis is based on data showing that both GRP78 and gp96

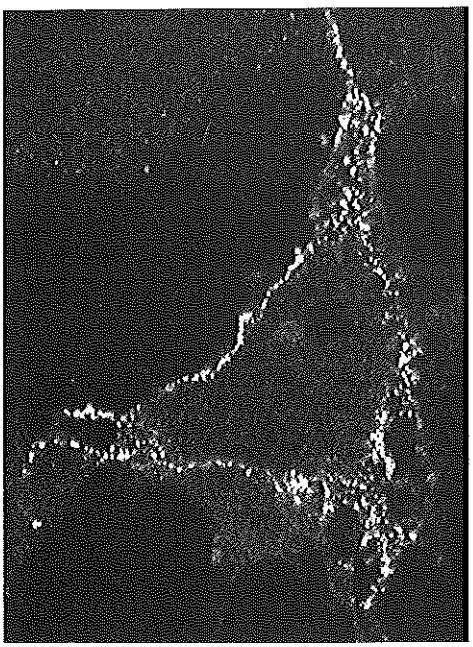


Figure 5. Immunocytochemistry of COS cells transiently transfected with p-gp96$^{\Delta KDEL}$. Transfection of COS cells and immunocytochemistry were performed as described in "Experimental Procedures". The results were examined by the use of confocal laser microscopy, and clearly demonstrate localization at the cell border, presumably at the plasma membrane.

expression is induced by the presence of malfolded proteins in the ER [Kozutsumi *et al.*, 1988] and that both proteins associate with unassembled immunoglobulin chains [Melnick *et al.*, 1992, 1994]. Besides, the promoters of both GRP78 and gp96 have several regulatory elements in common and the genes have been shown to be coordinately expressed [Chang *et al.*, 1989; Liu and Lee, 1991; McCauliffe *et al.*, 1992]. Others have implicated gp96 in antigen presentation [Li and Srivastava, 1993, Srivastava *et al.*, 1994]. Dechert *et al.* [1994] isolated porcine gp96 from brain microvessels and showed that the protein is a protein kinase. Its function is unknown.

The identification of an HDL-binding protein as an ER localized protein is unexpected. However, several lines of evidence indicate that expression of gp96 is not restricted to the ER, but is also present in the plasma membrane, where it might exert a different function. Gp96 could be immunoprecipitated from cell surface radiolabeled cells and purified from isolated plasma membranes [Srivastava *et al.*, 1986; Maki *et al.*, 1990]. In rat pancreas, gp96 was shown to be present both in ER and plasma membranes by immunoblotting and electronmicroscopical immunohistochemistry [Takemoto *et al.*, 1992]. Hayes *et al.* [1994] showed that gp96 purified from rat liver or chicken liver or oviduct does not react with an anti-KDEL-antibody, suggesting that the KDEL-sequence has been removed or modified. Several KDEL-containing proteins have been

described to escape ER retention [Nilsson and Warren, 1994], including rat growth hormone, the a subunit of chorionic gonadotropin [Zagouras and Rose, 1989] and protein disulfide-isomerase [Takemoto *et al.*, 1992]. In the case of at least one protein, the plant cysteine endopeptidase SH-EP, the KDEL sequence is removed during processing of the mature protein [Okamoto *et al.*, 1994].

Our data show that gp96 is indeed localized at the cell surface in porcine liver [De Crom *et al.*, 1992; 1994] and in human liver (Fig. 3b). In Appendix Paper 4, we describe the ultrastructural localization of gp96 in porcine liver, which is confined to the plasma membrane and the endosomal/lysosomal compartment, while the protein was hardly detectable in the ER. In light microscopic immunohistochemistry studies in human liver sections, patterns that might represent ER localization were observed in some cells (Fig 3b, insert). The plasma membrane localization might be tissue dependent, as has been demonstrated for protein disulfide-isomerase [Takemoto *et al.*, 1992].

The dual and possibly tissue dependent localization pattern of gp96 suggest multi-functionality. Recently, a number of proteins have been assumed to be multi-functional, including proteins involved in lipoprotein metabolism like the low density lipoprotein (LDL) receptor related protein (LRP) [Krieger and Herz, 1994]. We propose that cell surface localized gp96 in liver acts as an HDL-binding protein. Unfortunately, transient expression in COS-1 cells failed to give a conclusive answer to the question whether or not the induction of gp96-expression enhances HDL-binding of cells. Expression of gp96 in transfected COS-1 cells was almost exclusively intracellular, presumably ER-localized, since the immunocytochemical pattern perfectly matches the pattern shown by Pelham and coworkers to be ER in COS-1 cells [Lewis and Pelham, 1992]. Macrophage scavenger receptors, that are localized at the plasma membrane in macrophages, have also been found to localize in the ER in transiently transfected COS-1 cells [Naito *et al.*, 1991].

Expression of a $gp96^{\Delta KDEL}$ construct gave rise to exclusively plasma membrane localized expression, albeit in a very low percentage of cells. Probably, plasma membrane localization can only be found in cells with the necessary regulatory processes. The nature of the plasma membrane association is unknown. The putative transmembrane domain postulated by Mazzarella and Green [1987] is a relatively hydrophobic domain recognized in a Kyte and Doolittle plot, but is not rated as a transmembrane domain by the PSORT program for the prediction of protein localization sites. Moreover, if this sequence would represent a truly transmembrane domain, and the N-terminal sequence is a signal sequence, the protein would end up

as an ER transmembrane protein with its C-terminus at the cytoplasmic side. In this situation the KDEL sequence cannot function as an ER retention signal [Alberts *et al.*, 1994]. This is not in agreement with our data showing that the KDEL sequence does effectuate ER localization in COS cells. Therefore it is more likely that gp96 is a plasma membrane associated protein without a transmembrane domain, but with a functional signal sequence. This membrane association is a strong one, however, since the protein has been purified by detergent treatments of an isolated plasma membrane enriched cellular fraction [De Crom et al., 1992]. Mazzarella and Green [1987] postulate a second transmembrane domain region that would result in the localization of the C-terminus in the lumen of the ER. This possibility seems unlikely however, because of the weak hydrophobicity of this domain.

We believe that the identification of a signal sequence at the N-terminus is correct, since the PSORT program positively identifies this signal sequence and predicts a cleavage site between amino acid 21 and 22, which is in perfect agreement with the N-terminal amino acid sequences that we determined.

Although expression studies in COS-1 cells do not allow the direct demonstration that expression of gp96 leads to (increased) HDL-binding by cells, a number of observations confirm the identification of the HDL-binding protein in porcine liver as gp96: N-terminal amino acid sequencing of purified proteins or CNBr fragments did not show the presence of any contaminations with other proteins; HDL-binding proteins that migrate at 90, 110, and 180 kDa in SDS-PAGE under non-reducing conditions all have the N-terminus of gp96; the newly raised antisera show a histological localization at the borders of the cells and the sinusoids in liver as was shown before with antisera raised against purified preparations of HDL-binding proteins [De Crom *et al.*, 1992, 1994].

Gp96 is known to occur as a homodimer. The cysteine residue at position 138 has been shown to be involved in dimerization [Qu *et al.*, 1994]. This cysteine residue is conserved in all species, including pig (Fig. 2).

Recently, Acton *et al.* [1996] identified SR-BI as an HDL-binding protein in the liver. SR-BI is probably also a multifunctional protein, since it can bind a number of other ligands, including native or chemically modified LDL and anionic phospholipids [Acton *et al,* 1994; Rigotti *et al.*, 1995]. These findings do not exclude the presence of other HDL-binding proteins in the liver, since the *in vivo* contribution of SR-BI to HDL or HDL-cholesterol uptake by the liver remains to be determined. SR-BI binding mediates selective uptake of HDL-cholesterol esters *in vitro* (i.e. without parallel uptake of HDL-protein)

[Acton *et al.*, 1996]. It is also possible that there is redundancy of HDL-binding proteins in the liver. This has been shown for several proteins, including proteins involved in lipid metabolism: apolipoprotein (apo) E mediated uptake of lipoproteins by the liver can occur via the apoB/E-receptor (LDL-receptor) or by a specific apoE-receptor, which is possibly LRP [Krieger and Herz, 1994]. It has been suggested that for vital functions as cholesterol metabolism redundancy of functional proteins is predictable [Barter and Rye, 1996b].

Further research including the generation of transgenic animals expressing gp96 is needed to evaluate the role of plasma membrane gp96 in the liver and in HDL metabolism.

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Appendix Paper 4

Ultrastructural Localization of gp96/GRP94, a Putative HDL-Binding Protein, in Porcine Liver

Rini P.G. de Crom,
Rob Willemsen,
Rien van Haperen,
Rick Janssens,
Arthur W.M. van der Kamp

MGC-Department of Cell Biology and Genetics,
Erasmus University Rotterdam,
3000 DR Rotterdam,
The Netherlands

submitted

Two major high density lipoprotein (HDL)-binding proteins of 90 and 180 kDa can be detected in porcine and human liver by ligand blotting. Immunocytochemical studies using light microscopy with antibodies recognizing specifically the 90 kDa protein or the 180 kDa protein, showed that the two proteins have a distinct localization. The 90 kDa protein is localized within the hepatocellular plates, while the 180 kDa protein can be found at the linings of the sinusoids. The 90 and 180 kDa proteins could be identified as the monomeric and homodimeric conformations of gp96/GRP94, a protein of unknown function that is localized both in the endoplasmic reticulum and at the plasma membrane, maybe in a tissue dependent way. To date, there is only limited evidence for the plasma membrane localization of gp96. Nothing is known about differences in function or localization between monomeric and homodimeric gp96. In this paper we study the ultrastructural localization of gp96 in porcine liver. We demonstrate that the homodimeric form is located at the basolateral plasma membranes of the hepatocytes, and at the plasma membranes of endothelial cells and Kupffer cells. The 90 kDa protein was abundantly present at the membranes of vesicles of the endosomal/lysosomal system as well as at the apical hepatocyte membranes, comprising the bile canaliculi. The monomeric protein is scarcely present at the basolateral membrane of the hepatocytes, but could be demonstrated in coated pits, suggesting involvement in receptor-mediated endocytosis. Labeling of the endoplasmic reticulum was virtually absent.

Many epidemiological studies have established a strong inverse correlation between plasma levels of HDL and the incidence of atherosclerosis [Forte and McCall, 1994]. The protection of HDL against the development of atherosclerosis is widely assumed to reside in its capability to transport cholesterol from peripheral tissues to the liver for excretion, a process called reverse cholesterol transport [Pieters *et al.*, 1994; Fielding and Fielding, 1995].

The last step in this process, the delivery of HDL-cholesterol to the hepatocytes, still remains elusive, but HDL-binding proteins that have been described in liver by several groups are possibly involved in this process [Fielding and Fielding, 1995; Acton *et al.*, 1996].

We described two HDL-binding proteins in porcine liver of 90 and 180 kDa that have a strikingly different localization. As shown by light-microscopic immunocytochemistry studies, the 90 kDa HDL-binding protein

is located within the hepatocellular plates, while the 180 kDa HDL-binding protein is located at the lining of the sinusoids [De Crom *et al.*, 1992]. In a later study it was shown that these two proteins and a third, minor 110 kDa HDL-binding protein, are structurally related. We concluded that the 180 kDa HDL-binding protein is a homodimer of the 90 kDa HDL-binding protein [De Crom *et al.*, 1994]. Subsequently, we identified the 90, 110 and 180 kDa HDL-binding proteins as gp96/GRP94, a protein known to occur both in a monomeric and in a dimeric conformation [Appendix Paper 3]. The function of this protein is unknown. Although it is generally believed to be an endoplasmic reticulum (ER) resident protein, where it probably acts as a chaperonin [Melnick *et al.*, 1994], several lines of evidence indicate that this protein is (also) localized at the plasma membrane, maybe in a tissue dependent way, where it possibly has other functions [discussed in Appendix Paper 3].

In this paper, we present the ultrastructural localization of the 90 and 180 kDa HDL-binding protein in porcine liver. The 90 kDa protein is predominantly found at the membranes of vesicles of the endosomal/lysosomal system and at the plasma membranes comprising the bile canaliculi. The 180 kDa protein is found at the basolateral plasma membrane of the hepatocytes, where the cell faces the space of Disse, and at the plasma membrane of both endothelial cells and Kupffer cells.

Experimental Procedure

Porcine liver specimens were immediately fixed in 3% paraformaldehyde and prepared for ultracryotomy as described previously [Willemsen *et al.*, 1988]. Ultrathin cryosections (60 nm) were immunolabeled with either rabbit polyclonal antibodies against the 90 kDa protein or against the 180 kDa protein [De Crom *et al.*, 1992]. Antibodies directed against cathepsin D were kindly provided by Dr. H. Aerts, University of Amsterdam. Antigen-antibody complexes were visualized with goat anti-(rabbit Ig) conjugated with 10 nm colloidal gold particles. To localize two antigens simultaneously, the first antibody was labeled with a 6 nm protein A–gold probe and the second with a 10 nm probe, according to Geuze *et al.* [1981]. Sections were stained with uranyl salts and embedded in 1.5% methylcellulose and examined with a Philips CM100. The specificity of the labeling procedure was checked by incubation of the sections with normal rabbit serum as substitution for the primary antibody, or with the different gold probes alone. Background labeling was negligible.

Results

The ultrastructural localization of the 90 kDa protein was studied by indirect immunogold labeling of electron microscopic frozen sections of porcine liver using an antibody specifically recognizing the 90 kDa protein [De Crom *et al.*, 1992]. Most label was found along the membranes of intracellular vesicles belonging to the endosomal/lysosomal compartment and along the

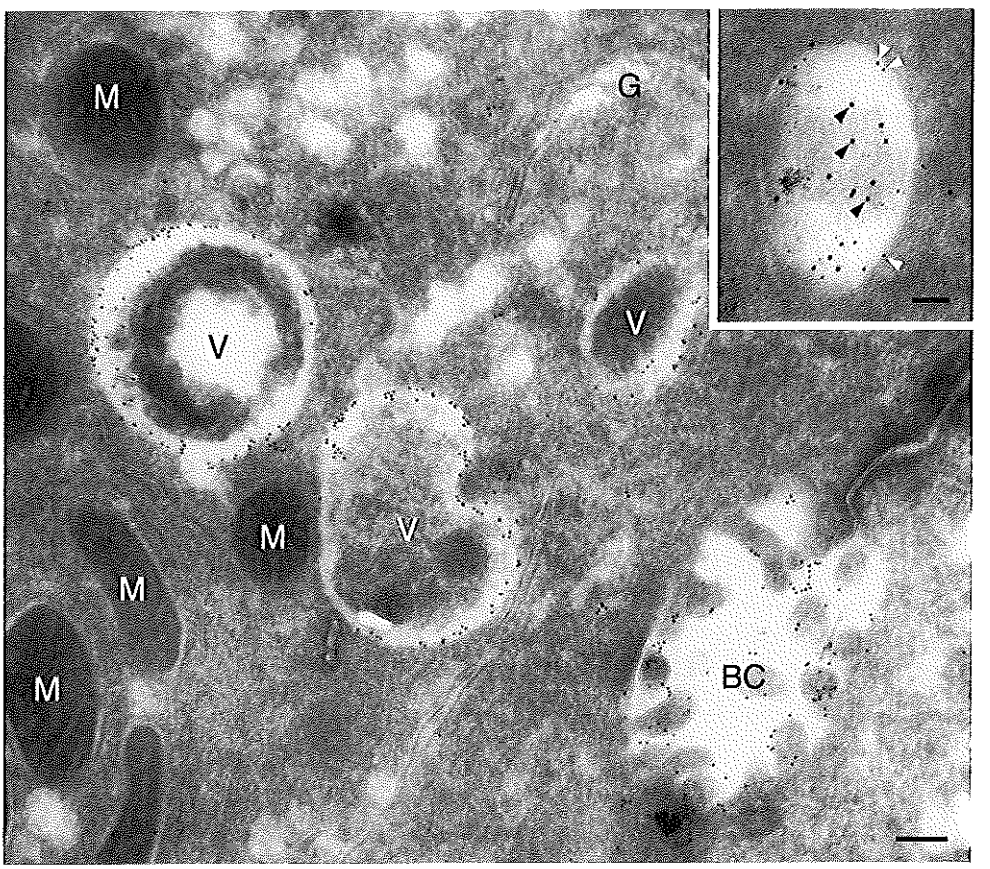


Figure 1. Subcellular localization of the 90 kDa HDL-binding protein in porcine liver. Immunocytochemistry was performed as described in "Experimental Procedure". The 90 kDa protein is present in high amounts at the membrane of the bile canaliculus (BC) and at the membranes of the vesicles of the endosomal/lysosomal system (V). G: Golgi complex; M: mitochondrion. Magnification: 43,000 ×; bar = 0.25 mm.

Insert: colocalization of the 90 kDa HDL-binding protein (6 nm gold particles; white arrowheads) and cathepsin D (10 nm gold particles; black arrowheads) in an endosomal/lysosomal vesicle. Magnification: 78,000 ×; bar = 0.1 mm.

membranes of the microvilli of the bile canaliculi (Fig. 1). Using an antibody directed against cathepsin D, a lysosomal enzyme, it was confirmed in a double labeling procedure that the 90 kDa protein is co-localized with cathepsin D (Fig 1, insert). Cathepsin D label is mainly found in the lumen of the vesicles, while the 90 kDa protein label is found preferentially localized at the limiting membranes of the vesicles, suggesting that it is a membrane-associated protein. High amounts of label were found associated with the apical membrane of the hepatocytes, comprising the bile canaliculi.

Apart from these two sites, no intracellular label was observed in the hepatocytes. Special attention was paid to the ER, since gp96 is reported to be an ER protein. However, only very few gold particles could be detected in the ER (Fig. 2).

At the basolateral membrane of the hepatocytes, facing the space of Disse, the 90 kDa protein is present in very low quantities. However,

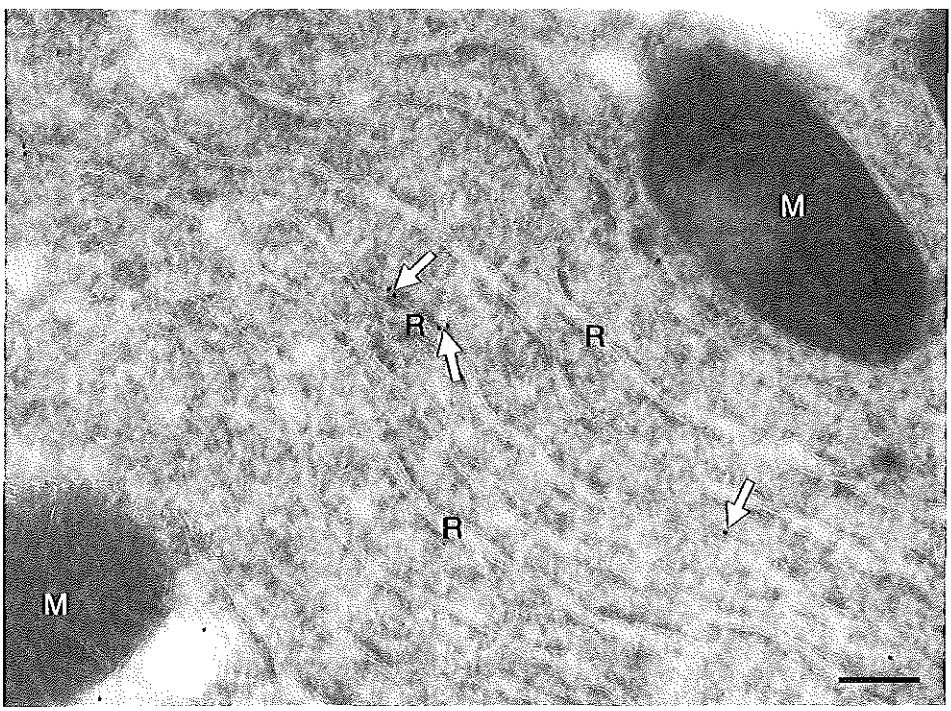


Figure 2. Subcellular localization of the 90 kDa HDL-binding protein in porcine liver. Immunocytochemistry was performed as described in "Experimental Procedure". Only few gold particles are present in the rough endoplasmic reticulum (R), indicating that the protein is expressed there in minor quantities (arrows). M: mitochondrion. Magnification: 60,000 ×; bar = 0.25 mm.

labeling in coated pits is observed (Fig. 3).

The distribution of the 180 kDa protein was studied in a similar way as the localization of the 90 kDa protein, using an antiserum specifically recognizing the 180 kDa protein [De Crom *et al.*, 1992]. The 180 kDa protein appeared to be localized primarily at the plasma membrane of both endothelial cells (Fig. 4a) and Kupffer cells (Fig. 4b). Gold particles can be found at the sinusoidal side and at the side facing the space of Disse. Labeling is also present at the microvilli of the hepatocytes protruding in the space of Disse. Intracellular labeling of the hepatocytes was found only occasionally. No ER labeling was found (not shown).

Using an antiserum recognizing both the 90 and 180 kDa proteins [De Crom *et al.*, 1992], a combination of both localization patterns was found (bile canalicular membranes, membranes of vesicles of the endosomal/lysosomal system, basolateral membranes of the hepatocytes and plasma membranes of endothelial cells and Kupffer cells), while

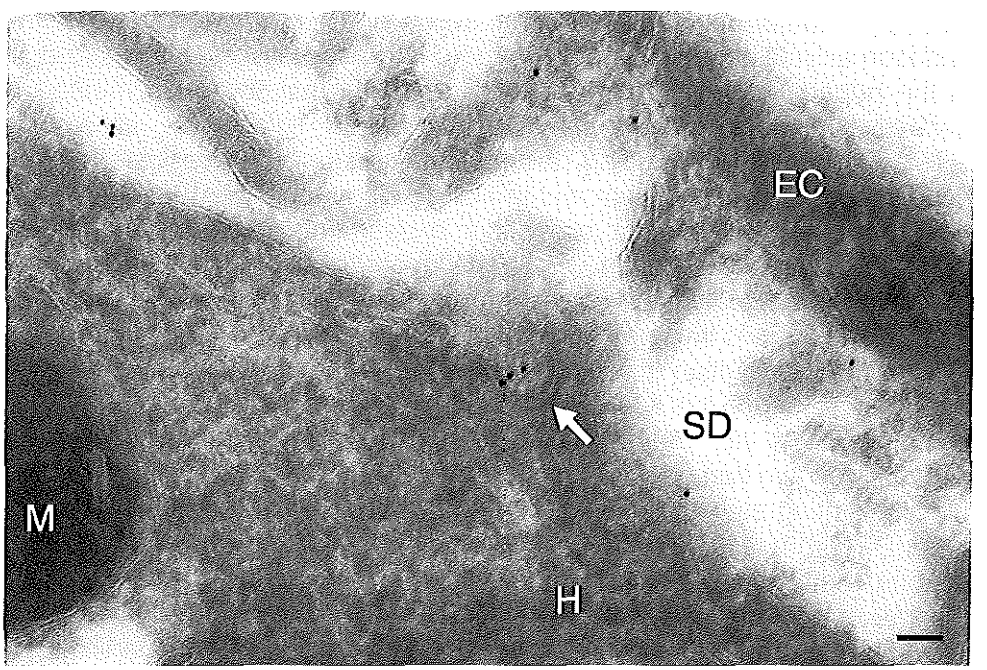


Figure 3. Subcellular localization of the 90 kDa HDL-binding protein in porcine liver. Immunocytochemistry was performed as described under "Experimental Procedure". The 90 kDa HDL-binding protein is detected in a coated pit (arrow) in the basolateral plasma membrane of the hepatocyte (H), facing the space of Disse (SD). EC: endothelial cell. Magnification: 84,000 ×; bar = 0.1 mm.

labeling of other intracellular compartments, including the ER, was virtually absent (not shown).

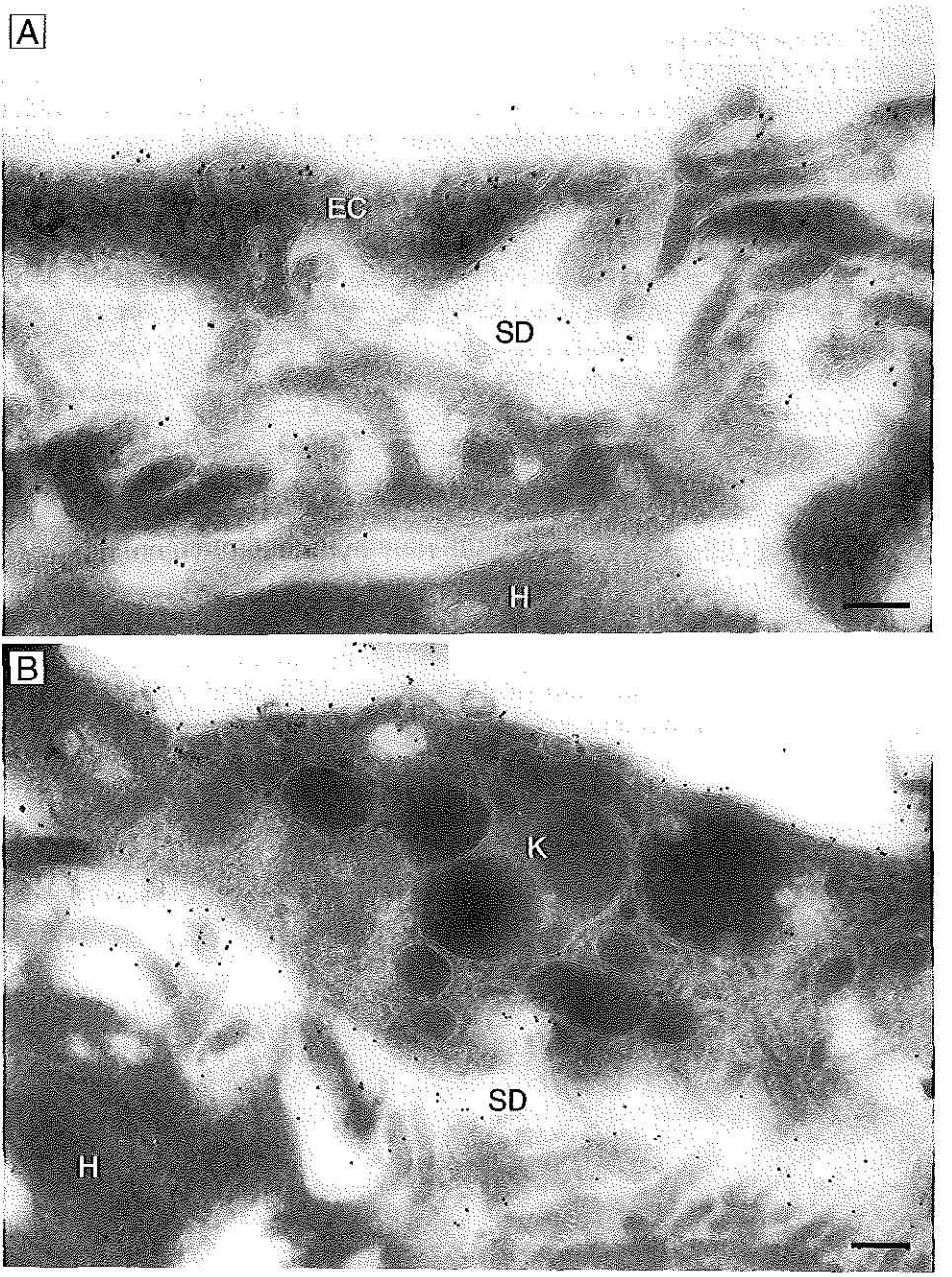
Discussion

The ultrastructural localization of the 90 and 180 kDa HDL-binding proteins in porcine liver is in agreement with the previously described bimodal localization pattern found in light microscopic studies [De Crom *et al.*, 1992]. The 180 kDa protein is a homodimer of the 90 kDa protein [De Crom *et al.*, 1992], which both have been identified as gp96/GRP94 [Appendix Paper 3]. Although gp96 has a C-terminal KDEL sequence and is generally considered as a resident ER protein, several lines of evidence from different groups indicate that gp96 also occurs as a plasma membrane bound protein, possibly in a tissue dependent way (discussed in Appendix Paper 3). The results in this paper show that gp96 is actually localized at the plasma membrane in porcine liver and in vesicles of the endosomal/lysosomal system, while there is no appreciable ER localization in the hepatocytes. Previously, we have shown that the used method is perfectly suited to detect immunocytochemical localization at several cellular organelles, including the plasma membrane, vesicles of the endosomal/lysosomal system and the ER [Willemsen *et al.*, 1988]. We have performed a large number of distinct experiments in which livers from different animals have been used. The photographs for the figures have been carefully selected and represent the localization pattern that has been repeatedly found in these experiments.

The distinct localization of monomeric versus dimeric gp96 in porcine liver is intriguing. Although it is known that gp96 occurs both as a monomer and as a dimer [Qu *et al.*, 1994], nothing is known about functional differences between the two forms.

Ligand blotting studies suggest a role of gp96 in HDL metabolism by the liver [De Crom *et al.*, 1992]. The presence of the dimeric protein at the plasma membrane of the hepatocytes in the space of Disse and at the plasma membrane of endothelial cells and Kupffer cells possibly facilitate the interaction of hepatic lipase with HDL, uptake of HDL particles, or

Figure 4. Subcellular localization of the 180 kDa HDL-binding protein in porcine liver. Immunocytochemistry was performed as described in "Experimental Procedure". The 180 kDa protein is found at the plasma membrane of endothelial cells (EC), Kupffer cells (K) and hepatocytes (H). SD: space of Disse. Magnification: 52,000 × (A), 45,000 × (B); bar = 0.25 mm.


A
EC
SD
H
B
K
SD
H

selective uptake of HDL-cholesterol esters, via an anchoring or docking mechanism, as has been suggested for another putative hepatic HDL-receptor, SR-BI [Acton *et al.*, 1996]. It is also possible that the dimeric protein represents excreted gp96 since Takemoto *et al.* [1992] showed that gp96 in pancreas cells is both plasma membrane bound and secreted. The protein found associated with endothelial cells and Kupffer cells could be hepatocyte-derived and, in analogy with the situation of hepatic lipase [Bensadoun and Berryman, 1996], bound to glycosaminoglycans, since we found that gp96 strongly associates with heparin (unpublished results). On the other hand, the finding of 90 kDa protein in coated pits in hepatocytes strongly suggest the involvement of receptor-mediated endocytosis, a process that has been described for a number of receptors, including the low density lipoprotein receptor [Brown and Goldstein, 1986]. Conformation of these speculations awaits further research.

The monomeric protein found in the endosomal/lysosomal system and bile canaliculi could be a degenerative of the (functional) dimeric protein, without any function in HDL metabolism. On the other hand, it is possible that the monomeric protein guides HDL along its intracellular route. The localization in the bile canaliculi is in agreement with this, since HDL/apolipoprotein (apo) A-I can be found in bile canaliculi and apoA-I has been suggested to inhibit cholesterol crystal formation [Kibe *et al.*, 1984]. Future experiments, including analysis of HDL-binding and uptake by hepatocytes in culture, are needed to evaluate the intracellular fate of HDL and apoA-I and the involvement of HDL-binding proteins in these processes.

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Appendix Paper 5

Expression of gp96/GRP94, a Putative HDL-Binding Protein, in Cultured Cells

Rini P.G. de Crom,
Rien van Haperen,
Frank G. Grosveld,
Arthur W.M. van der Kamp

MGC-Department of Cell Biology and Genetics,
Erasmus University Rotterdam,
3000 DR Rotterdam,
The Netherlands

Gp96/GRP94 is able to bind HDL *in vitro*. It is an endoplasmic reticulum resident protein, that is also localized at the plasma membrane in some tissues. In order to evaluate the HDL-binding capacities of this protein, stably transfected cells were generated, using several cell types. However, protein expression appeared to be highly variable (COS, CHO cells) or absent (L cells). In later passages of protein expressing cells, invariably the non-expressing cells appeared to predominate. We tranfected MEL cells with a construct containing the β-globin locus control region, allowing selection of growing cells without expression of gp96/GRP94. Protein expression occurs upon induction of differentiation of the MEL cells. A high number of single transfected cells were isolated and grown. The level of protein expression upon induction of these clonal cell lines showed considerable variation. In none of these cell lines, a stable protein expression in later passages could be observed. Fluorescence activated cell sorter analyses of some of the most highly expressing clonal MEL cells indicated that the increase in expressed protein was exclusively located intracellularly. Therefore, clonal MEL cell lines were isolated that had been transfected with constructs in which the C-terminal KDEL tetrapeptide from gp96 had been deleted. Finally, two clonal lines expressing relatively high levels of gp96/GRP94 were selected and subjected to HDL-binding studies. One of them showed a marked increase of HDL-binding activity compared with the control cells, the other cell line showed no increase. This suggests that gp96/GRP94 is able to mediate HDL-binding. However, this finding has to be confirmed in further studies.

In previous studies, we have detected proteins with the ability to bind HDL *in vitro* in porcine and human liver by ligand blotting [De Crom *et al.*, 1992; 1994]. These proteins have been identified as conformational forms of gp96/GRP94 [De Crom *et al.*, 1996a], a known ER resident protein. However, this protein is also localized at the plasma membrane, as has been reported by several groups [discussed in De Crom *et al.*, 1996a], and as has been shown by electronmicroscopical immunohistochemistry in our laboratory [De Crom *et al.*, 1996b].

Attempts to demonstrate the HDL-binding capactity of gp96 by transfecting cDNA transiently to COS cells failed because only low percentages of expressing cells could be obtained and because expressed gp96 was exclusively localized in the ER. Expression of a gp96 mutant with a deletion of the C-terminal tetrapeptide KDEL, that is a known ER retention

signal, resulted in plasma membrane localization, but in a very low percentage of the cells (< 1%). As a consequence, HDL-binding studies to these cells did not lead to any conclusive results [De Crom *et al.*, 1996a].

Therefore it was decided to isolate stably transformed cultured cells.

Experimental Procedures

Cultured cells

COS-1 cells, Hep G2 cells, CHO-9 cells, L cells and MEL cells were cultured in DMEM/F10$^+$ (1:1) supplemented with 2 mM L-glutamine, 100 IU penicillin/ml, 100 mg streptomycin/ml and 10% FCS at 37 °C in a 5% CO_2 athmosphere. All cell types were transfected with Lipofectin™ (Promega) according to the manufacturer's instructions, except for MEL cells, that were transfected by electroporation using a BioRad Gene Pulser™ (settings: 117 V, 1200 mF, 10 msec). The next day following transfection, cells were selected on culture medium containing 800 mg/ml G418. MEL cells were induced by adding 2% dimethylsulfoxide to the medium four days after transfection and were harvested for analysis after 24 hours [Antoniou, 1991].

Constructs

For cotransfection to COS, CHO and HepG2 cells, pCD-X-neo was used, containing a neomycin resistance gene into a derivative of the mammalian expression vector pCD-X [Okayama and Berg, 1983] and pCD-X–p-gp96 or pCD-X–h-gp96 containing the porcine or human gp96 cDNA respectively as described before [De Crom *et al.*, 1996a].

L cells were transfected with a mammalian expression vector pJG1 containing the CMV promoter and the TK-neo gene (gift of Jacky Guy, Rotterdam), and the porcine or human gp96 cDNA cloned into the polylinker (pJG1–p-gp96 and pJG1–h-gp96 respectively).

MEL cells were transfected with a vector named pEV3, which is a derivative of pEC3 [Needham *et al.*, 1992], containing the β-globin locus control region and parts of the β-globin gene for mRNA stability, into which porcine or human gp96 cDNA was cloned (pEV3–p-gp96 and pEV3–h-gp96 respectively). Alternatively, a cDNA encoding p-gp96 with a deletion of the C-terminal tetrapeptide KDEL [De Crom *et al.*, 1996a] was cloned in this vector (pEV3–p-gp96$^{\Delta KDEL}$).

DNA and RNA analysis

DNA and RNA isolation from cells, as well as Southern and Northern blotting were performed using standard techniques [Sambrook *et al.*, 1989]. Blots were hybridized with radioactively labelled probes as indicated in the figure legends. Hybridizing bands were visualized by autoradiography or using a Phosphor Imager (Molecular Dynamics).

Immunoblotting and immunocytochemistry

Immunoblotting and immunocytochemistry was performed exactly as described before [De Crom *et al.*, 1996a].

HDL-binding to MEL cells

Isolation and radiolabelling of human HDL with ^{125}I was performed exactly as described before [De Crom *et al.*, 1992]. 200 ml of MEL cell suspension in medium containing 10% lipoprotein deficient serum (LPDS) was incubated with the indicated amounts of ^{125}I–HDL at 37 °C for 2 h. Cells were washed four times in PBS containing 5% LPDS-FCS, 2 times in PBS and resuspended in 0.5 ml PBS. Associated radioactivity was determined in a gamma counter. Then cell protein was dissolved by adding NaOH to a final concentration of 0.1 M and determined according to Lowry [1951]. Aspecific cell association was measured in the presence of a 25-fold excess of unlabelled HDL. Specific cell association was calculated by subtracting the aspecific cell association from the total cell association. All measurements were performed in triplicate.

Fluoresence activated cell sorting analysis

Aliquots of MEL cells were either fixed (4% paraformaldehyde; 100% methanol) or unfixed and incubated with an antiserum directed against porcine [De Crom *et al.*, 1994] or human [De Crom *et al.*, 1996a] gp96 (1 h, room temperature; dilution 1:250), and subsequently washed and incubated with a secondary antibody (goat-anti-rabbit) conjugated to FITC (Nordic; 1 h, room temperature; dilution 1:80). Samples were analyzed on a FACScan (Becton Dickinson).

Results and Discussion

COS-1 cells, HepG2 cells and CHO-9 cells were co-transfected with 1 mg pCD-X–neo and 10 mg of either pCD-X–h-gp96 or pCD-X–p-gp96. The following numbers of G418 resistant clones were isolated: COS cells: 60 clones transfected with h-gp96, 60 with p-gp96; HepG2 cells: 20 clones transfected with h-gp96, 20 with p-gp96; CHO-9 cells: 95 clones transfected with h-gp96, 95 with p-gp96. DNA was isolated from each of these clones and analyzed by Southern blotting: ~20% of the COS cells and ~25% of the CHO cells contained gp96 DNA incorporated into the chromosomes. None of the HepG2 cells had gp96 incorporated into the chromosomes. It appeared to be much more difficult to isolate G418 resistant HepG2 cell clones than COS or CHO clones. Moreover, the surviving cells grew slowly and showed an aberrant morphology, probably indicating a bad condition of the cells. Therefore, HepG2 cells were considered unsuitable for this kind of experiment.

CHO and COS cell clones that had incorporated gp96 into their chromosomes, were analyzed for protein expression by immunocytochemistry. Each of the clones showed protein expression, but the percentage of expressing cells varied considerably, from ±5% to ±50%. However, when later passages of the cells in culture were rescreened, percentages of expressing cells had dropped to < 5% in all cases, indicating

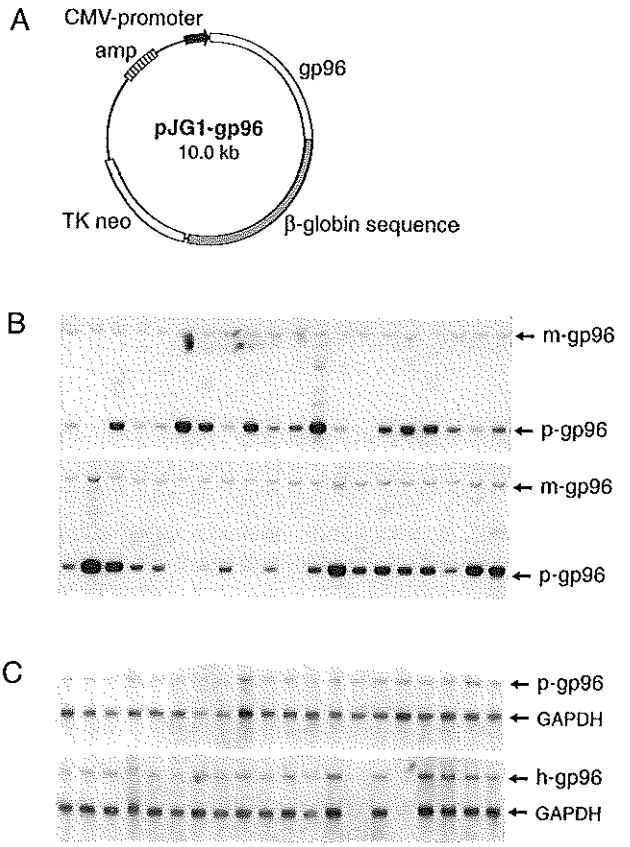

Figure 1. Transfection of L cells. A. L cells were transfected with the vector pJG1 in which human or porcine gp96 cDNA was cloned. The β-globin sequence represents exon II and III from human β-globin and intronic sequences, and is derived from the vector pEC3 [Needham *et al.*, 1992]. B. DNA was isolated from G418 resistant L cell clones, digested with EcoRV and BamHI and subjected to Southern blotting. The upper panel contains DNA from cells transfected with pJG1–p-gp96, the lower panel contains DNA from cells transfected with pJG1–h-gp96. Blots were hybridized with full length h-gp96 cDNA. A band representing endogenous gp96 in L cells (m-gp96) is visible in every lane. C. RNA was isolated from the same L cell clones and Northern blotting was performed. GAPDH served as an internal standard.

that there is no stable expression. Probably, the non-expressing cells quickly overgrow the expressing cells.

We decided to make a construct containing both the neomycin resistance gene and the gp96 cDNA (porcine or human), for which we used

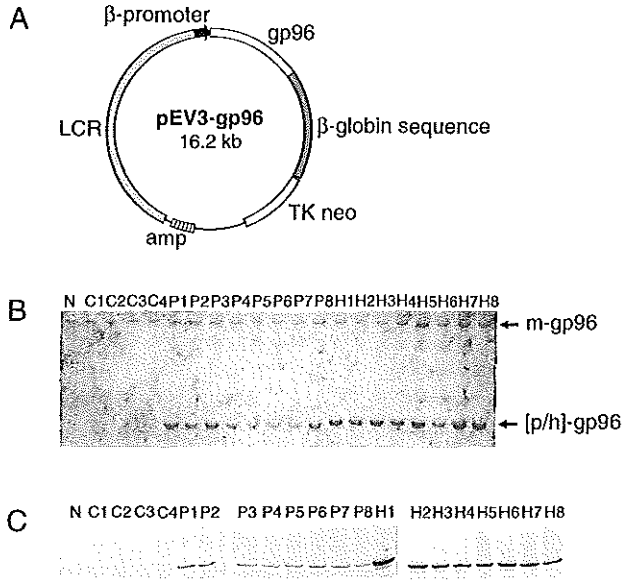

Figure 2. Transfection of MEL-cells. A. MEL-cells were transfected with the vector pEV3 in which human or porcine gp96 cDNA was cloned. The β-globin sequence represents exon II and III from human β-globin and intronic sequences, and is derived from the vector pEC3 [Needham *et al.*, 1992]. B. DNA was isolated from G418 resistant MEL-cell clones, digested with EcoRI and subjected to Southern blotting. N: non-tranfected MEL cells; C1–C4: populations transfected with pEV3; P1–P8: populations transfected with pEV3–p-gp96; H1–H8: populations transfected with pEV3–h-gp96. Blots were hybridized with full length h-gp96 cDNA. A band representing endogenous gp96 in MEL-cells (m-gp96) is visible in every lane. C. Protein was isolated from the same MEL-cell clones and immunoblotting was performed. An antiserum directed against human p96 was used [De Crom *et al.*, 1996a], which cross-reacts with porcine gp96.

a plasmid that contained also exon II and III of the human β-globin gene plus intronic sequences (pJG1–gp96; Fig. 1A). The β-globin sequences are incorporated to give rise to mRNA with intronic sequences, that should lead to higher stability. We transfected mouse L cells with 10 mg of either pJG1–h-gp96 or pJG1–p-gp96 and isolated 48 G418 resistant clones for each construct.

Southern blotting showed that these clones had taken up 0 to 20 copies of the plasmid (Fig. 1B). Northern blotting studies showed no increase in RNA (Fig. 1C). Not surprisingly, we were not able to detect any protein by Western blotting in any of the clones.

Possibly expression of gp96 interferes with cellular proliferation. This would result in a selection of cells that do not express, or somehow switch

off the expression of the protein. Therefore, we decided to use murine erythroleukemia (MEL) cells. These cells can be transfected with a plasmid containing the neomycine resistance gene and the DNA of interest under the control of a minimal promoter and the β-globin locus control region (LCR). This DNA will not be expressed in undifferentiated cells, but will be expressed when the MEL cells are induced by agents like dimethylsulfoxide to undergo erythroid differentiation, that will activate the β-globin gene [Needham *et al.*, 1992]. This allows the selection of cells that have taken up the transfected DNA under conditions in which the protein of interest is not expressed. In this way, an eventual selective effect of the disadvantageous influence of gp96 expression may be avoided.

We cloned either porcine or human gp96 cDNA in this plasmid (pEV3–[h/p]-gp96; Fig. 2A), that also contains the β-globin sequences present in pJG1–gp96. MEL cells were transfected with pEV3 (4 independent populations: C1–C4), pEV3–p-gp96 (8 independent populations: P1–P8), pEV3–h-gp96 (8 independent populations: H1–H8) by electroporation and grown in the presence of G418. Cells were induced by dimethylsulfoxide and analyzed. Southern blotting showed that each of the cell populations P1–P8 and H1–H8 had incorporated gp96 cDNA (Fig. 2B). Each of the cell populations expressed gp96 protein, as demonstrated by immunoblotting (Fig. 2C). HDL-binding was studied to C4, P3 and H5 (Fig. 3). Binding to the h-gp96 expressing cells appeared to be considerably higher than to control cells, while p-gp96 expressing cells bind equal amounts of HDL as control cells. However, in a second experiment using newly induced cells derived from the same cell populations, no difference in HDL-binding activity of control cells, p-gp96 or h-gp96 expressing cells was observed. Therefore, it

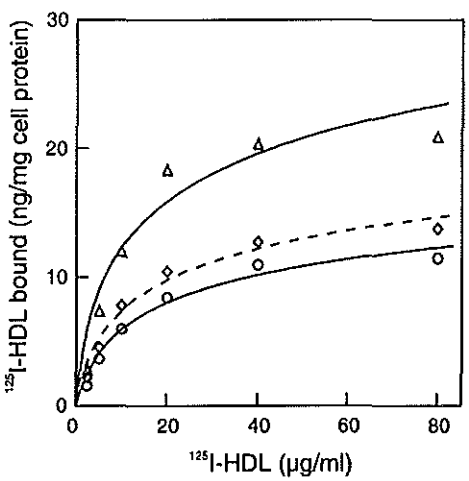

Figure 3. Cell association of ^{125}I–HDL to transfected MEL cell populations. MEL cells were incubated in medium containing 10% lipoprotein deficient serum (LPDS) at 37 °C for 2 h. Cells were washed with PBS and specific cell association was determined as described under Experimental Procedures. Values are means of triplicate measurements. ◊, dashed line: MEL cells transfected with pEV3 (C4); ○, solid line: MEL cells transfected with pEV3–p-gp96 (P3); Δ, solid line: MEL cells transfected with pEV3–h-gp96 (H3).

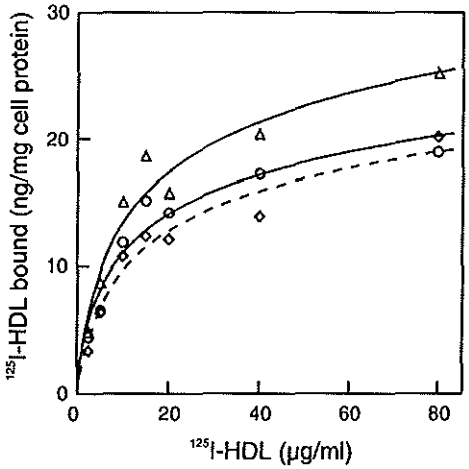

Figure 4. Cell association of ^{125}I-HDL to clones of transfected MEL cells. MEL cells were incubated in medium containing 10% lipoprotein deficient serum (LPDS) at 37 °C for 2 h. Cells were washed with PBS and specific cell association was determined as described under Experimental Procedures. Values are means of triplicate measurements. ◊, dashed line: MEL cells transfected with pEV3 (C4); ○,Δ, solid line: MEL cells transfected with pEV3–p-gp96$^{\Delta KDEL}$.

was decided to select single cell clones. 100 clones were selected transfected with p-gp96 and 100 clones with h-gp96. Each of these clones was tested by immunocytochemistry. The number of protein expressing cells varied from ~5% to ~75%. Clones with the highest numbers of expressing cells were selected. 27 clones transfected with p-gp96 were selected and 27 clones transfected with h-gp96. Cells from these clones were subjected to a second round of induction and immunocytochemistry. Finally, four individual clones (2 for each construct) were selected for HDL-binding. No difference in HDL-binding activity was found.

Using FACS analyses, it appeared that in cells transfected with h-gp96 incubated with anti-gp96 no difference was found with control cells when unfixed cells were used. However, in fixed cells, there was a two-fold increase in signal in gp96 transfected cells, indicating that most of the protein is located intracellularly.

Therefore, MEL cells were transfected with pEV3–p-gp96$^{\Delta KDEL}$. Expression was 10 to 15%. 200 subclones were isolated and tested. About 80 clones were expressing the protein. After a second round of isolation and induction, only 20 clones appeared to express the protein. Hence the clones were not stable. Finally, two subclones were selected. These clones were tested for HDL-binding (Fig. 4). One of the two clones showed a two-fold increase in HDL-binding activity, but the other clone did not.

In conclusion, we were not able to obtain a cell line stably expressing gp96, despite many attempts. It also proved to be very difficult to obtain a stable cell line of MEL cells showing a high level of gp96 protein expression upon induction. In MEL cells most of the expressed protein is located intracellularly. Transfection with a ΔKDEL construct resulted in two selected

clones with a high level of gp96 expression. HDL-binding was increased to the cells of only one of these clones. This observation suggests that gp96 mediates binding of HDL to cells. However, the transfection experiments of gp96 to cultured cells appeared to be troublesome. Therefore, they hardly allow definitive conclusions. Further experiments, including the generation of transgenic mice are needed to evaluate the function of gp96.

Acknowledgments

We thank Ton Verkerk for FACS analyses; and Mirko Kuit for preparation of the figures.

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Appendix Paper 6

Transgenic Mice Expressing gp96/GRP94, a Putative HDL-Binding Protein

Rini P.G. de Crom,
Rien van Haperen,
Teus van Gent*,
Rick Janssens,
Arie van Tol*,
Frank G. Grosveld,
Arthur W.M. van der Kamp

MGC-Department of Cell Biology and Genetics,
and *Department of Biochemistry,
Erasmus University Rotterdam,
3000 DR Rotterdam,
The Netherlands

Although gp96/GRP94 has been reported to be an endoplasmic reticulum localized protein, several lines of evidence indicate that at least part of the expressed protein is localized at the plasma membrane. This plasma membrane localized gp96/GRP94 is a putative HDL-binding protein in liver, because the protein has the ability to bind HDL *in vitro*. We generated transgenic mice expressing gp96/GRP94 in order to evaluate the involvement of this protein in HDL metabolism. We used three different constructs with different promoters and regulatory sequences in order to obtain distinct patterns of protein expression. First, we used a construct including the mouse albumin enhancer and promoter that should result in liver-specific expression. Five lines of transgenic mice were generated. Protein expression appeared to be relatively low, that is not much higher than the expression of murine gp96/GRP94. No differences were found when HDL-binding activity to isolated liver plasma membranes was compared between transgenic mice and non-transgenic control litter mates. HDL decay studies did not show any differences either. However, the level of the transgene might be too low to allow the detection of any effect on HDL metabolism. Second, transgenic mice were generated using a construct including the human CD2 promoter and locus control region, resulting in T-cell specific expression. No differences were found in HDL-binding activity to isolated thymus plasma membranes of trangenic mice compared to controls. In fluorescence activated cell sorter analyses it appeared that gp96 is expressed exclusively intracellularly in trangenic mice. Third, a construct was used including the promoter of mouse H-2K^b, a major histocompatibility class I gene. This should result in expression in virtually all types of cells. In order to obtain protein expression localized at the plasma membrane, a mutation was introduced in the gp96 encoding cDNA resulting in the deletion of the C-terminal tetrapeptide KDEL. However, in two lines of transgenic mice made with this construct we were unable to detect any protein expression at all. In conclusion, several lines of transgenic mice did not allow us to obtain conclusive evidence concerning the role of gp96/GRP94 in HDL metabolism. Introduction

Gp96/GRP94 is a protein that has 50% homology with hsp90 and is therefore classified as a stress protein [Gething and Sambrook, 1992]. The protein contains the C-terminal tetrapeptide KDEL, which is an ER retention signal [Pelham and Munro, 1993; Nilsson and Warren, 1994]. Indeed, the protein is localized in the ER in a variety of tissues, where it possibly acts as

a chaperonin [Melnick *et al.*, 1994], although other functions have been suggested [Srivastava *et al.*, 1994].

At least a part of gp96 escapes ER retrieval in some tissues [Takemoto *et al.*, 1992]. Several groups found evidence for plasma membrane localization of gp96, where it might have a distinct function (discussed in De Crom *et al.*, 1996a). We showed by electron microscopic immunocytochemistry that in the liver, gp96 is to a large extend localized at the plasma membrane [De Crom *et al.*, 1996b].

In the liver, gp96 is possibly implicated in HDL metabolism, since it has *in vitro* HDL-binding activity, as shown by ligand blotting [De Crom *et al.*, 1996a].

We wanted to evaluate this hypothesis by the generation of transgenic mice expressing gp96/GRP94. We used three different constructs that were anticipated to result in three distinct patterns of protein expression.

Experimental Procedures

Constructs

Liver expression. Full length human gp96 was fused to human β-globin sequences from the vector pEC3 [Needham *et al.*, 1992] as described before [De Crom *et al.*, 1996a]. This was cloned into the *Bam*HI site of the vector 2335A-1 (kindly donated by Dr. R.D. Palmiter, Washington, U.S.A.), 3¢ of a 2.3 kb fragment encompassing the mouse albumin enhancer and promoter (equivalent to construct NB in Pinkert *et al.*, 1987). A 7.6 kb DNA segment devoid of vector sequences was used for the generation of transgenic mice (Fig. 1A).

T-cell expression. Full length human gp96 fused to human β-globin sequences (see above) was cloned into the *Eco*RI site of the T-cell specific expression vector VA hCD2 (kindly donated by Dr. D. Kioussis, London). This vector contains the promoter and locus control region (LCR) of the human CD2 gene [Zhumabekov *et al.*, 1995]. A 16.8 kb DNA segment devoid of vector sequences was used for the generation of transgenic mice (Fig. 2A).

Generalized expression. A 4.2 kb *Eco*RI-*Nru*I fragment encompassing the mouse major histocompatibility complex H-2K^b class I promoter (kindly donated by Dr. D. Kioussis, London) was ligated to a porcine gp96 cDNA with a premature stop codon eliminating the C-terminal KDEL tetrapeptide (p-gp96$^{\Delta KDEL}$) fused to human β-globin sequences (De Crom *et al.*, 1996a). This promoter should give rise to expression in a broad range of tissues [Jat *et al.*, 1991]. A 9.5 kb DNA segment devoid of vector sequences was used for the generation of transgenic mice (Fig. 4).

Transgenic mice

Transgenic mice were produced essentially according to Hogan *et al.* [1994]. In short, a solution of DNA from which vector sequences had been removed by digestion with endonuclease restriction enzymes was purified by the use of an Elutip-d column (Schleicher and Schuell). This preparation, containing 1–4 ng/ml DNA was injected into the fertilized eggs from superovulated FVB females that had been mated with FVB

males. Injected eggs were surgically transferred to BCBA pseudopregnant females. Tail DNA was isolated from newborn mice 10 days after birth and was analyzed by Southern blotting. Transgenic mice were mated with FVB wild types for at least two generations. F2 transgenic mice were used for experimental analyses and compared with non-transgenic littermates.

Cholesterol measurements, decay studies
Human HDL was isolated and radiolabeled with ^{125}I–HDL as described before [De Crom *et al.*, 1992]. Labeling of HDL with [^{3}H]cholesteryl linoleyl ether and cholesteryl [^{14}C]oleate was performed exactly as described before [Groener *et al.*, 1989]. Mice were starved overnight. Blood was collected via eye punctions and plasma levels of cholesterol were determined enzymatically using a commercial kit (Boehringer). For HDL decay studies, mice were injected intravenously in the tails. Blood was collected by eye punctions at the indicated time intervals. Animals were killed and organs were collected by dissection. Radioactivity was determined in a gamma counter (^{125}I) or by scintillation counting (^{3}H, ^{14}C).

Antisera, immunoblotting, FACS analysis
A polyclonal antiserum directed against human gp96 has been described before [De Crom *et al.*, 1996a]. A polyclonal antiserum directed against murine gp96 has been obtained in essentially the same way. For the immunization of rabbits, bacterially expressed protein was used, purified by preparative polyacryl amide gel electrophoresis in the presence of SDS. For bacterial expression, a cDNA fragment comprising nucleotides 64-560 from murine gp96 was obtained by PCR and cloned in pET11.

Immunoblotting and FACS analysis using these antisera were performed as described before [De Crom *et al.*, 1992; De Crom *et al.*, 1996c].

HDL-binding studies
HDL-binding studies to plasma membrane preparations of different organs were performed as described before [De Crom *et al.*, 1989].

Results and Discussion

Liver specific expression
Five lines of transgenic mice were generated using a construct containing the mouse albumin enhancer and promoter, human gp96 cDNA and exon II and III plus intronic sequences from the human β-globin gene (Fig. 1A). The β -globin sequences were added in order to obtain an mRNA with intronic sequences for stability and high level expression [Needham *et al.*, 1992]. The five lines were encoded ABH1 to ABH5. Livers were isolated from these mice and subjected to immunoblotting using two different antisera: an antiserum raised against a bacterially expressed fragment of human gp96 (Fig 1B, left panel) and an antiserum raised aginst a bacterially expressed fragment of murine gp96 (Fig 1B, right panel). Protein expression of h-gp96

varied considerably, with the highest expression present in the lines ABH1 and ABH5. Although the two immunoblots cannot be compared quantitatively, the results suggest that h-gp96 expression in the ABH1 or ABH5 mice is in the same order of magnitude as the endogenous m-gp96 expression. Plasma membrane preparations from livers of each of the ABH lines were analyzed for HDL-binding activity using a fixed concentration of ^{125}I–HDL. None of the lines showed a marked increase of HDL-binding compared to controls (Fig. 1C).

One of the lines, ABH1, was analyzed in some detail. Cholesterol levels in plasma were different in males and females, but not in non-transgenic versus transgenic animals (Table I). Liver plasma membranes were isolated and HDL-binding activity was studied (Fig. 2A). The binding curves did not differ significantly between ABH1 mice and control littermates. HDL decay was studied by injecting mice with ^{125}I–HDL. No significant differences were found (not shown). Uptake of HDL cholesterol in the liver was studied by injecting mice with HDL labeled with [^{3}H]-cholesteryl ether and [^{14}C]-cholesteryl ester (Fig. 2B). Cholesterol ethers are non-degradable analogs of cholesterol esters. As a consequence, cholesterol ethers become trapped after intracellular uptake and are therefore suitable tracers to monitor this process. In contrast, cholesterol esters will be degraded and its associated label will therefore dissociate from the cells that have taken up the HDL particle [Groener *et al.*, 1989]. As expected, the disappearance from plasma after injection for both labels is similar, while the amount of label found in the liver is higher for [^{3}H]-cholesteryl ether than for [^{14}C]-cholesteryl ester. No differences were found between transgenic mice and control littermates (Fig. 2B).

In conclusion, transgenic mice expressing h-gp96 under the control of the mouse albumin enhancer and promoter do not show appreciable overexpression of the transgene in the liver. We were not able to find any effect of the moderate liver expression of h-gp96 in the ABH mice on HDL metabolism. However, the level of expression might be too low to enable us to measure such effects. Therefore, the ABH mice are not a suitable model to evaluate the hypothesis that gp96 is involved in HDL metabolism.

T-cell expression

We generated transgenic mice for gp96 with a construct that should give rise to expression in T-cells. The thymus has been reported to be among the organs with the lowest uptake of HDL in rats [Glass *et al.*, 1985]. We confirmed these results for mice (Table II). The reason for the generation of these mice was that we anticipated that effects of expression of a protein

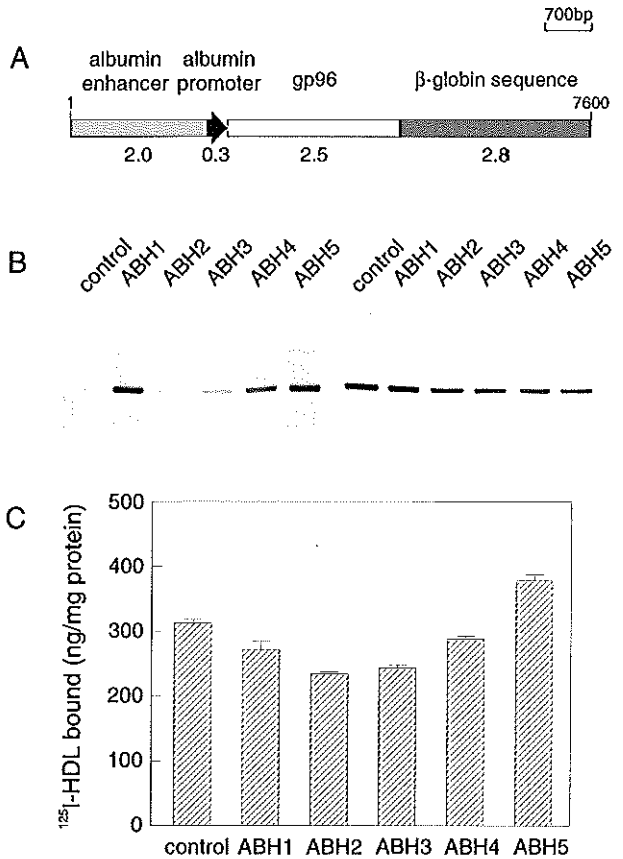

Figure 1. Liver specific expression. A. Construct ABH used for the generation of transgenic mice with liver specific expression. Sequences encompassing the mouse albumin enhancer and promoter were fused to human gp96 cDNA and human β-globin sequences (exons II and III plus intronic sequences and polyadenylation signals). The numbers present the sizes of the different elements in kilobasepairs. The total length of the construct is given in basepairs. B. Immunoblots showing the expression of gp96 in liver homogenates from five independent lines of ABH transgenic mice using an antiserum specific for human gp96 (left panel) or an antiserum recognizing murine gp96 (right panel). C. Binding of human ^{125}I–HDL (10 μg/ml) to plasma membrane preparations of livers from the five ABH lines. See "Experimental Procedures" for details.

that is involved in HDL metabolism would be most evident in an organ with a low basal activity of HDL metabolism. A construct was used containing the LCR, promoter and some intronic sequences of the human CD2 gene, in which we cloned human gp96 cDNA and exon II and III plus

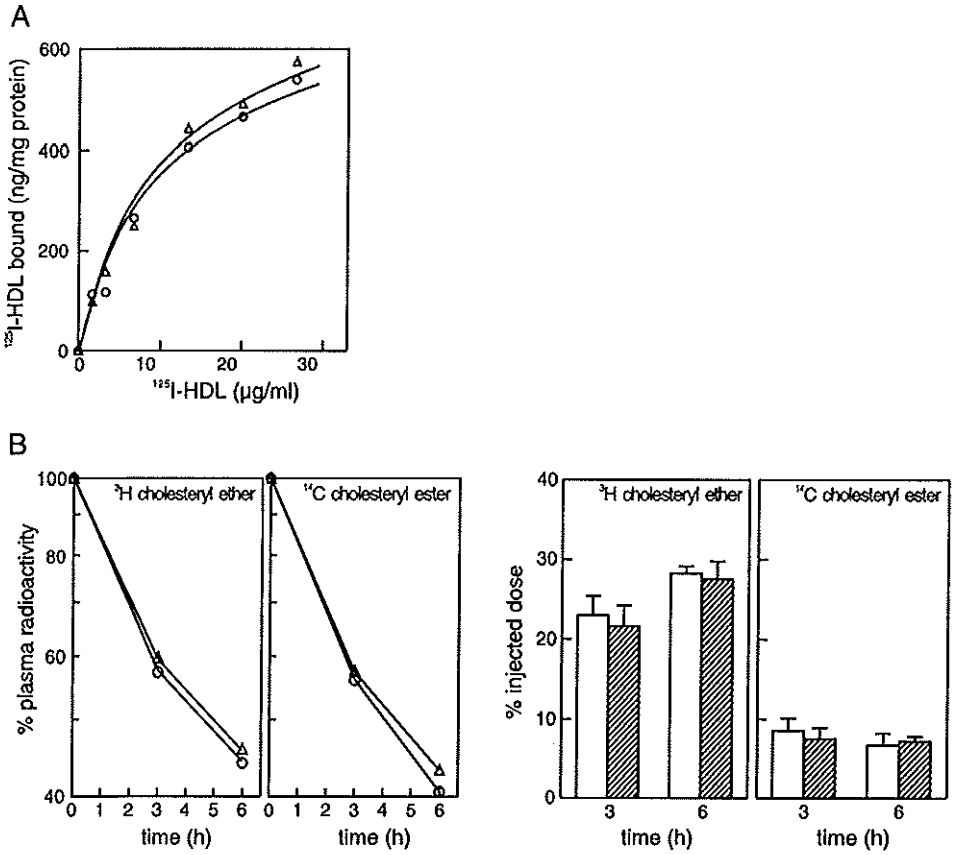

Figure 2. HDL metabolism in ABH mice. A. Binding of human ^{125}I–HDL to plasma membrane preparations of livers from ABH mice (Δ) and control littermates (O). B. *Left:* Plasma decay of [^{3}H]cholesteryl linoleyl ether and cholesteryl [^{14}C]oleate in ABH mice (Δ) and control littermates (O), following injection with 0.2 μl of human HDL (total cholesterol 0.585 mM; 555 × 10^{3} dpm/ml [^{3}H]cholesteryl linoleyl ether and 237 × 10^{3} dpm/ml cholesteryl [^{14}C]oleate). *Right:* Liver uptake of [^{3}H]cholesteryl linoleyl ether and cholesteryl [^{14}C]oleate in ABH mice (hatched bars) and control littermates (open bars).

intronic sequences from the human β-globin gene (Fig. 3A). Two lines were generated encoded CDH1 and CDH2. The thymus was isolated from these mice and subjected to immunoblotting using antisera directed against either human or murine gp96 (Fig. 3B). Both lines expressed the human protein; the line CDH1 in rather high amounts. Higher molecular weight forms are

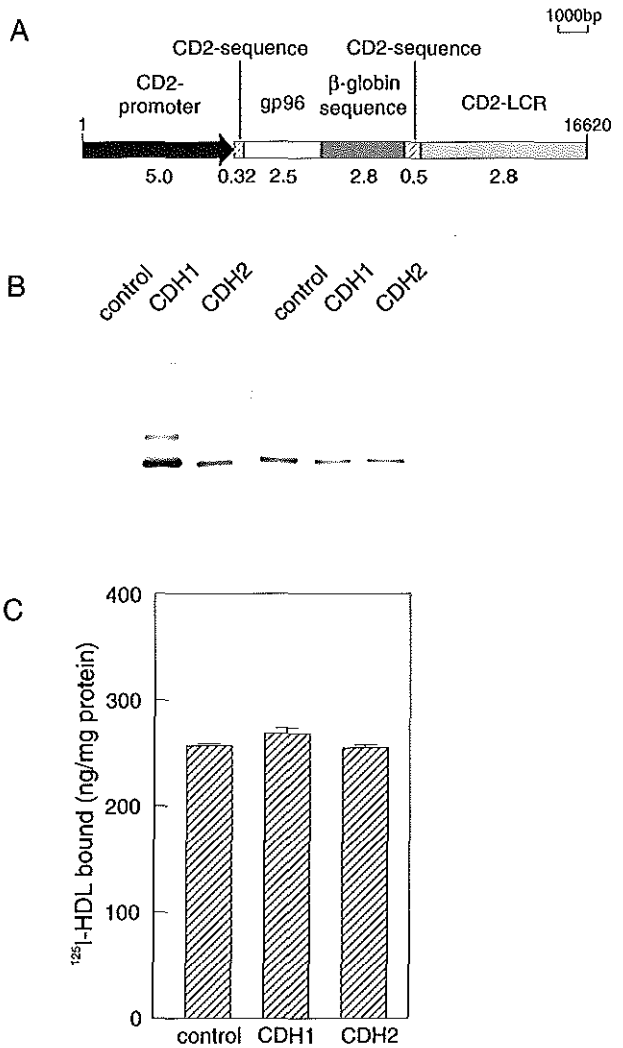

Figure 3. T-cell specific expression. A. Construct CDH used for the generation of transgenic mice with T-cell specific expression. Sequences encompassing the human CD2 promoter, LCR and small parts of the CD2 gene were fused to human gp96 cDNA and human β-globin sequences (exons II and III plus intronic sequences and polyadenylation signals). The numbers present the sizes of the different elements in kilobasepairs. The total length of the construct is given in basepairs. B. Immunoblots showing the expression of gp96 in thymus homogenates from two independent lines of CDH transgenic mice using an antiserum specific for human gp96 (left panel) or an antiserum recognizing murine gp96 (right panel). C. Binding of human ^{125}I–HDL (10 μg/ml) to plasma membrane preparations of the thymus from the two CDH lines. See "Experimental Procedures" for details.

present in these mice, corresponding to the 110 and 180 kDa proteins found in porcine liver [De Crom *et al.*, 1992, 1994]. However, when HDL-binding activity was studied, no difference could be found between the non-transgenic mice and the two transgenic mouse lines (Fig. 3C). In fluorescence cell sorter analyses fixed and unfixed thymocytes were compared. No differences were found when unfixed thymocytes of CDH mice and controls were compared. However, fixed thymocytes of CDH mice showed a two-fold increase in signal compared to cells from control litter mates using an antiserum directed against h-gp96. These results indicate that the expressed proteins are present exclusively intracellularly. As a consequence, analyses of the CDH mice do not lead to any conclusive results with regard to the supposed role of gp96 in HDL metabolism.

Generalized expression

We generated transgenic mice using a construct with the H-2K^b promoter, which is a major histocompatibility class I promoter [Jat *et al.*, 1991]. With this construct we aimed to induce expression in virtually all types of cells, that would obviously affect HDL metabolism, if gp96 is involved in it to any extend. In order to achieve plasma membrane expression, we used a gp96 cDNA construct in which the C-terminal KDEL sequence had been deleted (Fig. 4). Two lines of transgenic mice were generated with this construct. From these mice, plasma membrane preparations were isolated from liver, kidney, lung, spleen, thymus and testis and analyzed for protein expression by immunoblotting. No expressed gp96 protein could be detected in any of the tissues analyzed. As expected, HDL-binding to plasma membrane preparations from livers and kidneys from these mice did not show any difference compared to non-transgenic littermates.

In conclusion, extensive studies in several lines of transgenic mice generated with three different constructs that should result in different

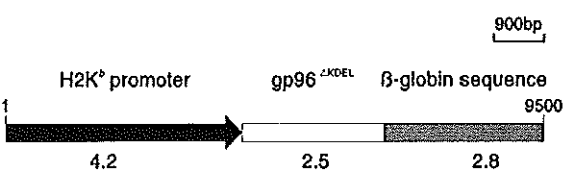

Figure 4. Generalized expression. Construct used for the generation of transgenic mice with gneralized expression. Sequences encompassing the mouse H-2K^b promoter were fused to human gp96$^{\Delta KDEL}$ cDNA and human β-globin sequences (exons II and III plus intronic sequences and polyadenylation signals). The numbers present the sizes of the different elements in kilobasepairs. The total length of the construct is given in basepairs.

patterns of protein expression did not result in a significant overexpression of gp96. For this reason, the results from experiments and measurements in these mice do not contribute to our understanding of the role of gp96 in HDL metabolism. The apparent inability to achieve gp96 overexpression might be attributable to the use of cDNA. Therefore we will isolate a cosmid containing the human gp96 gene for the generation of transgenic mice. Next to this, we will concentrate on the generation of mice genetically modified to lack gp96 expression.

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