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Mutational Analysis of Ligand - LDL-Receptor Interactions

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ABSTRACT

Lipoprotein receptors are cell surface-exposed receptors that are specific for the binding and uptake of lipoproteins from the circulation into hepatic and extrahepatic tissues. This process is termed receptor-mediated endocytosis and is largely dependent upon the interaction between members of the so-called Low Density Lipoprotein (LDL) receptor family and plasma lipoproteins. The best characterized receptor of this family is the LDL receptor (LDLR). It plays a major role in the regulation of plasma cholesterol levels by mediating the binding and uptake of apolipoprotein B- (apoB) and -E (apoE) -containing plasma lipoprotein particles.
To gain further insight into the molecular mechanism of LDL receptor- ligand interactions, I performed the present study in the domesticated chicken (Gallus gallus), which serves as an excellent model organism for research on lipid metabolism. Various surface receptors homologous to members of the mammalian LDLR family have been discovered in this species, and are recognized as important in the maintenance of systemic lipid homeostasis and embryongenesis including follicle development and oocyte growth. In 2003, the first avian LDL receptor ortholog was characterized and revealed a high degree of conservation during evolution, since the LDLR's hallmark properties are already present in the chicken protein. The numerous avian and mammalian relatives of the receptor family share characteristic similarities, both in structural and functional terms. Via highly conserved cysteine-rich repeat elements in the extracellular ligand-binding domain the receptors are able to bind many unrelated proteins, such as apolipoproteins, proteases, signaling molecules, and several other groups of proteins. Acting as cargo transporters, members of this receptor family are not only capable of transporting macromolecules, but also of signal transduction.

According to previous studies, certain chicken receptors belonging to the LDLR family are able to bind apolipoprotein E, a protein of mammalian origin, which is not produced in the avian species, while other receptors like the chicken LDLR do not have binding affinity for apoE. In agreement with previous investigations, this observation indicates that certain variations in the ligand-binding domain of the receptor contribute to the recognition of a variety of heterogeneous ligands. In the present study I focused on the investigation of structural differences between apoE-binding competent and apoE-binding incompetent receptors that are pivotal for ligand-binding. To address these issues I generated receptor fragments of the chicken LDLR consisting of the ligand-binding region. Based on previous findings derived from extensive studies of the human LDLR, mutations in putatively important receptor domains that were characterized as critical for apoE binding were introduced. Following bacterial expression, the receptor mutants were purified using affinity chromatography. Since the functionality of the LDLR depends on the correct folding including disulfide bond formation, the main challenge was to re-fold the recombinant receptor fragments into a binding-active conformation. This was achieved by the assistance of the receptor-associated protein (RAP), a molecular chaperone especially acting on LDLR

family members. The refolding procedure was performed in the presence of RAP in an environment that allows disulfide bond formation and Ca^{2+} incorporation. Finally, the mutated receptors were characterized with regards to the ligand-binding properties towards apolipoprotein B and -E containing lipid particles. The data obtained from solid phase binding assays revealed that the first LA repeat is not required to bind apoB- containing lipoproteins. However, mutations in the linker region separating repeat 4 and 5 as well as mutations in LA repeat 5 dramatically impaired the binding of apoB-containing lipoproteins. Initial investigations examining the binding activity of the mutant receptors towards apoE-containing particles did not lead to reproducible results and thus require further investigations.

These data obtained in an oviparous species contribute to the understanding of LDLR-ligand interactions and are in agreement with previous investigations, in which the importance of LA5 for ligand recognition of the human LDLR was established.

ZUSAMMENFASSUNG

Lipoproteinrezeptoren sind Plasmamembranproteine, welche für die spezifische Bindung und Aufnahme der im Blutkreislauf zirkulierenden Lipoproteine in hepatische und extrahepatische Gewebe verantwortlich sind. Dieser physiologische Vorgang wird als rezeptorvermittelte Endozytose bezeichnet und basiert auf der Interaktion zwischen Mitgliedern der sogenannten Low Density Lipoprotein (LDL) Rezeptor Familie und Lipoproteinen im Plasma. Der als erster entdeckte und am besten charakterisierte Lipoproteinrezeptor ist der LDL Rezeptor (LDLR). Dieser Rezeptor spielt eine wichtige Rolle in der Regulation der Cholesterinspiegel durch die Bindung und Internalisierung von Lipoproteinen, welche Apolipoprotein- B (apoB) und /oder -E (apoE) als Proteinkomponente besitzen.

In der vorliegenden Arbeit wurde das legende Huhn, ein gut etablierter Modellorganismus zur Erforschung des Lipidstoffwechsels, herangezogen um die Interaktion des LDL Rezeptors mit Apolipoprotein B- und -E hältigen Lipoproteinen näher zu charakterisieren. Früheren Studien zufolge gibt es zahlreiche Oberflächenrezeptoren im Huhn, die eine deutliche Homologie zur Familie der LDL Rezeptorproteine von Säugern aufweisen. Der zum menschlichen LDL Rezeptor homologe Rezeptor im Huhn wurde erstmals im Jahr 2003 beschrieben und weist die klassischen Strukturmerkmale der Säuger-LDL Rezeptoren auf, was auf eine hohe evolutionäre Konservierung deutet. Alle Mitglieder der LDL Rezeptorfamilie besitzen einen charakteristischen modularen Aufbau aus vier bis fünf unterschiedlichen Domänen. Die amino-terminale Region enthält die Ligandenbindungsdomäne des Rezeptors und besteht aus mehreren cysteinreichen Abschnitten, den sogenannten LA-repeats, welche die Interaktion mit verschiedenen Proteinen, wie Apolipoprotein, Proteasen und Signalproteinen vermitteln. Mutationsanalysen zeigen, dass die einzelnen Repeats unterschiedliche funktionelle Bedeutungen bei der Ligandenbindung besitzen.

Bindungsstudien im Hühnersystem ergaben, dass einige Rezeptoren der LDLR Familie Apolipoprotein E binden, ein in Säugern, jedoch nicht im Huhn produziertes Protein. Andere Rezeptoren wiederum besitzen diese Bindungseigenschaft nicht. Dieses unterschiedliche Verhalten weist darauf hin, dass bestimmte Variationen in der ligandenbindenden Domäne des Rezeptors einen entscheidenden Einfluss auf die Ligandenbindung besitzen. In der vorliegenden Arbeit habe ich mich damit beschäftigt, die strukturellen Unterschiede zwischen ApoE-bindenden Rezeptoren und Rezeptoren, welche die Fähigkeit ApoE zu binden nicht besitzen, zu ermitteln. Gemäß den Ergebnissen aus früheren Mutationsanalysen wurde die Ligandenbindungsdomäne des Hühner- LDLR an vermeintlich wichtigen Bindungsregionen mutiert. Die bakteriell exprimierten mutanten Rezeptorproteine wurden durch Affninitätschromatographie gereinigt und, um die physiologische Funktionsweise zu erlangen, einem Faltungsprozess unterzogen, welcher durch das molekulare Chaperon RAP (Receptor-associated Protein) unterstützt wurde. Schließlich wurden die Rezeptormutanten hinsichtlich der Ligandenbindung von Apolipoprotein B- und -E -hältigen Lipoproteinen

untersucht. Diese Bindungsanalysen ergaben, dass das erste cysteinreiche LA Repeat (LA1) nicht an der Binding von Apolipoprotein B beteiligt scheint. Indessen führten Mutationen in den Repeats 4 und 5 (LA4, LA5), sowie in der Linker-Region zwischen diesen Repeats zu erheblichen Einschränkung bezüglich der Bindung von ApoB-hältigen Lipoproteinen. Bindungsanalysen zur Erforschung der Interaktion zwischen ApoE und den Rezeptormutanten lieferten bisher keine reproduzierbaren Ergebnisse, und erfordern daher weitere Untersuchungen.

Die Daten, die im Zuge dieser Arbeit durch Experimente im Huhn ermittelt wurden, tragen zum Verständnis des molekularen Mechanismus der LDL Rezeptor-Ligand Interaktion bei und stehen im Einklang mit Untersuchungsergebnissen am menschlichen LDL Rezeptor, welche eine große Bedeutung des fünften LA Repeats (LA5) für die Ligandenbindung beschreiben.

1. INTRODUCTION

Lipoproteins are macromolecular structures that facilitate the transport of water-insoluble lipids and other lipophilic components in the aqueous environment of the circulatory system. While free fatty acids are bound to albumin, cholesterol, triglycerides, and phospholipids are packaged into lipoprotein complexes in order to allow the transport to various tissues and cells requiring lipids. Various distinct lipoproteins can be classified, which differ in size, lipid composition and protein content and account for distinct physiological functions.

1.1 THE MAJOR PLASMA LIPOPROTEINS

In general, all lipoproteins share a common structural organization and are widely conserved across distinct species. The spherical particles are composed of a neutral lipid core containing triacylglycerols (TG), cholesteryl-esters (CE) and small amounts of unesterified cholesterol. To allow water solubility, the inner core is surrounded by a surface monolayer consisting of amphiphatic lipids, mostly phospholipids, and unesterified cholesterol, and apolipoprotein molecules. Based on their size, density, as well as lipid- and apolipoprotein composition, lipoproteins can be categorized into five classes: chylomicrons (CM), very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). The density of these lipoproteins is inversely correlated with the particle's lipid content and direct proportional to the protein contents. These protein components are characteristic for each lipoprotein class and their distribution among various classes are indicated in figure 1.1.

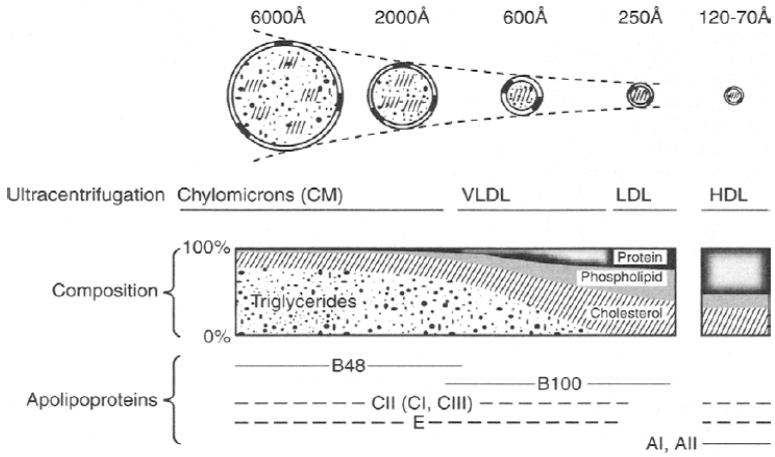

Figure 1.1 Major Lipoprotein classes and their composition

The major lipoprotein classes are categorized according to their density. Particle diameters range from 6000 Å for CM to 70 Å for HDL. The various classes of lipoproteins are characterized by varying contents of triglycerides, cholesterol, phospholipids and apolipoproteins. (Jonas, A. and Phillips, M.C. In *Biochemistry of Lipids, Lipoproteins and Membranes*, (D.E. Vance and J.E. Vance), 5th Edition, 2008)

The processes of lipoprotein metabolism have been intensively studied. In the following I will briefly summarize the main aspects of the underlying pathways. Chylomicrons (CM) and VLDL are the major carriers of triglycerides and are synthesized and secreted by the intestine (CM) and liver (VLDL). When these particles enter the blood system, they are immediately hydrolyzed by the enzyme lipoprotein lipase (LPL). Thereby, free fatty acids are released, and so-called remnant particles are formed. Free fatty acids bound to albumin are transported to adipocytes and muscle cells, where they are catabolized or restored as triglycerides, respectively. Remnant particles are rapidly cleared from the blood trough receptor-mediated endocytosis by the liver or are further acted on by lipases, leading to LDL particles that are finally removed by the liver.
Another class of lipoproteins, high density lipoproteins (HDL), are synthesized and assembled in the liver and intestine and play an important role in mediating the so-called reverse cholesterol transport. They are responsible for the removal of excess cholesterol from peripheral tissues and for the transport to the liver and steroidogenic tissues, either for metabolism or for exretion (reviewed by Fielding, C.J. and Fielding, P.E. 2008).

1.2 APOLIPOPROTEINS AND ROLES IN METABOLISM

Lipoprotein metabolism is regulated and controlled by specialized apolipoprotein constituents residing on the distinct lipoprotein classes. These apolipoproteins are involved in the transport and redistribution of lipids and determine the cellular uptake of lipoproteins by interaction with various cell surface lipoprotein receptors. Apolipoproteins act as a scaffold for the assembly of lipoproteins and contribute to the structural stability of the particles. Some apolipoproteins act as cofactors and regulate the activity of enzymes that modify lipoproteins in the circulation (Mahley R.W., Innerarity, T.L. et al. 1984). For an overview, see Table 1.1.
The apolipoproteins found in the plasma can be classified into two types: the non-exchangeable and the exchangeable or soluble apolipoproteins. Apolipoprotein B (ApoB-100 and apoB-48) is a very large and water-insoluble protein belonging to the group of non-exchangeable proteins. ApoB-100 and apoB-48 are the principal protein components of LDL, VLDL, and chylomicrons (CM), and after synthesis and assembly they enter the circulation, where the lipoproteins are processed and cleared from the circulation via specific receptors (Jonas, A. and Philips, M.C. 2008). In contrast, the exchangeable apolipoproteins including apolipoproteins A, -CI-III, and -E, are secreted in lipid-free or lipid-poor forms and can acquire lipids in the circulation (Pownall, H.J. and Gotto, A.M. 1992). They are soluble, amphihilic proteins that have much smaller molecular masses compared to apoB, thus facilitating the transfer and exchange among various lipoprotein particles in the plasma (Saito, H., Lund-Katz, S. et al. 2004).

Apolipoprotein	Chromosomal localization	MW (kDa)	Primary source	Lipoproteins	Function
ApoA-I	11	28	liver, intestine	CM, HDL	reverse cholesterol transport, LCAT activator
ApoA-II	1	17	liver	HDL	potential Inhibitor of LCAT
ApoA-IV	11	45	liver, intestine	CM	activator of LCAT
ApoA-V	11	40	liver	CM, VLDL, HDL	regulator of plasma TG-level
ApoB-48	2	242	intestine	CM	TG transport
ApoB-100	2	512	liver	VLDL, IDL, LDL	TG-and cholesterol transport, interaction with LDLR
ApoC-I	19	7	liver	CM, VLDL, HDL	activation of LCAT
ApoC-II	19	9	liver	CM, VLDL, HDL	activation of LPL
ApoC-III	11	9	liver	CM, VLDL, HDL	inhibitor of LDL
ApoE	19	34	liver, brain	CM, VLDL, HDL	reverse cholesterol transport, interaction with LDLR + other family members

Table 1.1 Summary of human apolipoproteins and their functional roles

(Jonas, A. and Philips, M.C. In *Biochemistry of Lipids, Lipoproteins and Membranes*, (D.E. Vance and J.E. Vance), 5th Edition, 2008)

1.3 APOLIPOPROTEIN E (APOE)

1.3.1 Gene Organization, Synthesis and Expression Profile

Apolipoprotein E was discovered in the early 1970's as a protein of triglyceride-rich lipoproteins in the plasma (Utermann, G. 1975). Immediately it became evident that it is an important determinant in lipid metabolism and cholesterol homeostasis. The encoding gene has been mapped to the human chromosome 19 (19p13.2), where it is located at the 5' end of the gene cluster with apoCI, apoCII and apoCIV. The apoE gene contains four exons separated by three introns and specifies for a secretory protein that follows the classical pathway for secretory protein synthesis and release. Its complete amino acid sequence was elucidated by Rall et al. 1982 (Rall, S.C., Weisgraber, K.H. et al. 1982) and revealed apoE as a polypeptide of 299 amino acids with a molecular mass of 34 kDa. The primary translation product is 317 amino acids long and contains an 18 amino acid signal peptide that directs the nascent polypeptide to the endoplasmatic reticulum (ER). The protein is then transported to

the Golgi compartment, where it undergoes O-linked glycosylation with the addition of carbohydrate chains containing sialic acid prior to secretion (reviewed by Greenow, K., Pearce, N.J. et al. 2005).
Unlike other apolipoproteins, apoE is synthesized by various tissues including kidney, spleen, lung, ovaries, adrenals, skin, and muscle, but its highest concentration can be found in the liver and the brain (Elshourbagy, N.A., Liao, W.S. et al. 1985). Belonging to the family of exchangeable apolipoproteins, apoE distributes in the circulation and associates with chylomicrons, chylomicron remnants, VLDL, and a subset of HDL lipoproteins.

1.3.2 Structural Properties

Structural analyses have revealed that apoE is composed of two structurally and functionally independent domains, a 22 kDa N- terminal domain (residues 1-191) and a 10 kDa C-terminal domain (residues 216-299) linked by a protease-sensitive hinge region (figure 1.2). These two domains have been characterized to fold independently and to account for distinct functions: While the N-terminal domain is responsible for low density lipoprotein (LDL) receptor binding, the C-terminal domain contains the major lipid-binding region and is responsible for association with lipoproteins.
The amino-terminal domain displays an ordered structure forming a bundle of 4 elongated α-helices, in which the helices are arranged in an antiparallel manner with their hydrophobic faces oriented towards the center of the bundle. This molecular architecture provides the structural basis for the considerable solubility of this protein in its lipid-free state in the aqueous phase.

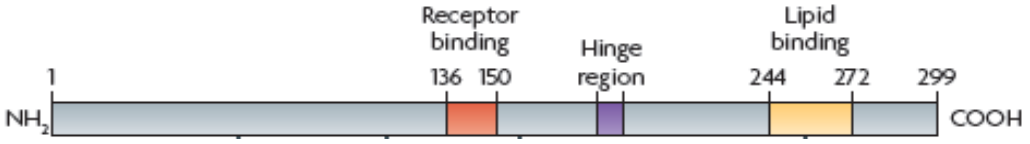

Figure 1.2 Schematic overview of human apoE

The 299-amino acid apolipoprotein E is composed of two independently folded domains: an N-terminal domain (residues 1-191) including the receptor-binding region and a C-terminal domain (residues 216-299) that contains the major lipid-binding domain. The two domains are linked by a protease-sensitive hinge region. (modified from Bu, G. 2009)

A cluster of basic amino acids between residues 136 and 150, located in the fourth helix of the N-terminal domain globular helix bundle structure has been elucidated to mediate receptor binding. The positively charged lysine- and arginine-residues have been implicated in the interaction with the negatively charged residues within the LDL receptor. ApoE has two heparin-binding sites (residues 129–169, 202–243) mediating the high affinity binding to heparan-sulphate proteoglycans (HSPG) (Weisgraber, K.H., Rall, S.C. et al. 1986).

In contrast to the N-terminus, the structural organization of the C- terminal domain of apoE is only poorly defined. This domain is predicted to be arranged in three amphiphatic α-helical structures featuring properties typical of other soluble and exchangeable apolipoproteins for switching reversibly between a lipoprotein-bound and a lipid-free state. In the absence of lipids, apoE exists as a tetramer, and this tetramerization is thought to be mediated by the C-terminal domain. Mutational analysis revealed the highly hydrophobic residues 267-299 to be responsible for the self-association of apoE (Westerlund, J.A. and Weisgraber, K.H. 1993). The lipid binding region ranges from residues 244-272 and is responsible for the association apoE with lipids, which is required for its high affinity binding to the LDL receptor.

Upon lipid binding, apolipoprotein E adopts a different, favourable conformation for receptor-binding by exposing the hydrophobic faces of its amphiphatic helices for interaction with lipid molecules. A number of studies indicated that apoE displays a high conformational heterogeneity after complexation with lipoproteins of varying size and shape. Therefore, the receptor binding affinity of apoE-containing lipoproteins depends on particle size, lipid composition, and the presence of other apolipoproteins (reviewed by Hatters, D.M., Peters-Libeu, C.A. et al. 2006).

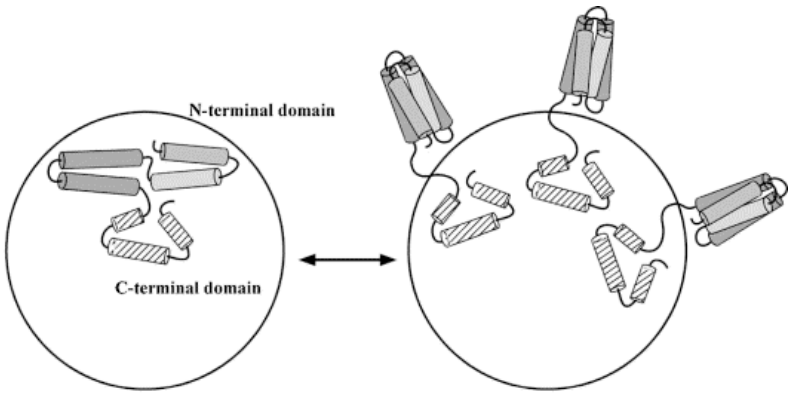

Figure 1.3 Model of two possible conformations of lipid-associated apoE

The initial binding step of apoE to lipid particles occurs through amphiphatic α-helices in the C-terminal domain or the N-terminal helix bundle (left). The second conformation is achieved by the opening of the helix bundle resulting in a structure, which allows receptor interactions. (Saito, H., Lund-Katz, S. et al. 2004).

ApoE displays two distinct lipid-bound states with the N-terminal four-helix bundle in either open, receptor-active or closed, receptor-inactive conformations (figure 1.3). Recently it has been sugggested that apoE-lipid binding involves a two-step mechanism, in which the initial binding occurs through amphipatic α-helices in either the C- terminal domain or N-terminal helix bundle. In the second step, the N-terminal domain undergoes a conformational opening, converting hydrophobic helix-helix interactions to helix-lipid interactions and resulting in a receptor-accessible structure. In this confirmation the positive electrostatic potential in the receptor-binding region is increased, thus enhancing the interaction with acidic elements of the LDLR (Saito, H., Lund-Katz, S. et al. 2004).

This model is compatible with the nature of exchangeable apolipoproteins, as the initial binding is readily reversible and allows rapid dissociation and exchange. Since the LDLR does not recognize apoE in its closed conformation, it is expected that only molecules that achieve the above described step2-binding will act as effective ligands for the LDLR (Nguyen, D., Lund-Katz, S. et al. 2009).

1.3.3 Physiological Roles

ApoE and Plasma Lipoprotein Metabolism:

ApoE is involved in many steps of lipoprotein homeostasis. Its primary role is to transport and deliver lipids from one tissue or cell type to another through the uptake via receptor-mediated endocytosis. ApoE is synthesized primarily in the liver as a component of VLDL, and as an exchangeable apolipoprotein it redistributes to enrich other lipoprotein particles. Serving the transport of dietary triglycerides and cholesterol, chylomicrons are synthesized by the small intestine, a tissue that does not produce apoE. During circulation through the capillaries both lipoproteins, VLDL and chylomicrons are lipolyzed on the surface of endothelial cells by the action of lipoprotein lipase (LPL) releasing fatty acids serving as energy source. In the course of this process a spectrum of particles of decreasing size and TG-content is produced including VLDL- remnants, CM- remnants and intermediate density lipoprotein particles. During lipolysis these remnants become enriched in apoE. Acting as a ligand for the LDL receptor and its family members, apoE promotes the uptake of cholesterol- enriched plasma lipoproteins by the liver, especially of VLDL and CM-remnants (Mahley, R.W. and Rall, S.C. 2000).

ApoE was assigned an important atheroprotective function via facilitating the reverse cholesterol transport by allowing cholesteryl-ester (CE)-rich core expansion in HDL. ApoE can be found on certain HDL subclasses, either as sole apolipoprotein or in combination with apoA-I. While apoA-I - containing HDL can accommodate only a limited amount of cholesteryl-esters (CE) in its core, the size and the CE- content of apoE- containing HDL can be significantly increased. This core expansion is attributable to the different interaction of apoE and apoA-I with lipids (Mahley, R.W, Huang, Y. et al. 2006). Since apoE is an effective ligand for LDLR, these apoE-enriched HDLs can deliver cholesterol from the peripheral cells to the liver via hepatic LDL receptors.

In addition to its ligand function, apoE can affect other aspects of lipoprotein metabolism. High levels of apoE restrict VLDL lipolysis by displacing the lipoprotein lipase activator protein CII from the particles. ApoE may also influence the activity of enzymes involved in lipoprotein metabolism such as hepatic lipase (HL), cholesteryl ester transfer protein (CETP) and lecithin-cholesterin-acyltransferase (LCAT) (reviewed by Getz, G.S. and Reardon, C.A. 2009). Independent of its role in lipoprotein clearance, apoE plays an important regulatory role in the assembly and secretion of VLDL by the liver in that overexpression and accumulation of hepatic apoE cause hypertriglyceridemia by stimulating hepatic VLDL-triglyceride secretion (Mensenkamp, A.R., Havekes, L.M. et al. 2001).

ApoE in Neurobiology:

The largest extrahepatic source of apoE is the brain, where it acts as a principal lipid transport protein in the cereprospinal fluid. ApoE plays an important role in the central nervous system. Since Corder and colleagues (Corder, E.H., Saunders, A.M. et al. 1993) observed that apoE4, one of the three apoE isoforms, is the major susceptibility gene associated with 40-65% of cases of sporadic and familial Alzheimer's disease (AD), the basis for the role of apoE and its polymorphisms in neuronal function and AD has become the subject of intense research (Mahley, R.W. and Rall, S.C. 2000).
ApoE- containing lipoprotein particles are predominantly produced by astrocytes and to some extent by microglia (Pitas, R.E., Boyles, J.K. et al. 1987; Uchihara, T., Duyckaerts, C. et al. 1995) to deliver cholesterol and other essential lipids to neurons through members of the LDL receptor family.
According to current views, neuronal expression of apoE is likely induced during peripheral nerve injury due to various toxic stimuli including oxidative stress, trauma, and ischemia. ApoE is involved in the repair process by redistributing lipids to regenerate neurons and Schwann cells, additionally it modulates neurite extension and cytoskeletal function (Greenow, K., Pearce, N.J. et al. 2005).

ApoE and Immunoregulation:

Muliple studies indicate that apoE can modulate key elements of the immune response, either positively or negatively (Getz, G.S. and Reardon, C.A. 2009). ApoE $^{-/-}$ mice are more susceptible to bacterial infections and display an impaired immune system. There is increasing evidence that apoE has an effect on the susceptibility to parasitic and viral infections (Mahley,R.W. 2009). Several viruses bind to cell surface heparan sulfate proteoglycans (HSPGs) as an initial event of infection. It seems possible that the ability of apoE to prevent infection can be explained by the competition of apoE for HSPGs (Mahley, R.W. and Rall, S.C. 2000). In HIV- positive patients, apoE4 homozygosity was shown to accelerate progression to AIDS and to increase susceptibility to opportunistic infections (Mahley, R.W., Weisgraber, K.H. 2009). Further studies are underway to gain a better insight in the underlying molecular mechanism.

1.3.4 ApoE Polymorphisms and Pathophysiological Implications

Human apoE exists in 3 isoforms, E2, E3 and E4, which differ by single amino acid variations at positions 112 and 158 (Fig. 1.4 and Table 1.2). ApoE3, the most common form, contains cysteine at position 112 and arginine at position 158, whereas E2 contains cysteines and E4 arginines, respectively, at both sites. The most common phenotype in the human population is E3 with an allelic frequency of 78%, followed by E4 with 15% and E2 with 7 %. These single amino acid substitutions alter the protein's structure and influence its lipid association and receptor binding. All 3 isoforms adopt a four helix bundle motif, but there are subtle

differences in the side-chain conformations and salt-bridge arrangements of the isoforms that have profound effects on their structure and biophysical properties.

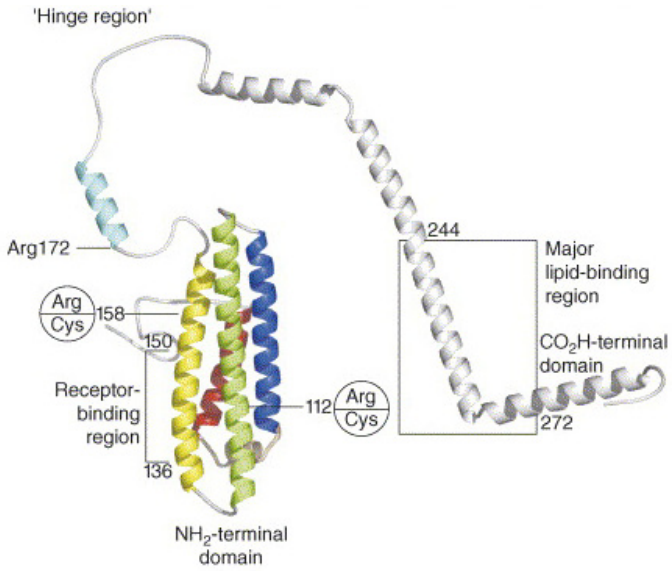

Figure 1.4 Model of the structure of lipid-free apoE

The N-terminal domain consists of a four helix-bundle (1, red; 2, blue; 3, green; 4, yellow). The LDLR-binding region resides on helix 4. The N-terminus also contains the polymorphic positions 112 and 158 that distinguish the three common isoforms. The C-terminal domain (gray) contains the main lipoprotein-binding elements. (Hatters, D.M., Peters-Libeu, C.A. et al. 2006)

The various isoforms were determined to have distinct lipid binding properties. ApoE2 and E3 preferentially bind to small, cholesterol-rich high density lipoproteins (HDL), whereas apoE4 prefers lower density lipoproteins, such as VLDL and LDL (Dong, L-M. and Weisgraber, K.H. 1996). This difference in lipoprotein binding appears to be determined by interactions of the C- and the N-terminal domains. In apoE4, these domains interact with each other in a unique manner unlike they do in no other isoform. The arginine at position 112 causes a rearrangement in the side chain of arginine at position 61 within the N-terminal domain of apoE4. This shift in the side chain orientation allows Arg61 to interact with an acidic residue (Glu255) within the C-terminus. This domain interaction mediates a closer contact between the N-and C-terminal domains, which does not occur to the same extent in apoE2 or apoE3, where the different conformation of the Arg61 side chain is less accessible for salt bridge formation. (Mahley, R.W. and Rall, S.C. 2000)

Several studies investigating the chemical and thermal stability of apoE revealed isoform-specific effects. ApoE isoforms differ considerably in the stability of the N- terminal domain with apoE2 showing the highest, apoE3 intermediate, and E4 only low resistance against chemical and thermal denaturation. This observation indicates that replacing cysteine- by arginine residues results in a cumulative decrease in the stability of the helical bundle (Acharya, P., Segall, M.L. et al. 2002).

Additional isoform-specific variations of apoE concerning its own expression and that of plasma lipids were assessed. ApoE2 is associated with decreased levels of apoB and cholesterol and increased levels of apoE and triglycerides (Mahley, R.W. and Rall, S.C. 2000). On the other hand, apoE4 tends to be associated with increased apoB and cholesterol levels and decreased apoE levels. While the variation in cholesterol levels can be attributed to the different affinities of the isoforms for the LDLR, the mechanisms causally related to these changes are currently poorly understood (Mahley, R.W. and Rall, S.C. 2000). For a summary of the main properties of the apoE isoforms, see Table 1.2.

Isoform	Allelic frequency	Amino acid variation		LDL-receptor affinity	Lipoprotein-binding preference	Associated disorders
		112	158			
ApoE2	7 %	Cys	Cys	Low	HDL	Type III hyperlipoproteinemia
ApoE3	78 %	Cys	Arg	High	HDL	unknown
ApoE4	15 %	Arg	Arg	High	VLDL, LDL	Alzheimer's disease

Table 1.2 Prevalence of human apoE isoforms and their key differences
(modified from Hatters, D.M., Peters-Libeu, C.A. et al. 2006)

A number of studies have revealed pathophysiological implications attributed to apoE polymorphism. In apoE2 the cysteine to arginine substitution leads to the elimination of the salt bridge between Arg158 and Asp154, but simultaneously a new salt bridge between Arg150 and Asp154 is formed. This salt bridge rearrangement lowers the positive electrostatic potential of the receptor-binding region, reducing the ability of apoE2 to interact effectively with the LDL receptor. The receptor binding activity of apoE2 was observed to be 50- to 100- times weaker compared to apoE3 and apoE4 (Schneider ,W.J., Kovanen, P.T. et al. 1981). As a result, apoE2 is associated with type III hyperlipoproteinemia, a lipid disorder characterized by increased plasma levels of cholesterol and triglycerides, xanthomas and premature cardiovascular disease (Mahley, R.W. and Rall, S.C. 2001).
Another pronounced pathological effect attributable to apoE polymorphism is the association of apoE4 with neurodegenerative diseases, particularly with Alzheimer's disease (AD). As a major risk factor for AD it not only increases the degree of risk, but also lowers the age of onset of AD (Saunders, A.M., Strittmatter, W.J. et al. 1993). Domain interaction and reduced stability are suggested to contribute to the association of apoE4 with disease (Dong L.-M. and Weisgraber, K.H. 1996; Morrow, J.A. Segall, M.L. et al. 2000), but the detailed mechanism is yet unclear.

1.3.5 Apo E Receptors

ApoE fulfills its function in lipid transport and delivery mainly by two receptor pathways. The first is the well characterized LDL receptor pathway in both hepatic and extrahepatic cells (see chapter 1.4.1). Via this pathway, the LDLR mediates the removal of LDL and remnant

lipoproteins from the circulation by binding to apoB100- and apoE-containing particles. Since apoE acts as ligand for all members of the LDLR family, it was anticipated that apoE plays a major role in the regulation of plasma cholesterol levels both in the plasma and in the central nervous system (Innerarity, T.L. and Mahley, R.W. 1978).

However, the finding that the absence of normal LDLR activity led to the accumulation of LDL, but not of apoE-enriched remnants pointed to an additional mechanism for the hepatic clearance of these remnant particles independent of the LDLR (Rubinsztein, D.C., Cohen, J.C. et al. 1990). This function has been attributed to a receptor with a structural similarity to the LDLR molecule and was therefore termed LDL receptor-related protein 1 (LRP1) (Herz, J., Hamann, U. et al. 1988). This second, receptor pathway is proposed to take place mainly in the liver in order to clear apoE- containing remnant lipoprotein particles that are generated in the circulation by lipolytic processing of intestinally derived CM and hepatically derived VLDL. As these remnants are highly atherogenic, the clearance of these particles from the plasma is of great importance.

Mahley and Huang have suggested cell-surface heparan sulfate proteoglycans (HSPGs) to be involved in an alternative pathway. (Mahley, R.W. and Huang, Y. 2007). They hypothesize that the lipoproteins are metabolized in a multistep process. First, remnant particles enter the perisinusoidal space (space of Disse) through the fenestrated endothelium, where they are captured by interaction with HSPG that are abundant in the matrix of the space of Disse and on the surface of hepatocytes. This binding is supposed to occur between the sulfate groups of proteoglycans and apolipoprotein E. Next, the remnants may undergo lipolytic processing by surface- or lipoprotein- bound lipases preparing the particles for uptake. Finally the processed particles reach the hepatocyte cell surface to bind to receptors, predominantly LRP1, that will internalize them. Several findings indicate that HSPG's on the hepatic cell surface are involved in the process of remnant lipoprotein uptake either by transferring the remnant particles to LRP1 or the LDLR or by directly serving as an acceptor for remnant lipoprotein uptake. To date in vivo HSPG-mediated clearance of remnant particles has not been demonstrated (MacArthur J.M., Bishop J.R. et al. 2007).

1.4 THE MAMMALIAN LDL RECEPTOR FAMILY

The LDL receptor family is an evolutionarily conserved group of cell- surface receptors. The last 30 years of research have led to detailed insights into function and structure of lipoprotein receptors, which is now one of the best understood aspects in receptor biology. Since the molecular characterization of the LDLR by Brown and Goldstein, an increasing number of related proteins have been discovered.
Characteristic hallmarks of LDLR family members are common structural elements, which show high degrees (70-100%) of sequence identity. Despite their structural homology, the different receptors are involved in a wide range of physiological functions in different tissues ranging from cargo transport function of a variety of ligands, ligand uptake through clathrin-mediated mechanisms, to participation as receptors and co-receptors in cellular signalling

events. The structural organization of all LDL receptor family members is depicted in figure 1.5. For detailed information on individual members of the LDLR supergene family see review by Schneider, W.J. In *Biochemistry of Lipids, Lipoproteins and Membranes*, (D.E. Vance and J.E.Vance), 5th Edition, 2008.

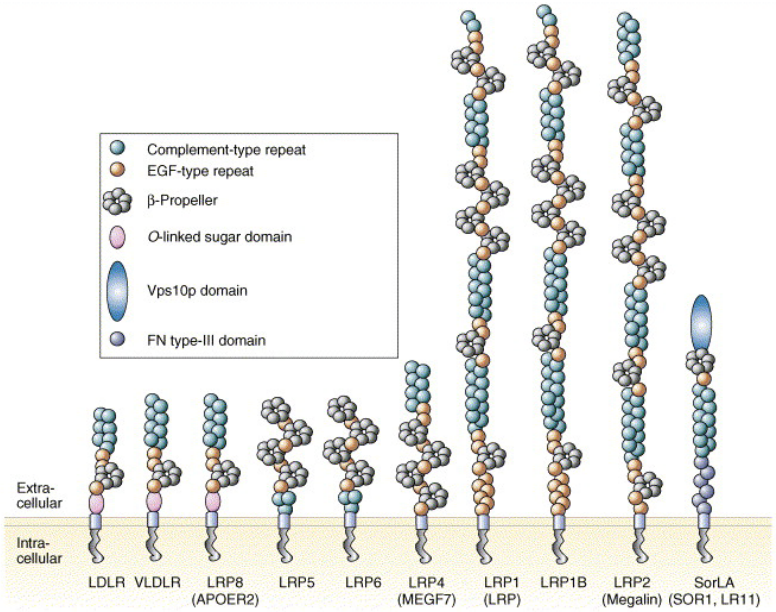

Figure 1.5 Domain structures of the members of the LDL receptor family

The LDLR family includes various cell-surface receptors with similarities in their structural organizations. Characteristic hallmarks are an extracellular domain (containing complement-type-, EGF-type repeats and O-linked sugar domains) and a short intracellular domain (harbouring the internalization sequence for endocytosis). Some family members also contain vacuolar protein-sorting 10 protein (Vps10p) domains and fibronectin (FN) type-III domains (SorLA). (Andersen, O. and Willnow, T. 2006).

1.4.1 The LDL receptor

1.4.1.1 Physiological Role

The LDL receptor was the first family member discovered; it plays a central role in systemic and cellular lipoprotein metabolism. Due to the pioneering work of Brown and Goldstein and their colleagues (Goldstein, J.L., Brown, M.S. et al. 1985) the role and mode of action of the LDLR in lipoprotein metabolism has been elucidated in great detail. The LDLR is an important determinant in the homeostasis of cholesterol by binding apolipoprotein E- and apolipoprotein B-100- containing lipoprotein particles and mediating their cellular uptake by endocytosis. The uptake of lipoproteins by the LDLR serves a dual role in lipid homeostasis. It delivers essential lipids required for the maintenance of cellular functions and it regulates the concentration of cholesterol-rich lipoproteins in the circulation. The importance of these functions is indicated by the large number of mutations in the LDL receptor gene leading to a

syndrome called familial hypercholesterolemia (FH) (Goldstein, J.L. and Brown, M.S. 1974). Patients with FH exhibit an increase in levels of circulating lipoproteins: on average, homozygous gene defects result in a four-fold and heterozygous defects in a two-fold elevation of mean plasma cholesterol levels. As a consequence, the hypercholesteremic FH patients suffer from premature atherosclerosis and coronary artery disease.

The uptake of cholesterol via receptor-mediated endocytosis of LDL is one of the best characterized mechanisms of macromolecular transport across the plasma membrane of eukaryotic cells (Goldstein, J.L., Brown, M.S. et al. 1985). This cellular mechanism is termed LDLR pathway and is depicted in figure 1.6.

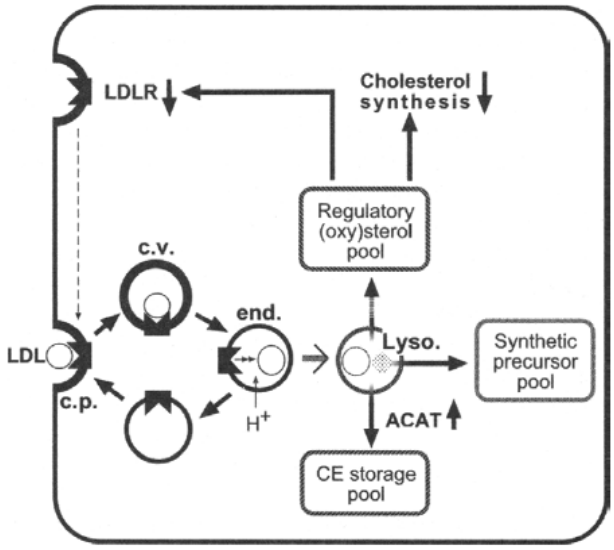

Figure 1.6 The LDL receptor pathway and regulation of cellular cholesterol homeostasis

LDL particles bind to the LDLR located in clathrin-coated pits (c.p.) and are internalized in coated-vesicles (c.v.). These vesicles become uncoated and acidified by protons (H^+), resulting in endosomes (end.). The low pH triggers the dissociation of the ligand –receptor complex: While the LDLR is recycled to the cell surface, LDLs are broken down in lysosomes (lyso.) releasing cholesterol via the hydrolysis of cholesteryl esters. This LDL-derived cholesterol is either subjected to the CE storage pool, the synthetic precursor pool or the regulatory oxysterol pool. For a detailed description see text. (Schneider, W.J. In Biochemistry of Lipids, Lipoproteins and Membranes, (D.E. Vance and J.E. Vance), 5th Edition, 2008)

The interaction of circulating lipoproteins with the LDL receptor occurs at the cell surface, where the receptor molecules are localized in so-called clathrin-coated pits. Clathrin is a protein that facilitates the formation of small vesicles within the cytoplasm and thus, supports the uptake of endocytic receptors. LDL particles bound to LDLR are internalized in clathrin-coated vesicles, which are acidified by protons resulting in endosomes, where the bound lipoprotein dissociates from the receptor due to the low pH. The receptor is then

returned to the cell surface in a process called receptor recycling. The LDL particle is targeted to lysosomes, where apolipoproteins are broken down into amino acids, lipids and cholesterol. The LDL- derived cholesterol has three main fates: (a) for further storage it is reconverted to cholesteyl esters by stimulating of acyl-CoA:cholesterol acyltransferase (ACAT), (b) it is used as biosynthetic precursor for bile acids, steroid hormones, and membrane synthesis, and (c) it serves several regulatory functions (mainly after conversion to oxysterols).

1.4.1.2 Regulation of the LDLR

The expression of the LDLR is controlled by a feed-back control mechanism that senses intracellular cholesterol concentrations. The sterol-regulated transcription factors SREBP (sterol response element binding proteins) play a key role in this regulatory mechanism (Brown, M.S. and Goldstein, J.L. 1999). Unlike other transcription factors, the SREBPs are synthesized as membrane-bound proteins attached to the ER. When cholesterol levels are low, the membrane-bound precursors of SREBPs are transported from the ER to the Golgi, where upon a two-step proteolytic cleavage, SREBPs are released from the Golgi membrane and translocated to the nucleus. There they bind to sterol regulatory elements in promoters and activate the transcription of various genes including those encoding the LDL receptor and the key enzymes for cellular cholesterol biosynthesis, HMG-CoA synthase and -reductase. When cholesterol levels are elevated, the transport of SREBPs to the Golgi complex and proteolytic release of active transcription factors are prevented. Consequently, the transcription of the target genes declines, and the cell produces less cholesterol and LDL receptor molecules, thereby preventing cellular cholesterol overload (Horton, J.D. Goldstein, J.L. et al. 2002).

1.4.1.3 Structural Organisation

The LDL receptor is a highly conserved membrane glycoprotein of 839 residues encoded by a gene of 45 kb located on the human chromosome 19 (19p13.1-13.3).
The mature receptor is a type I transmembrane protein consisting of five functionally and structurally defined modules (see Fig. 1.7). In order of appearance from the amino-terminus, these domains are characterized as follows:

1) **The ligand-binding domain:** This domain consists of seven cysteine-rich repeats (R1-R7), each about 40 amino acids in length. These modules are referred to as LDL receptor type A (LA) repeats, or occasionally, complement-type A repeats. Nuclear magnetic resonance (NMR) and crystal structures of the distinct repeats revealed a two-loop conformation established by three disulfide bonds, characteristically between cysteine residues I+III, II+V and IV+VI within a LA repeat (reviewed by Gent, J. and Braakman, I. 2004). In the C-terminal part of each LA-repeat, conserved clusters of negatively charged residues (with

the signature tripeptide Ser-Asp-Glu, SDE) are found. These clusters are thought to interact with corresponding clusters of positively charged residues in the receptor binding regions of apoB-100 and apoE. The seven adjacent LA-repeats are connected by short flexible linkers contributing to the flexibility of the LDLR and enabling the LDLR to accommodate its shape to bind a variety of heterogeneous lipoprotein particles (of different particle shape and diameter). In general, the individual ligand-binding modules are linked by 4-5 residues with exception of the LA-repeats 4 and 5, which are connected by a 12- residue linker.

2) **EGF (epidermal growth factor) precursor homology domain:** The EGFP homology domain lies next to the ligand-binding site and is characterized by the high degree of homology with the precursor to epidermal growth factor (EGFP).The domain consists of two EGF-like modules (EGF-A and EGF-B), followed by a 6-bladed β-propeller domain with a YWTD consensus motif at the core, and a third EGF-like repeat (EGF-C). This part of the LDLR is implicated in the acid-dependent dissociation of the ligands in endosomes. The acidic environment triggers a conformational change in the LDLR receptor leading to a closed structure between the β-propeller and the binding repeats LA4 and LA5, thereby releasing the ligand (Rudenko, G., Henry, L. et al. 2002).

3) **O-linked sugar domain:** The O-linked sugar domain, located just outside the plasma membrane, is 58 amino acids long and enriched in serine and threonine residues. Most of the 18 hydroxylated amino acid side chains are glycosylated. The O-linked oligosaccharides undergo posttranslational elongation in the course of receptor maturation. Although the structure of this region is very well known, its specific function remains somewhat unclear (Davis, C.G., Elhammer, A. et al. 1986).

4) **Transmembrane domain:** A hydrophobic domain of 24 amino acids anchors the LDLR in the lipid bilayer. The sequence of this domain is the least conserved of all receptor domains in seven mammalian species. This finding speaks against a unique function other than anchoring. Deletion of this domain, both in naturally occurring mutations, for instance, the internalization-defective receptor form – FH 274 (Lehrman, M.A., Schneider, W.J. et al. 1985) or by generated by site-directed-mutagenesis, leads to secretion of truncated receptors from the cells.

5) **Cytosolic domain:** The cytoplasmic domain of the LDLR constitutes a short stretch of 50 amino acid residues and is involved in the targeting of the LDLR to clathrin-coated pits. Mutational analysis revealed a dominant role for the NPxY motif (Asn-Pro-Xxx-Tyr, where x denotes any amino acid; in the LDLR and most frequently it is valine) as internalization signal recruiting a variety of adaptor- and scaffolding proteins. Recently, the cytoplasmic domains of the LDLR and its relatives have come into renewed focus due to their involvement in signalling pathways (Willnow, T.E. 1999).

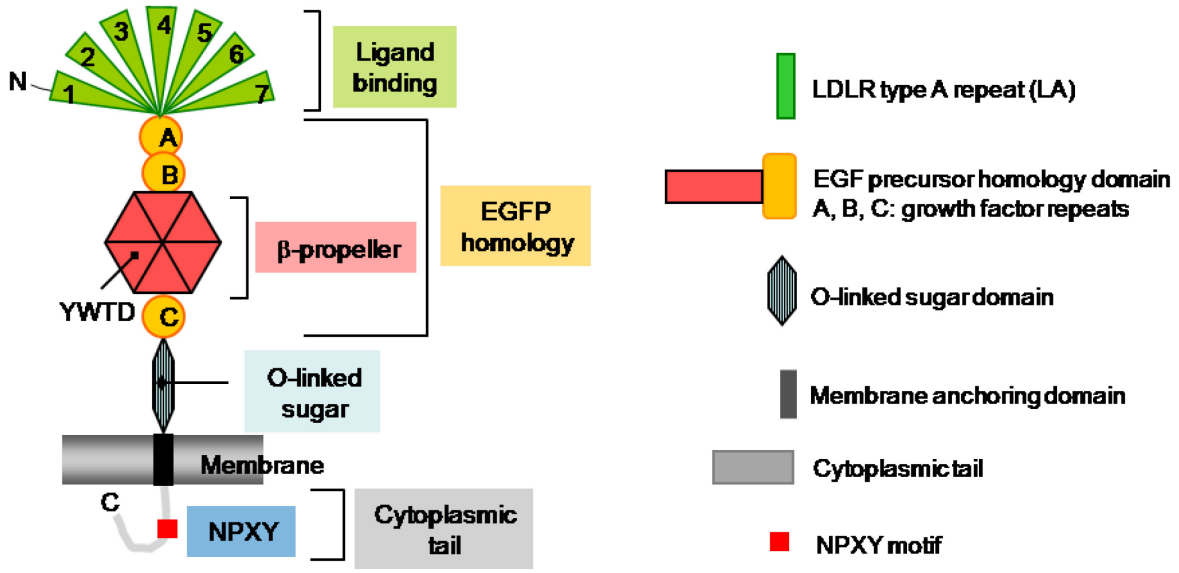

Figure 1.7 Schematic model of the LDL receptor

The domains of the LDL receptor from the amino-terminus (N) to the carboxy-terminus (C) are: a ligand-binding domain consisting of 7 cysteine-rich repeats, the Epidermal Growth Factor-Precursor homology domain (EGFP), the O-linked sugar domain, a short transmembrane domain and a cytoplasmic tail containing the internalization signal for endocytosis (NPXY). A more detailed description is given in the text.

1.4.1.4 Biogenesis and Folding of the LDLR

The LDLR is synthesized by the ribosomal machinery and the precursor of the mature receptor is postranslationally folded in the endoplasmatic reticulum (ER). Upon transport to the Golgi network, the LDLR undergoes extensive O-linked glycosylation giving rise to the mature 160 kDa form of the LDLR, which is finally transported to the cell surface. Because of the modular structure and the observation that individual modules can fold into their native structures, both in vivo and in vitro, folding of the LDL-receptor domains has long been considered to occur independently and sequentially, starting with the most N-terminal cysteine-rich repeat. However, it was revealed that folding of the LDLR in the ER occurs in a non- vectorial manner (Gent, J. and Braakman, I., 2004; Jansens, A., van Duijn, E. et al. 2002). Early in the folding process, the newly synthesized polypeptide chains fold rapidly into compact structures by the formation of non-native disulfide bonds most likely between cysteines of different domains. This rapid collapse may prevent unfavorable aggregation and interaction with other cysteine-containing proteins or LDL receptor ligands. Continuing LDLR folding, these non- native disulfides are reshuffled, allowing expansion of the molecule. Finally, in the native conformation, disulfide bonds only exist between cysteine-residues within individual repeats. Studies investigating the requirements for formation of native disulfide bonds showed that calcium ions were required for proper folding. Highly conserved

acidic residues near the C- terminal end of repeat 5 (R5) were identified to stabilize the structure by participating in the incorporation of a calcium ion. (reviewed by Gent, J. and Braakman, I. 2004).

Chaperone-assisted folding in the endoplasmatic reticulum (ER)

Through its Ca^{2+}-rich environment and unique oxidizing potential, the endoplasmatic reticulum provides an optimized environment for folding, disulfide bond formation, maturation, and assembly of proteins. The proper folding of the LDLR is thought to be supported in the ER by the help of a variety of molecular chaperones and folding factors. These may include general chaperones like BiP, calnexin, calreticulin, as well as so-called private chaperones, which in the case of the LDL receptor family, is the receptor-associated protein (RAP). Other chaperones, e.g. Boca and Mesd, are implicated in the structural stabilization and facilitation of the correct folding of receptor proteins.

General chaperones

Numerous molecular chaperones function in the ER to generally ensure proper synthesis of proteins. This ER quality control system for newly synthesized proteins includes the retention of unfolded proteins in the ER, proteasome-mediated degradation of persistently misfolded proteins, the retrieval of misfolded proteins from later compartments of the secretory pathway back to the ER, and the rerouting of proteins from the Golgi apparatus to lysosomes.

The well known chaperone BiP (binding immunglobuline protein) is the most abundant ER-chaperone that interacts non-selectively with various substrates thereby providing support in protein folding. BiP was identified to bind transiently to the LDLR and to retain misfolded mutants in the ER (Jørgensen, M.M., Jensen, O.N. et al. 2000).

Calnexin and Calreticulin are lectin-like ER chaperones that interact specifically with monoglycosylated N- linked glycoproteins and play an important role in glycoprotein quality control. The oxidoreductases of the protein disulfide isomerase (PDI) family catalyze and monitor the correct formation of disulfide bonds, which is a major event in LDLR folding. Thus, the enzyme PDI was proposed to play a role in contributing to LDLR folding (reviewed by Gent, J. and Braakman, I. 2004).

Private Chaperones

The hallmark of private chaperones is that they provide support only for selected proteins or protein families. The receptor-associated protein (RAP) is a specialized chaperone that assists LDLR family members to fold correctly and traffic safely along the secretory pathway. It was initially discovered as a 39-kDa protein that was co-purified with LRP1 from human placenta (Bu, G. and Marzolo, M.P. 2000). Immunofluorescent studies demonstrated that RAP was primarily localized intracellularly with 70% in the endoplasmatic reticulum and 24% in Golgi compartments. This observation of an intracellularly active, ER-resident protein proposed RAP as a trafficking chaperone/escort protein for LRP1.

The important role of RAP was supported by studies in RAP-knockout mice, in which LRP1 was retained as aggregates in the ER. Simultaneously, the amount of functional LRP1 in the liver and brain was reduced significantly (Willnow, T.E., Rohlmann, A., Horton, J. et al. 1996).

It was revealed that RAP functions as a chaperone and escort protein in the course of LRP1 biogenesis. During the biosynthesis of the receptor, RAP associates with the receptor via the ligand-binding region and assists in the proper folding of the receptor. Following the subsequent trafficking, the function of RAP is to prevent the premature interaction of the receptor with ligands that are expressed in the same cellular compartment and thus might interfere with the proper folding of the receptor. Upon escorting the receptor to Golgi-compartments, RAP dissociates from the receptor as a result of the low pH and recycles back to the ER.

The role of RAP as a folding chaperone for lipoprotein receptors could be extended to other members of the LDLR family (including particularly LDLR, LRP2 and the VLDLR). Interestingly, RAP-assisted folding was observed to be differential among individual receptors. While RAP deficiency led to an impaired folding process of LRP1 and VLDLR, the folding of the LDLR remained largely unaffected. This finding was consistent with the lower affinity of RAP binding to the LDL receptor when compared with that to LRP1 and the VLDLR. It is possible that for an efficient folding process of the LDLR, other chaperones such as BiP or yet unidentified chaperones are able to compensate for RAP (Medh, J.D., Fry, G.L. et al. 1995). The finding that in RAP-deficient mice, the level of LDLR expression was unaffected in the liver (Willnow, T.E., Rohlmann, A., Horton, J.et al. 1996), but was downregulated in the brain (Veinbergs, I., Van Uden, E.et al. 2001) suggests that RAP-assisted LDLR folding has a different importance in various cells and tissues.

Recently an evolutionarily conserved ER- protein was identified, named Boca in Drosophila and Mesd (Mesoderm development) in the mouse, acting as chaperones for LDLR receptor relatives in these species (Culi, J. and Mann, R.S. 2003; Hsieh, J.C., Lee, L. et al. 2003). In Drosophila, it was shown that Boca is essential for membrane localization of Arrow, the homologue of LRP5 and LRP6 in vertebrates, and of Yolkless (yl), the receptor required for vitellogenin uptake into the Drosophila's oocyte (Schonbaum, C.P., Lee, S. et al. 1995; Schonbaum, C.P., Perrino, J.J., et al. 2000). Moreover, Boca/Mesd have been proposed as chaperones that are required for the correct assembly and folding of the β-propeller domain and of the EGF precursor domain of LRs (Culi, J., Springer, T.A. et al. 2004; Hsieh, J.C., Lee, L. et al. 2003).

1.4.1.5 Ligand Recognition by the LDL Receptor

Mutational analysis and binding studies revealed that the seven tandemly repeated LA modules are significant in the contribution to the binding of the LDLR to various ligands. Recombinant receptors containing only the seven LA repeats fully retain the ability to bind LDL, showing that the rest of the protein is not required for ligand-binding (Simmons, T., Newhouse, Y.M. et al. 1997). The primary sequences of repeats LA1-LA7 are ~ 40-50 % identical. As outlined above, each module contains three disulfide bonds and a calcium ion that is tightly coordinated by four conserved acidic residues at the C-terminal end of the module.

The LDL receptor binds two apolipoprotein ligands - apolipoprotein E and apolipoprotein B-100. The receptor exhibits high-affinity binding of LDL particles, which contain a single copy of apolipoprotein B-100 and of β-VLDL particles containing multiple copies of apolipoprotein E. Although these apolipoproteins do not share structural similarities, sequence elements rich in positively charged amino acids are present in both. Together with the fact that highly conserved acidic residues within the LDLR are implicated in receptor binding, it has been postulated that the LDLR-ligand interaction occurs via electrostatic interactions between acidic residues in the receptor and basic residues within the ligand (Weisgraber, K.H. 1994).
Mutational analysis of the LDL receptor provided evidence for a functional difference between distinct LA repeats (Esser, V., Limbird, L.E. et al. 1988). These investigations suggested that LA1 is not required for binding of either LDL or β-VLDL particles, while LA repeats 2+3 and LA 6+7 as well as the EGF-A repeat are required for maximal binding of LDL, but not of β-VLDL. LA repeat 5 was found to be crucial for the binding of both ligands. Further investigations established that although LA5 is required for association with apoE- and apoB-100 containing ligands, LA5 alone is not sufficient to mediate binding. However, the pair LA4 and LA5 was determined as the minimal receptor module retaining the ability to bind apoE- containing particles (Fisher, C., Abdul-Aziz, D. et al. 2004). Recent investigations by Yamamoto et al. provide support for this finding by revealing that for high affinity binding, the LA5 module must also exist in the correct context with respect to the other LA repeats within the ligand-binding region (Yamamoto, T. and Ryan, R.O. 2009).
Moreover, the importance of LA5 is underscored by a large number of mutations localized to this repeat that lead to FH. In general, the LA5 repeat seems to be particularly important for the binding and release of LDLR ligands. This repeat contains several amino acids that are not present at the same location in any of the other repeats. One of the unique attributes is that the cluster of serine-aspartatic acids-glutamic acid (SDE) is followed by an additional glutamate, creating a sequence of 3 negatively charged amino acids, whereas all the other repeats have only two (Russell, D.W., Brown, M.S. et al. 1989). It may be possible that this extra negatively charged residue contributes to the specific function of LA5.

Recently, another ligand that binds to the extracellular domain of the LDL receptor was identified, termed proprotein convertase subtilisin-like kexin type 9 (PCSK9) (Abifadel, M., Varret, M.et al. 2003). Several naturally occurring mutations in PCSK9 were observed, which lead either to a gain or loss of protein function. Loss of function mutations are associated with hypocholesterolemia and protect from heart disease, while gain of function mutations in PCSK9 lead to autosomal dominant hypercholesterolemia (ADH), a rare form of familial hypercholesterolemia (FH).
The property of PCSK9 is based on the sequence-specific interaction with the EGFP repeat A (EGF-A) of the LDLR. As a secreted factor, PCSK9 interacts with the LDLR followed by the subsequent internalization of the PCSK9-receptor complex into the endosome. The binding of PCSK9 apparently interferes with the acid-dependent conformational change essential for receptor recycling. Thus, the PCSK9-bound LDLR is targeted to lysosomal degradation rather than recycling to the plasma membrane, which in turn results in decreased amounts of LDLR on the cell surface accompanied by elevated LDL-cholesterol levels.
The precise mechanism of PCSK9 action is not fully understood yet, and is currently under extensive investigation. Since PCSK9 is proposed to be a new determinant in cholesterol

homeostasis, it constitutes a promising target for the treatment of hypercholesterolemia and atherosclerosis (reviewed by Lambert, G., Charlton, F. et al. 2009).

1.5 THE CHICKEN LDL RECEPTOR FAMILY

To date, various avian LDL receptor relatives homologous to members of the mammalian LDLR family have been discovered (reviewed by Schneider, W.J. 2009). Members of the LDL receptor gene family play crucial roles in chicken embryo development, but are also important in other physiologic processes in the avian species.
In oviparous species, the developing embryo is absolutely dependent on nutrients stored in the egg yolk. The main components of the yolk are vitellogenin (VTG) and VLDL, which are produced in and secreted from the liver under the control of estrogen (Tata, J.R. 1986). These two yolk precursors are taken up into the growing oocyte by means of endocytosis mediated by receptors belonging to the LDLR family, most notably LR8, as described below.

1.5.1 The chicken VLDL Receptor (LR8)

A 95 kDa protein in the oocyte plasma membrane was identified as the major receptor for deposition of yolk precursors in the laying hen (George, R., Barber, D.L. et al. 1987; Stifani, S., Barber, D.L. et al. 1990; Nimpf, J. and Schneider, W.J. 1991). Molecular characterization of this protein revealed that it is a member of the LDLR supergene family featuring all structural hallmark properties except for the presence of an additional (eighth) LA-repeat in the ligand-binding domain, from which it derives its name.
LR8 was initially anticipated to be a cell-type specific LDLR isoform, as antibodies against mammalian LDL receptors showed cross reactivity against the chicken oocyte receptor (Hayashi et al. 1989). Since the detection of the mammalian VLDL receptor (Takahashi et al. 1992), which is characterized by an eight-repeat ligand-binding domain and the determination of 84% sequence identity between LR8 and the VLDLR, LR8 was confirmed to be a homologue of the VLDLR rather than of the LDLR (Bujo, H., Hermann, M. et al. 1994).
The gene for LR8 is located on the chicken sex chromosome Z (Bujo, H., Hermann, M. et al. 1994). All VLDLRs exist in alternative forms generated by differential splicing (Bujo, H., Lindstedt, K.A. et al. 1995). Indeed, in chicken somatic cells, LR8 is expressed as the larger isoform, LR8+, which contains the O-linked sugar domain, whereas the oocyte expresses the LR8- isoform lacking the O-linked sugar domain. The physiological relevance for this dichotomy is not known; interestingly, the level of expression of LR8 in oocytes is 3 orders of magnitude larger than that of LR8+ in somatic cells. On the other hand, mammalian VLDL receptors are predominantly found in tissues with active metabolism of fatty acids, such as skeletal muscle, heart, adipose tissue, and brain; however, the exact range of functions performed by VLDLR in mammals is not known. In contrast to mammals, at least the function of the LR8- isoform in avian species is well established. It serves as a multifunctional receptor which transports lipoproteins and other components not for immediate catabolism, but for storage and later use as energy supply for the developing embryo. The functional

importance of LR8- is demonstrated by the infertile, non-laying chicken strain named R/O (Restricted Ovulator) carrying a single point mutation in the LR8 gene. Because of the failure to deposit VLDL and VTG into the oocyte, these chickens develop severe hyperlipidemia and atherosclerotic lesions (Nimpf, J,. Radosavljevic, M.J. et al. 1989; Bujo, H., Lindstedt, K.A. et al. 1995). The larger somatic cell variant LR8+ is likely to perform similar functions in mammals and oviparous species, as this isoform is expressed in the same tissues in these species. The tissue distribution may also simply suggest a role for the receptor in the transport and uptake of triglyceride-rich lipoproteins into metabolically active tissues (Hussain, M.M., Strickland, D.K. et al. 1999).

Chicken LR8 shows high affinity binding of the major yolk precursors VTG via the lipovitellin domain (Stifani, S., Barber, D.L. et al. 1990) and VLDL via apolipoprotein B-100 (Nimpf, J,. Radosavljevic, M.J. et al. 1989). In doing so, the affinity of VTG for the oocyte receptor appears to be higher than for apoB. In addition, LR8 was shown to interact with RAP, a molecular chaperone for members of the LDLR family (see chapter 1.4.1.4).
In 1990 Steyrer et al. showed that apolipoprotein E is an additional ligand for LR8. This is surprising and interesting, as apoE is an apolipoprotein of mammalian origin, which is absent in avian species (Steyrer, E., Barber D. L. et al. 1990). Since LR8 transports VTG in addition to VLDL, it has been suggested that VTG may represent a functional analogue of apoE. Its biochemical properties, the presence of regions with sequence homology to apoE and, most of all, binding to lipoprotein receptors that recognize apoE, strongly support this notion (Steyrer, E., Barber D. L. et al. 1990). According to the current knowledge, LR8 is hypothesized to be a product of ancient genes with the ability to interact with many, if not all, ligands of more recent members of the LDLR family.

1.5.2 The chicken LDL Receptor

Besides the massive transport of VLDL and VTG from the liver to the growing oocyte, there must be a way for somatic cells to independently control and maintain cholesterol homeostasis. In 1989, Hayashi et al. revealed that chicken fibroblasts express a different apoB- specific receptor than that identified in oocytes (Hayashi, K. Nimpf, J. et al. 1989). However, low levels of expression and sluggish regulation by sterols and statins were likely responsible for the delayed characterization of this receptor as bona-fide LDLR. Lately, Hummel et al. delineated this 130 kDa receptor as the first avian LDLR ortholog (Hummel, S., Lynn E. G. et al. 2003). It displays all hallmark properties of the LDLR family, which implicates that the overall structure of the LDLR has been conserved in evolution. Comparing the primary protein sequence between mammalian and chicken LDLR reveals only subtle differences: while the human LDLR contains a linker region with 12 residues in length between the LA repeat 4 and 5, this linker consists of 28 amino acids in the chicken LDLR. In addition, there are variations in the arrangement of negatively charged residues in the amino-terminal LA- repeat, in the sequence of the internalization signal, and also in the cytoplasmic receptor tail. LDL receptor protein expression was found to be restricted to adrenal glands, estrogen-induced liver, and somatic cells of the ovarian follicle. Unlike the function of the receptor in mammalian metabolism, in the laying hen the delivery of LDL-

derived cholesterol to tissues involved in steroid synthesis, such as adrenal and ovary appears to be particularly, or even exclusively, important.
Ligand-binding studies demonstrated that the chicken LDLR binds to apoB-100 containing lipoproteins particles (VLDL, LDL), while the receptor adopts a preference of LDL over VLDL particles. This is consistent with its main role in providing lipoprotein-derived cholesterol for steroid production in somatic cells (Hummel, S., Lynn E. G. et al. 2003). In comparison to the chicken oocyte-specific receptor, and with mammalian LDLRs, the avian somatic LDLR cannot bind apoE or vitellogenin, thereby constituting the only known LDLR that interacts with apoB but not apoE (Bujo, H., Hermann, M. et al. 1997).

1.6 AIMS OF THE THESIS

In laying hens, the targeting of hepatically synthesized lipoproteins into oocytes and somatic tissues occurs simultaneously. This dual task is performed by efficient lipoprotein transport systems including receptors of the LDLR supergene family. The laying hen expresses two different apoB-specific receptors, of which the oocyte receptor LR8 is responsible for the massive uptake of yolk precursors from the plasma into the growing oocyte, and the somatic cell receptor (LDLR) is part of a regulatory system for maintenance of systemic cholesterol homeostasis.
Although both avian receptors bind apoB-100- containing lipoproteins, LDL and VLDL, only the oocyte-specific receptor LR8 recognizes vitellogenin and the mammalian apoE. This indicates that in spite of the high homology between these two receptors, the ligand-binding domain of the ggLDL receptor seems to have a different structure that accounts for this unique binding property. The aim of this thesis is to reveal the structural determinants critical for the LDLR-apoE interaction and responsible for the differential ligand specificity of chicken LDLR and LR8.
To elucidate these critical elements, I introduced a series of mutations in putatively important domains within the ligand-binding region of the chicken LDL receptor. The mutated receptors were further characterized regarding ligand-binding properties with the objective to reveal structural determinants important for apoE binding.

2. MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Chemicals and general reagents commonly used in the experimental protocols of this thesis were obtained from: Amersham Biosciences, Amresco, Applichem, Bio-Rad, Calbiochem, Fermentas, Fluka, Gerbu, Invitrogen, Merck, Pierce, Roche, Roth, Sigma Aldrich, Star Lab and Zymed. All restriction enzymes were purchased from Fermentas Life Sciences.

2.2 BACTERIAL STRAINS AND VECTOR SYSTEMS

Bacterial Strain	XL10-Gold Ultracompetent Cells
Genotype	Tet^R Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173* *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB* $lacI^q$ZΔ*M15* Tn*10* (Tet^R) Amy Cam^R]
Reference	Stratagene

Table 2.1 Properties of E.coli XL10-Gold

Bacterial Strain	One Shot BL21 Star(DE3) Chemically Competent Cells
Genotype	*F- ompT hsdSB (rB^- mB^-) gal dcm rne131 (DE3)*
Reference	Invitrogen

Table 2.2 Properties of E.coli BL21

The commercial plasmid pMalc2x (New England Biolabs) was modified as described by Ronacher, B., Marlovits, T.C. et al. (2000) by the introduction of a novel multiple cloning site and a hexa- histidine tag provided at the C- terminal end of the resulting fusion protein.

Vector	pMalc2b
Size	6710 basepairs
Genotype	*lacI promoter, lacI coding sequence Multiple Cloning sites (NcoI- HindIII), His-Tag, MBP-Tag, M13 origin, pBR origin, ampicillin resistance*
Reference	pMalc2 from New England Biolabs

Table 2.3 Properties of pMalc2b expression vector

2.3 PRIMERS

The designed oligonucleotides were synthesized by MWG-Biotech AG.

Name	Sequence
LA7m_fwd	5' -GAC GGC AGC GAT GAG TCG CCC GAA ATG TGC CGC- 3'
LA7m_rev	5' -GCG GCA CTC CGT GCC GCC ATC GCA GAC CC- 3'
LA7m5_fwd	5’ -GAT GGG TCC GAC GAG GAA GGA TGC GAC CCC CCC- 3’
LA7m5-rev	5’ -GGG GGG GTC GCA TCC TTC CTC GTC GGA CCC ATC- 3’
LA7del_fwd	5’ -GAA CGG AGC CCG ACG TGC GGG GTC GTC CCC CGG CCC- 3’
LA7del_rev	5’ -GGG CCG GGG GAC GAC CCC GCA CGT CGG GCT CCG TTC- 3’

Table 2.4 Primers used for site-directed mutagenesis

2.4 ANIMALS

Mature Derco brown laying hens (30-40 weeks old) were purchased from Heindl Co. (Vienna, Austria) and maintained on layer’s mash with free access to water and feed under a daily light period of 16 hours. For estrogen treatment, a rooster was injected with 17β-OH estradiol (10 mg/kg body weight) into the breast muscle. The liver was taken from the estrogenized rooster 48 h after injection.

2.5 ANTIBODIES

Protein	Antibody	Dilution
ggLR8	Rabbit anti-LR8 antibody	Conc. 10 µg/µl
ggLDLR	Rabbit anti-LA1-7 antibody	1: 1000
ggLDLR-LA1-7-His	Mouse anti-His antibody	1: 2000
hsLDLR	Rabbit anti-hLDLR2 antibody	1: 1000
ApoE	Mouse anti-ApoE antibody	1: 5000
His-RAP-myc	Mouse anti-myc antibody	1: 100
ggApoB	Rabbit anti-ggApoB antibody	1: 1000
Rabbit IgG	Goat anti-rabbit IgG	1: 50 000
Mouse IgG	Goat anti-mouse IgG	1: 1500

Table 2.5 Antibodies

2.6 CELL CULTURE

2.6.1 Cell lines

Chinese hamster ovary cells (CHO^{wt}, CHO^{ApoE2}, CHO^{ApoE3}, CHO^{ApoE4})

The CHO cell line is originally derived from the ovary of the Chinese hamster. Recombinant cell lines were produced as described in (Tagalakis, A.D., Graham, I.R. et al. 2001) and were a kind gift of Prof. Nimpf's lab. CHO cells lacking the dihydrofolate reductase (*DHFR*) gene were stably transfected with apoE expression plasmids (p7055.apoE2/3/4) encoding the selectable *DHFR* gene

Cell monolayers were cultured in Iscove's MEM supplemented with 5% dialysed FCS, 2mM L-glutamine, 1% non-essential amino acids, and 100 U penicillin and 100 μg streptomycin per ml. Cells were incubated in a 37°C incubator in an atmosphere of 7,5% Co_2 in air.

Mouse fibroblast cell line M4 Δ806

The simian virus 40 large-T-antigen- immortalized mouse fibroblasts (M4 cell line) used in this study were kindly provided by Prof. D. Blaas. The cell line M4 Δ806 originates from murine wild-type fibroblasts featuring disruptions in genes coding for the LDL-receptor and LRP, though expressing a truncated form of the human LDL receptor, where a stop codon was introduced at amino acid 807 to disrupt the receptor internalization signal. M4 cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 5 % FCS, 2mM L- glutamine and 100 U penicillin and 100 μg streptomycin per ml. Cells were incubated in a 37°C incubator in an atmosphere of 7,5% CO_2 in air.

2.6.2 Solutions, Media and Supplements

PBS (1x)
137mM NaCl
0.27 mM KCl
10 mM Na_2HPO_4
1.7 mM KH_2PO_4

DMEM- Dulbecco's modified essential medium (Invitrogen)
IMDM- Iscove's Modified Dulbecco's Medium (Invitrogen)
Opti-MEM- Reduced Serum Medium with GlutaMAX (Invitrogen)

Supplements:
L-Glutamine (100x; Invitrogen)
FCS (Fetal Calf Serum) (Invitrogen)
Penicillin/Streptomycin (100x; Invitrogen)
Trypsin/EDTA solution (Invitrogen)

2.6.3 Growth of Cells

Cells were grown as monolayers in tissue culture-treated dishes (IWAKI; 100 mm). All solutions added to cell cultures were pre-warmed to 37°C. Cellular growth and viability were supervised by phase contrast microscopy. After growing to subconfluency, cells were harvested from the culture dish by trypsin treatment: Cell monolayers were washed two times with PBS, trypsinized with 1 ml trypsin/EDTA solution and incubated at 37°C for several minutes. After detachment of the cells, the trypsin was neutralized with an appropriate amount of growth medium and the cells were splitted to fresh culture dishes in adequate dilution. All operations were performed under sterile conditions in a biohazard hood.

2.6.4 Freezing and Thawing of Cells

Freezing cells

After washing two times with PBS cells were harvested by the addition of trypsin solution and the trypsinized cells were diluted in ~ 5 ml culture medium. The cell suspension was pelleted by centrifugation at RT for 3 min at 300 x g and the pellet was cryopreserved by resupending the cell pellet with 10% DMSO in FCS. The cells were transferred to cryo tubes (Nunc) and immediately cooled to -80°C for at least 5 days and for long term storage the vial was transferred to liquid nitrogen (-196°C).

Thawing cells

Frozen cells were thawed quickly by incubation in a 37°C water bath. To deplete the cryoprotecting agent dimethyl sulfoxid (DMSO) cells were washed with 10 ml culture medium and pelleted by centrifugation at RT for 3 min at 300 x g. The pellet was then resuspended in 1 ml medium and the cells were seeded in a fresh 100 mm culture dish containing 9 ml supplemented growth medium.

2.6.5 Harvesting of ApoE- containing Lipoprotein Particles secreted from CHO Cells

CHO cells secreting human apolipoprotein E isoforms (apoE2, apoE3, apoE4) were used to obtain human apolipoprotein E- containing lipoprotein particles. For harvesting apolipoprotein E isoforms, the CHO cells were cultured in 150 mm dishes until a confluency of 80% and then the growth media was switched to harvesting media (OptiMEM, Invitrogen). Collections were performed after a 12 h conditioning period and the isoform conditioned media were concentrated using Amicon- Ultra concentration columns with a 30.000 kDa molecular weight cut off. ApoE isoforms were stored at 4°C.

2.6.6 Quantification of ApoE by Enzyme-linked Immunosorbent Assay (ELISA)

The concentrations of the apoE2, E3, E4 isoforms were measured by a sandwich ELISA (ApoE4/Pan-ApoE ELISA kit, MBL). The assay procedure was carried out at room temperature. Media samples from CHO^{apoE2}, CHO^{apoE3} and CHO^{apoE4} were diluted 1:100 in

assay buffer and incubated for 1 h in provided microwell strips coated with polyclonal antibodies against human apoE. After washing 4 times with 100 µl wash solution, a peroxidise-conjugated anti-apoE antibody diluted 1:100 in conjugate diluent was added and incubated for 1 h. After another washing step, 100 µl peroxidase substrate (TMB/H_2O_2) were added and incubated for 30 min until the enzyme reaction was stopped by adding 50 µl stop solution (0.5 mol/l H_2SO_4). The optical density of each well was then measured at 450 nm using a microplate reader.
A reference calibrator provided from the manufacturer was used to construct a standard curve to which the values of the samples were compared and calibrated.

2.6.7 Solubilization of M4 Δ806 Cells and Preparation of Total Protein Extracts

Cell monolayers were washed three times with ice cold PBS, harvested with a cell scraper, resuspended in 10 ml PBS and centrifuged at 2000 x g for 5 min (Haraeus Megafuge). Then the cell pellet was solubilized by the addition of buffer B (400 µl /150 mm dish). Insoluble material was removed by ultracentrifugation at 300.000 x g (Beckman TLA 100.1 rotor) for 40 min at 4°C. Protein concentration of the total protein extracts were determined using the Bradford method and the cell extracts were quickly frozen in liquid N_2 and stored at -80°C until use.

Buffer B
200 mM Tris/maleate, pH 6.5
2 mM $CaCl_2$
1.4% Triton X-100
Complete protease inhibitor cocktail (Roche)

2.7 MOLECULAR BIOLOGICAL METHODS: DNA

2.7.1 Site-directed Mutagenesis of Recombinant Plasmid DNA

Mutant strand synthesis

Mutations were introduced using QuikChange II XL Kit (Stratagene), which is an efficient polymerase chain reaction (PCR)- based site-directed mutagenesis method. This procedure employs a double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation. A high-fidelity DNA polymerase catalyzes the extension of the mutagenic primers without primer displacement generating mutated plasmids. Following amplification the PCR- product is treated with Dpn I endonuclease triggering the digestion of the parental non-mutated DNA template, thereby selecting for mutation-containing DNA.
The chicken ligand-binding domain (ggLA1-7) has been previously cloned by Bajari et al. (Bajari, T.M., Strasser, V. et al. 2005). The vector containing the recombinant receptor fragment was used as a template for the amplification reaction. Complementary mutagenic primers were designed according to the manufacturer's instructions and are listed in table 2.4.

The reaction mixture was prepared in a sterile PCR-tube as stated below and kept on ice:

5 µl of 10x reaction buffer
x µl (10 ng) of ds DNA template
x µl (125 ng) of forward primer
x µl (125 ng) of reverse primer
1 µl of dNTP mix
3 µl of QuikSolution reagent
µl ddH_2O to a final volume of 50 µl

This mixture was subjected to thermal cycling in a Thermocycler T3000 (Biometra) using the stated PCR program.

	Cycles	Temperature	Time
Initial Denaturation	1	95°C	1 minute
Denaturation	18	95°C	50 seconds
Annealing		60°C	50 seconds
Extension		68°C	8 minutes
Final Extension	1	68°C	7 minutes

Table 2.6 Program used for site-directed mutagenesis

Dpn I Digestion of amplification products

The PCR- products were then digested for 1 h at 37°C by the addition of 1 µl of the Dpn I restriction enzyme (10U/µl).

Transformation of XL10-Gold ultracompetent cells

For transformation an aliquot (45 µl/reaction) was gently thawed on ice and mixed with 2 µl of β- mercaptoethanole. The cells were incubated on ice with gently mixing every 2 min. Then 2 µl of the Dpn I- treated DNA were transferred to the pre-treated ultracompetent cells and the reactions were incubated on ice for 30 min. The competent cells were heat shocked for 30 sec at 42°C and immediately chilled on ice for 2 min. The transformation mixture was diluted with pre-heated (42°C) S.O.C. medium and incubated for 1 h at 37°C with shaking at 225-250 rpm. The transformed bacteria were plated on LB- ampicillin agar plates and grown o/n at 37°C.
Single ampicillin-resistant colonies were picked and inoculated in 5 ml LB medium supplemented with 100 µg/ml ampicillin and grown o/n at 37°C. Plasmid DNA was isolated using the Fast Plasmid Mini Kit (Eppendorf).

LB medium
10g tryptone
5g yeast extract
10g NaCl
ddH_2O ad 1000 ml

LB-Amp agar plates (100 µg/ml ampicillin)
10g Tryptone
5g Yeast Extract
10g NaCl
15g Agar-Agar
ddH_2O ad 1000 ml

2.7.2 Mini-Preparation of Plasmid DNA

To extract and purify plasmid DNA from bacteria the FastPlasmid Mini kit from Eppendorf was used. The kit employs a rapid, non-organic alkaline lysis method using a single solution for cell resuspension, lysis, and DNA binding.
The Complete Lysis Solution has been stored at 4°C prior to use. The procedure was performed at room temperature using a 5418 Eppendorf table centrifuge.
1.5 ml overnight culture was harvested by centrifugation at 13.000 rpm for 1 min. The bacterial pellet was resuspended in 400 µl of ice-cold Complete Lysis Solution and mixed thoroughly by constant vortexing at the highest setting (30 sec). The lysate was allowed to incubate a RT for 3 min. Afterwards the lysate was transferred to Spin Column Assembly and centrifuged for 1 min at 13.000 rpm. Bound DNA was washed by adding 400 µl diluted Wash Buffer and the column was again centrifuged 1 min at 13.000 rpm. The filtrate was discarded and the Spin Column was placed back into the waste tube and centrifuged once again in order to dry the Spin Column. The Spin Column was transferred into a provided Collection Tube and 50 µl Elution buffer was added directly to the center of the Spin Column membrane. To elute Plasmid DNA another centrifugation step for 1 min at 13.000 rpm was done. The eluted DNA was used immediately for downstream applications or stored at -20°C.

2.7.3 Midi-Preparation of Plasmid DNA

For the preparation of large scale plasmid DNA the QIAfilter Plasmid Midi Kit (QIAGEN) was used. All centrifugation steps were performed in Sorvall GSA- and HB-6 rotors. Picking a single colony a starter culture of 3 ml LB medium (100 µg/ml ampicillin) was inoculated and incubated approximately 8 h at 37°C under vigorous shaking. 500 µl of the starter culture were diluted in 100 ml fresh selective LB medium and growth was allowed o/n at 37°C under vigorous shaking. The bacterial culture was harvested by centrifugation at 6.000 x g for 15 min at 4°C. After removing the supernatant, the cell pellet was resuspended and vortexed in 5 ml buffer P1 until the pellet was completely dissolved. After the addition of 5ml Lysis buffer P2 the components were mixed by gently inverting 4-6 times and incubated at RT for 5 min. Proteins and chromosomal DNA were precipitated by adding 5 ml ice-cold buffer P3 and gently inverting 4-6 times. Then the cell lysate was poured into the barrel of a QIAfilter Cartridge and incubated at RT for 10 min. During the incubation a QIAgen-tip 100 column was equilibrated by applying 5 ml of buffer QBT. The lysate was cleared by filtration through the QIAfilter Cartridge and transferred to the pre-equilibrated binding column. The lysate was allowed to enter the resin by gravity flow. After washing the column two times with 10 ml buffer QC, the DNA was eluted with 5ml buffer QF in a sterile 15 ml Falcon tube. To precipitate the DNA, the eluate was mixed with 3.5 ml isopropanol and centrifuged immediately at 15.000 × g for 30 min at 4℃. The DNA pellet was washed with 2 ml of 70% ethanol, air-dried and re-dissolved in an appropriate amount of sterile ddH_2O. DNA concentration was determined with the NanoDrop Spectrophotometer ND-1000 (PeqLab) by measuring the absorption at 260 nm.

Buffer P1 (Resuspension buffer)
50 mM Tris-HCl, pH 8.0
10 mM EDTA
100 μg/ml RNase A

Buffer P2 (Lysis buffer)
200 mM NaOH
1% SDS (w/v)

Buffer P3 (Neutralization buffer)
3.0 M Potassium Acetate, pH 5.5

Buffer QBT (Equilibration buffer)
750 mM NaCl
50 mM MOPS, pH 7.0
15% Isopropanol (v/v)
0.15% Triton X-100 (v/v)

Buffer QC (Wash buffer)
1 M NaCl
50 mM MOPS, pH 7.0
15% Isopropanol (v/v)

Buffer QF (Elution buffer)
1,25 M NaCl
50 mM Tris-HCl, pH 8.5
15% Isopropanol (v/v)

TE-buffer
10 mM Tris-HCl, pH 7.5
1 mM EDTA

2.7.4 DNA-Sequencing

In order to confirm the efficiency and accuracy of site-directed mutagenesis the recombinant plasmid DNAs were subjected to sequencing conducted by VBC- Genomics Bioscience Research GmbH, Vienna, Austria.

2.7.5 Transformation into Competent E. coli Cells

Plasmids were transformed into chemically competent E. coli BL21 cells for high-level protein expression. Transformation of recombinant plasmids derived from Plasmid Preparation was done using the following protocol: An aliquot (50 µl/tube) of chemically competent E. coli BL21 cells was thawed on ice and mixed carefully with 5 µl plasmid minipreparation diluted 1:50. After an incubation of 30 min on ice, the bacterial cells were heat shocked for 30 sec at 42°C and immediately placed on ice again. For regeneration, 250 µl of provided pre-warmed S.O.C. medium was added and the vial was incubated at 37°C for 1 h under gentle agitation. 50 µl and 100 µl of the transformation mix were plated onto pre-warmed selective LB-agar plates containing 100 µg/ml ampicillin and incubated at 37°C overnight. The transformants were screened for the insert-containing vector by restriction analysis and agarose gel electrophoresis.

2.7.6 Restriction Enzyme Digestion

For restriction analysis of recombinant plasmids derived by Plasmid preparation the following components were mixed and incubated for 1 h at 37°C:

3 µl	miniprep DNA
5 units	appropriate restriction enzymes (EcoRI, XbaI (10 U/µl))
1 µl	appropriate 10x restriction buffer
x µl	ddH_2O to a final volume of 10 µl

The appropriate restriction buffer was chosen according to recommendations of the manufacturer. The restriction digest was checked by agarose gel electrophoresis.

2.7.7 Agarose Gel Electrophoresis

Restricted plasmid DNA was analyzed by agarose gel electrophoresis using 1% (w/v) agarose gels containing 1 µg ethidium bromide per ml of gel. The DNA fragments were separated with a constant voltage of 100 V for 30 min. DNA samples were mixed with 5x DNA loading buffer and loaded onto the gel together with 8 µl of Generuler DNA Ladder Mix (Fermentas) serving as a size marker. 1x TAE buffer was used as electrophoresis running buffer.
To visualize size-fractionated DNA molecules the agarose gel was illuminated with UV- light.

<u>50x TAE Buffer</u>
2 M Tris-HCl, pH 8.0
1 M Acetic acid
0.1 M EDTA

<u>5x DNA loading buffer</u>
5 ml 100% Glycerin
2 ml 0.5 M EDTA
12 ml 50x TAE
3 ml ddH_2O
Bromphenol blue

<u>Ethidium bromide stock solution</u>
10 mg/ml in ddH_2O

2.8 MOLECULAR BIOCHEMICAL METHODS: PROTEIN

2.8.1 Preparation of Total Protein Extracts

All operations were performed at 4°C. For total protein extracts, chicken tissues (either fresh or frozen at -80°C) were transferred to ice-cold homogenization buffer (4 ml/g wet tissue) and homogenized with an Ultra-Turrax T25 homogenizer 3 times for 20 sec. The homogenates were centrifuged for 10 min at 620 x g, 4°C, and Triton X-100 was added to the supernatant to a final concentration of 1%. After incubation for 30 min on ice, the supernatant was obtained by centrifugation at 300.000 x g for 1h and stored at –80°C until use. Protein concentration was determined using the method of Bradford.

Homogenization buffer
20 mM HEPES, pH 7.4
300 mM sucrose
150 mM NaCl
Complete protease inhibitor cocktail (Roche)

2.8.2 Preparation of Membrane Protein Extracts

For membrane protein extraction chicken tissues (small white follicles, estrogen-treated rooster liver) was placed in ice-cold buffer A (5 ml of buffer A/g wet tissue). The tissues were subjected to homogenization using an Ultra-Turrax T25 homogenizer (3x 20 sec). Cellular debris was removed by centrifugation at 5 000 x g for 5 min, 4°C in a SS34 rotor (Sorvall), and the resulting supernatant was poured over 4 layers of cheesecloth into a fresh tube. The filtrate was centrifuged at 100.000 x g for 1 h in a TLA 100.3 rotor (Beckmann). The accumulated supernatant was discarded and the membrane pellets were washed in 3 ml buffer A by aspiration through a 18- and a 22-gauge needle and re-sedimented by ultracentrifugation at 100.000 x g for 1 h, 4°C. Again the supernatant was discarded. In order to solubilize membrane proteins the pellets were resuspended in 1 ml of buffer B, flushing again through an 18-and a 22-gauge needle. Afterwards, reagents were added to adjust the suspension to a final volume of 2 ml containing the following components: First 80 µl 4 M NaCl (4% of the total volume) was added and the suspension was sonicated once for 30 sec using a Bandelin sonicator (Bandelin, Berlin, Germany). Then 520 µl ddH_2O (26% Vol.) and 400 µl 10% Triton X-100 (10% Vol.) were added to the samples. Undissolved material was removed by centrifugation at 100.000 x g for 1 h, 4°C. The clear supernatant, designated membrane extract, was aliquoted, quickly frozen in liquid nitrogen and stored at -80 °C until use. Protein concentration was determined using the Bradford method.

Buffer A
20 mM Tris-HCl, pH 8.0
2 mM-$CaCl_2$
150 mM-NaCl
Complete protease inhibitor cocktail (Roche)

Buffer B
250 mM Tris-maleate pH 6.0
2 mM $CaCl_2$
Complete protease inhibitor cocktail (Roche)

2.8.3 Preparation of Chicken Lipoproteins

VLDL and LDL were purified by preparative density-gradient centrifugation. Blood of a rooster was collected in a tube containing EDTA with a final concentration 1 mg/ml and plasma was obtained by centrifugation 15 min. at 3.500 rpm at 4°C using a Haraeus table centrifuge. In order to isolate VLDL and LDL fractions the plasma was adjusted to a density of 1.063 g/ml by the addition of solid KBr and subsequent ultracentrifugation at 50.000 rpm for 24 h at 4°C. Lipoproteins were fractionated by use of a SW41 Ti rotor in a Beckman XL 70 centrifuge. Chicken VLDL and LDL were recovered from the top, diluted with LDL-buffer and stored at 4°C until use. The collected fraction was analyzed for protein content via Bradford assay and the purity was evaluated by SDS-PAGE.

2.8.4 Expression and Purification of Recombinant Proteins

The chicken ligand-binding domain (ggLA1-7) has been previously cloned by Bajari et al. (Bajari, T.M., Strasser, V. et al. 2005). A pMal expression vector provides the recombinant receptor fragments with a C-terminal His and an N-terminal maltose-binding protein (MBP)-tag. Glycerol stocks of E. coli transformed with the expression vector pMalc2b harbouring the wt and mutated ggLA1-7s were used to prepare starter cultures of 3 ml LB medium (100 µg/ml amicillin), which were incubated o/n at 37°C with vigorous shaking. These overnight cultures were diluted in 500 ml of LB medium supplemented with ampicillin (100 µg/ml). The cultures were grown under gentle shaking until the optical density (OD_{600}) was 0.6. Then protein expression was induced by adding isopropyl ß-thiogalactopyranoside (IPTG) with a final concentration of 1 mM. The expression was continued for 3-4 h at 37°C under gentle agitation (145 rpm) and the cells were pelleted by centrifugation for 15 min at 4°C and 5000 rpm in a Sorvall centrifuge using a GS3 rotor. The cell pellet was resuspended in 6 ml lysis buffer and to achieve complete cell lysis, the cell suspension was sonicated 8 times for 30 sec. on ice. The lysates were centrifuged at 4°C at 4000 rpm for 15 min using a Haraeus table centrifuge. The supernatant was collected and stored at -20°C or was used immediately.
The recombinant His fusion proteins were purified by Ni–NTA affinity chromatography using the batch procedure according to the manufacturer's instructions (Qiagen). 1 ml Ni-NTA sepharose beads, previously washed in lysis buffer, were added to 6 ml lysate and filled up with lysis buffer to a volume of 50 ml. This lysate-Ni-NTA mixture was allowed to incubate o/n on a rotary shaker at 4°C. The beads were harvested by centrifugation for 4 min. at 2000 rpm and 4°C (Haraeus Megafuge). To remove unbound proteins the Ni-NTA beads were washed 4 times with 10 bed volumes of wash buffer (10 ml) and after each washing step the beads were harvested by centrifugation for 4 min. at 2000 rpm and 4°C.
To achieve the dissociation of the His-tagged proteins from the Ni-NTA resin buffers increased imidazole concentrations were used. All in all, 3 elution steps were performed by adding 1 ml elution buffer and rotating the beads for 4 min at 4°C on a shaker at the highest setting. The beads were again centrifuged to collect the fractions with the purified proteins in the supernatant. The elutions were pooled and dialyzed o/n against 0.5 l buffer F using SnakeSkin Pleated dialysis tubes with a MWCO of 7.000 kDa (Thermo Scientific). Samples were either subjected immediately to re-folding procedures or stored at -80°C until use.

Lysis buffer
50 mM Tris, pH 8.0
500 mM NaCl
1% Triton X-100
0,1% Tween 20
10% glycerol
20 mM imidazol
Complete protease inhibitor cocktail (Roche)

Elution buffer
50 mM Tris, pH 8.0
500 mM NaCl
1% Triton X-100
0,1% Tween 20
10% glycerol
250 mM imidazol
Complete protease inhibitor cocktail (Roche)

Wash buffer
50 mM Tris, pH8.0
500 mM NaCl
1% Triton X-100
0,1% Tween 20
30 mM imidazol
10% glycerol

Buffer F
50 mM Tris-HCl, pH 7.4
50 mM NaCl
0.1 % Tween 20
2 mM $CaCl_2$

2.8.5 Folding of Purified Proteins via RAP-Sepharose

Expression of recombinant His-RAP-myc

Rat RAP (receptor-associated protein) was subcloned into the expression vector pET-15b as described in (Bajari, T.M., Strasser, V. et al. 2005). The vector was transformed in E. coli BL21 cells featuring a C-terminal His- and a N-terminal myc-tag. A small amount of an E. coli glycerol stock was inoculated in 5 ml LB-medium (100 μg/ml ampicillin) and incubated overnight at 37°C under vigorous shaking. The overnight culture was diluted with 1l pre-warmed LB-medium (100 μg/ml ampicillin) and grown at 37°C until an optical density (OD_{600}) of 0.6. The expression of His-RAP-myc was induced by the adding isopropyl β-thio-galactopyranoside (IPTG) to a final concentration of 1 mM and shaking for 3 h at 37°C. The induced bacteria were harvested by centrifugation at 4000 x g for 10 min, resuspended in 2-5 ml/g wet weight of lysis buffer and sonicated on ice (8x 10sec). The lysate was centrifuged at 10.000 x g for 45 min at 4°C and the supernatant was used for the purification of His-RAP-myc by Ni-NTA affinity chromatography (see 2.8.4).

Preparation of RAP- Sepharose

To generate RAP-Sepharose the purified His-RAP-myc fusion protein was immobilized on CNBr- activated Sepharose 4B (Amersham Pharmacia Biotech). 1 g freezed-dried powder was supended in 30 ml 1 mM HCl and shaken on a rotary shaker for at least 30 min. at RT. The medium swelled immediately and resulted in about 3.5 ml final volume. To remove additives, the beads were washed on a glass filter funnel with 100 ml 1 mM HCl, added in several aliquots. In order to activate the sepharose the beads were rinsed with 20 ml of coupling buffer and the activated beads were incubated in the ligand to be coupled: 20 mg purified RAP fusion protein were dissolved in 20 ml coupling buffer containing protease inhibitors (Roche). The mixture was rotated gently o/n at 4°C on an end-over-end shaker.

Using the glass filter funnel excess ligand was sucked out and washed with a total of 150 ml coupling buffer. For blocking remaining active groups the beads were resuspended in 30 ml blocking buffer and rotated for 2-3 h at RT. Finally the sepharose was washed as described above with at least three cycles of alternating pH: 2 times with 50 ml wash buffer 1, 2 times with 50 ml wash buffer 2 and finally 4 times in alternating order. Until usage the coupled RAP- sepharose was stored in storage buffer at 4°C.

Coupling buffer pH 8.3
0.1 M $NaHCO_3$
0.5 M NaCl

Blocking buffer pH 8.3
1 M ethanolamine

Wash buffer 1 pH 8.0
0.1M boric acid
1 M NaCl

Wash buffer 2 pH 4.0
0.1 M sodium acetate
1 M NaCl

Storage buffer pH 7.4
25 mM Tris-HCl
0.01% EDTA
0.02% NaN3

Refolding of ggLA1-7 receptor fragments via RAP-coupled Sepharose

Purified, dialyzed ggLA1-7 receptor fragments were refolded by dialysis in the presence of 500 µl of RAP-coupled sepharose with 2 mM reduced glutathione and 1 mM oxidized glutathione. After 72 h dialysis at 4°C against 1l buffer F, the glutathione was gradually removed by replacing one-tent of the dialysis buffer volume with buffer F every 1.5 h until the whole buffer volume was exchanged. Finally the bound protein was eluted from the RAP-Sepharose with buffer G. The eluted proteins were immediately adjusted to a pH of 8.0 using 0.1 M HCl and then dialyzed against buffer F for 24 h to remove Ammonia.
The purity and folding of the purified gg-LA1-7 fragments was determined by subjecting the eluted protein fraction to SDS-PAGE on a 7.5% polyacrylamide gel under both reducing and non-reducing conditions.

Buffer G
25 mM Tris
75 mM NaCl
0.1 M NH_4OH

2.8.6 Determination of Protein Concentration via Bradford Protein Assay

To determine total protein concentration a dye-binding assay, based on the Bradford method was used (BioRad). This assay uses the dye Coomassie Brilliant Blue G-250, which binds selectively to basic and aromatic residues within peptides and proteins, and this binding is accompanied by a shift in absorbance maximum from 465 nm to 595 nm. The shift can be followed in a spectrophotometer by measuring the absorbance at 595 nm.

1 µl sample was mixed with 1 ml Bradford reagent, incubated for 5 min at RT and analyzed with a spectrophotometer at 595nm.
A provided BSA standard with a given concentration (2mg/ml) was used to create a dilution series and the Bradford values for the standards are then used to construct a standard curve to which the values of the samples were compared.

2.8.7 SDS-Polyacrylamide Gel Electrophoresis (SDS- PAGE)

Protein extracts and recombinant proteins were analyzed by one- dimensional SDS-Polyacrylamide Gel electrophoresis using 7.5% and 12 % polyacrylamide gels. Since a denaturing, discontinuous SDS-PAGE was performed two separately polymerized layers of polyacrylamide were prepared. First the ingredients of the separating gel (table 2.7) were mixed, poured into cleaned gel units (BioRad Mini gel system) and overlaid with isopropanol. The gel was allowed to polymerize for at least 15 min. After completely removing the isopropanol, the stacking gel was prepared and poured onto the polymerized separating gel. The combs were inserted and polymerization took place at least 15 min. At last the comb was removed, the slots were rinsed with ddH_2O and the gels were assembled to the electrophoresis chamber filled with 1x electrophoresis buffer.

Ingredients (1 gel)	Stacking gel (4%)	Separating gel (7.5%)	Separating gel (12%)
ddH_2O	1525 µl	2425 µl	1675 µl
1.5 M Tris-HCl pH 8.8	—	1250 µl	1250 µl
0.5 M Tris-HCl pH 6.8	625 µl	—	—
30% Polyacrylamide	325 µl	1250 µl	2325 µl
10% SDS	25 µl	50 µl	50 µl
10% APS	12.5 µl	25 µl	25 µl
TEMED	2.5 µl	5 µl	5 µl

Table 2.7 Ingredients of gels used for SDS-PAGE

Protein samples were analyzed under reducing (by adding 25 mM Dithiotreitol) or under non-reducing conditions according to Laemmli. In case of reducing conditions the samples were incubated for 10 min at 95°C before loading onto the gel.
The gel was run at first at 80 V until the samples have entered the stacking gel and then the electric current was increased to 180 V until the Bromphenol Blue dye front migrated from the bottom of the gel. 5 µl of PageRuler Plus Prestained Protein ladder (Fermentas) or 8 µl of Unstained Precision Plus Protein Standard (BioRad) were applied, serving as molecular weight markers.
Following electrophoresis, the gels were either subjected to Coomassie blue staining or to Western- and Ligand blot analysis.

Acrylamide stock
29.2% Acryamide
0.8% N,N'-Methylenbisacrylamide

4x Laemmli buffer
31.2% Glycerine
6% SDS
20 mM Tris-HCl pH 7.4-7.5
as reducing agent 25mM Dithiothreitol (DTT) was added

1x Electrophoresis buffer
25 mM Tris
0.192 M Glycine
1% SDS

2.8.8 Coomassie Staining

In order to stain proteins in SDS-PAGE gels, the polyacrylamide gel was soaked in Coomassie solution and incubated for at least 1 h at RT on a laboratory shaker. The gel is then destained for 2 h at RT or o/n at 4°C with frequent changes of destaining solution until the gel background is clear and protein bands could be clearly visualized. For the purpose of documentation the gels were dried in a gel vacuum dryer for 50 min at 80°C.

Coomassie solution
10% Acetic acid
25% Isopropanol
0.287 g Coomassie Brilliant Blue R250 in ddH_2O

Destaining solution
10% Acetic acid
30% Methanol in ddH_2O

2.8.9 Western Blot Analysis

Wet- (Tank-) Blotting

After electrophoresis the separated proteins were transferred to nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences) using wet blotting Mini Trans-Blot System (BioRad). Before blotting the stacking gel was removed and the provided support pads of the transfer unit and the Whatman papers were soaked in 1x transfer buffer. The blot was assembled in the following order: support pad, 3 Whatman papers, gel, nitrocellulose membrane, 3 Whatman papers and again support pad. The sandwich was placed in the electrotransfer unit with the membrane side of the sandwich positioned at the positive terminal. The transfer was performed at a constant current of 100 V for 1 h under chilled conditions. Following transfer, the blotting efficiency was checked by staining the membrane

with Ponceau S. Immobilized proteins and standards were visualized by rinsing the membrane with ddH_2O.

Detection

Signals were detected using the enhanced chemiluminescence (ECL) method (Pierce). This method depends on the incubation of the membrane with a substrate that produces luminescence when exposed to horseradish peroxidase (HRP)-conjugated to the secondary antibody.
At first the membrane was blocked at least 1 h with 5% non-fat dry milk in 1x TBS-T buffer (blocking solution). After removing the blocking solution, the membrane was incubated o/n at 4°C or at least 1 h at RT with primary antibody (diluted in blocking solution). The membrane was washed 3x 10 min in 1x TBS-T, followed by incubation with a secondary HRP-conjugated antibody (diluted in blocking solution). After 1 h incubation the membrane wash washed another 3 times for 10 min with 1x TBS-T. An equal amount of ECL solution 1 and 2 were mixed, spread over the blot and incubated for 3 min. The excess of ECL solution was drained off, the membrane was placed in an X-ray cassette and exposed to an autoradiography film (Pierce, CL-XPosure^{TM}Film) depending on the intensity of the signal and background.

1x Transferbuffer
25 mM Tris
192 mM Glycine

1x PBS
137 mM NaCl
0.27 mM KCl
10 mM Na_2HPO_4
1.7 mM KH_2PO_4

1x TBS-T
25 mM Tris-HCl, pH 7.4
140 mM NaCl
2.5 mM KCl
0.1 % Tween 20

Ponceau S
1x PBS
0.2% Ponceau S
3% Trichloracetic Acid

2.8.10 Ligand Blot Analysis

Ligand blot analysis is a technique derived from the standard Western blot method to detect protein-protein interactions in vitro.
Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane via wet-blotting according to the Western Blot protocol. Nonspecific binding sites on the membrane were blocked for 1 h at RT using 5% non-fat dry milk in 1x TBS-T + 2mM $CaCl_2$ (blocking buffer). In case of testing multiple interacting partners the membrane was cut into single strips, each probing with different ligands. The indicated ligand proteins were diluted in blocking buffer and the formation of specific protein-protein interactions was allowed o/n at 4°C under gentle agitation.
Unbound proteins were washed off 3 times for 20 min with 1x TBS-T+ 2 mM $CaCl_2$. After washing a ligand-specific antibody was diluted in 1x TBS-T + 2mM $CaCl_2$ and was added to the membrane. After 1h of incubation at RT under gentle agitation, the membrane was

washed another 3 times for 10 min with 1x TBS-T + 2 mM $CaCl_2$. An appropriate HRP-conjugated secondary antibody diluted in blocking buffer was added and incubated for 1 h at RT. Finally the membrane was rinsed 3 times for 10 min with 1x TBS-T + 2 mM $CaCl_2$ prior to chemiluminenescent detection using the ECL kit from Pierce.

2.8.11 Co-Immunoprecipitation

Co-Immunoprecipitation was carried out to precipitate protein complexes and to investigate protein- protein interactions. Receptor fragments (LA7wt, LA7m, LA7del, LA7dbl) obtained from the re-folding procedure were incubated with 15 µg bacterially expressed, purified His-RAP-myc for at least 2 h or o/n on a rotator at 4°C under gentle agitation. Following this incubation, a monoclonal anti-myc antibody was added and the samples were incubated for 2 hr at 4°C on a rotator under gentle agitation. After this incubation period, the Protein A Sepharose beads (50 µl wet volume) were added to the mixtures. Samples were incubated overnight at 4°C under gentle agitation as mentioned above. The next day, the samples were centrifuged for 2 min at 2000 rpm and 4°C. The supernatant was discarded and the beads were washed 3 times with 500 µl 1x TBST + 2 mM $CaCl_2$ + proteinase inhibitor (ROCHE). In between the washing steps, the beads were centrifuged for 2 min. at 2000 rpm and 4°C to remove the supernatant. After the final centrifugation, the supernatant was discarded and an appropriate amount of 4x Laemmli buffer + 25mM DTT were added to the beads. Afterwards, samples were heated up to 95°C for 10 min to separate the proteins from the beads. Samples were centrifuged and the supernatant was analyzed by SDS-PAGE and Western blotting.

2.8.12 ELISA- based Solid Phase Binding Assay

ELISA was performed in 96 flat-bottom wells (Greiner). The wells were coated either with tissue membrane protein extracts or recombinant purified proteins diluted in 1x TBS + 2 mM $CaCl_2$ (100 µl/well). The plate was incubated o/n at 4°C under gentle agitation and humid conditions to avoid the microtiter plate to run dry during incubation. After 2 washes with blocking solution, the wells were subsequently blocked for 1 h with 100 µl of blocking solution (2% (w/v) BSA in 1x TBS + 2 mM $CaCl_2$). Then the wells were incubated for 1 h with 100 µl of different concentrations of the respective ligands diluted in blocking buffer. The assay proceeded with 4 washing steps by adding and discarding 100 µl of blocking solution prior to 1 h incubation with the primary, ligand-specific antibody. Wells were again washed 4 times with 2% BSA solution and supplied with 100 µl of appropriate HRP-conjugated secondary antibody diluted in blocking buffer. After a series of final washes with 1x TBS the peroxidase activity was measured by the addition of 100 µl 3,3',5,5'-Tetramethylbenzidine (TMB; Sigma) as substrate solution. The reaction was stopped after 5-10 min by the addition of 50 µl of 1 M H_2SO_4. The enzyme activity was monitored by measurement of absorbance at 450 nm using a microtiter plate reader.

3. RESULTS

To gain better insights into the mechanism of apolipoprotein-receptor interactions and to reveal the critical structural requirements that are essential for interaction, this study was performed in the domesticated chicken (Gallus gallus), an established model organism for research on lipid metabolism in our research group. Avian and mammalian members of the LDL receptor family share characteristic similarities, both in structural and functional terms. This high degree of homology becomes apparent when the primary protein sequences of distinct family members are compared. Figure 3.1 depicts the alignment of the chicken LDLR, the chicken VLDLR, termed LR8, and the human LDLR.

The overall receptor architecture is highly similar including typical hallmarks of this receptor family. In general, the ggLDLR and the humLDLR are composed of a ligand-binding domain consisting of 7 LA- repeats, followed by EGF-like repeats, an O-linked sugar domain, a transmembrane- as well as a cytoplasmic domain. The chicken VLDL receptor displays almost the same build-up, though harboring an extra N-terminal LA- repeat and lacking the O-linked sugar domain. Comparing the protein sequences of between the human and the chicken LDLR reveals some subtle differences. These include that the linker region between the sixth cysteine in LA repeat 4 and the first cysteine of LA repeat 5 consists of 12 residues in the human, but 28 residues in the chicken receptor. Moreover, the arrangement of negatively charged residues in the C- terminal region of LA repeats, particularly that of LA1 and LA5, and the intracellular domain harboring the internalization signal show some differences (Hummel, S., Lynn, E.G. et al. 2003).

```
 chickenLR8     (1) MRSSRQRGDRSAATGGGCGARRWALPRCGALCLLLALGCLRTATDGAKAK (50)
chickenLDLR     (1) MAAWALLLGVLLSAA----------------------------------- (15)
  humanLDLR     (1) MGPWGWKLRWTVALLLAAAGT----------------------------- (21)

 chickenLR8    (51) CEESQFQCSNGRCIPLLWKCDGDEDCSDGSDESACVKKTCAESDFVCNSG (100)
chickenLDLR    (16) ---------------------------------TDVWGCDPEQFRCGDG (31)
  humanLDLR    (22) ---------------------------------AVGDRCERNEFQCQDG (37)

 chickenLR8   (101) QCVPNRWQCDGDPDCEDGSDESAELCHMRTCRVNEISCGPQS--TQCIPV (148)
chickenLDLR    (32) GCISATWVCDGGTECRDGSDEEPEMCRSLQCPAQHFDCGDAVGRERCVPL (81)
  humanLDLR    (38) KCISYKWVCDGSAECQDGSDESQETCLSVTCKSGDFSCGGRV--NRCIPQ (85)

 chickenLR8   (149) SWKCDGEKDCDSGEDEENCGNVTCSAAEFTCSSGQCISKSFVCNGQDDCS (198)
chickenLDLR    (82) SWRCDGHRDCRHGADEWGCEPPPCASDQQRCSDGSCVSRAFLCDGDRDCP (131)
  humanLDLR    (86) FWRCDGQVDCDNGSDEQGCPPKTCSQDEFRCHDGKCISRQFVCDSDRDCL (135)

 chickenLR8   (199) DGSDELECAPPT-CGVHEFQCKSSTCIPISWVCDDDADCSDHSDESLEQC (247)
chickenLDLR   (132) DGGDERDCPPPPPCPPASFRCPDGVCVDPAWLCDGDADCADGADERSPTC (181)
  humanLDLR   (136) DGSDEASCPVLT-CGPASFQCNSSTCIPQLWACDNDPDCEDGSDEWPQRC (184)

 chickenLR8   (248) G------------------RQPAPPVKCSTSEVQCGSGECIHKKWRCDG (278)
chickenLDLR   (182) AEATAAEAEAAEAEAEEGEGVVPRPAQRCPPLRVPCRSGGCVPRGWRCDG (231)
  humanLDLR   (185) RGL----------------YVFQGDSSPCSAFEFHCLSGECIHSSWRCDG (218)

 chickenLR8   (279) DPDCKDGSDEINCPSRTCRPDQFRCED-GNCIHGSRQCNGVRDCLDGTDE (327)
chickenLDLR   (232) SPDCSDGSDEDGCDPPLCPPEEFRCADDGRCVWGGRRCDGHRDCADGSDE (281)
  humanLDLR   (219) GPDCKDKSDEENCAVATCRPDEFQCSD-GNCIHGSRQCDREYDCKDMSDE (267)
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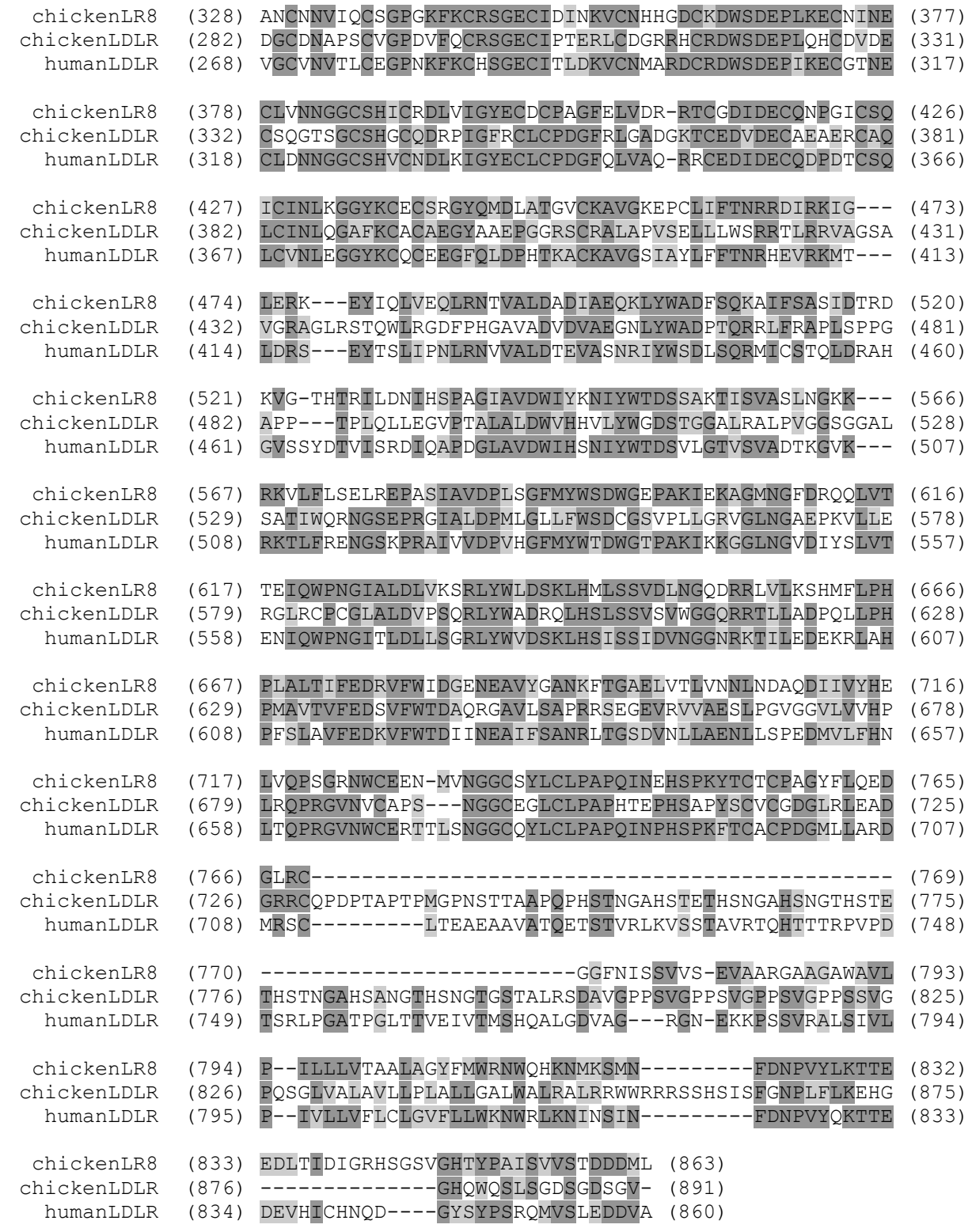

Figure 3.1 Protein sequence comparison of chicken LR8, chicken LDLR, and human LDLR

Numbering of the amino acid sequences starts at the methionine residue corresponding to the initiation codon. Identical and conserved residues are shaded in dark-, similar residues in light grey.

3.1 MOLECULAR CHARACTERIZATION OF human LDLR-APOE INTERACTION

Many previous and current studies are focusing on the ligand-binding properties of various members of the LDL receptor family. The LDL receptor is the best characterized member of this family, and the ligand recognition by this receptor has been studied extensively. The human LDLR achieves its physiological function by binding cholesterol-rich particles via apolipoprotein E and/or apolipoprotein B and thereby mediates their internalization and subsequent lysosomal degradation. Molecular characterization of the precise binding event revealed that the LDLR discriminates against distinct variants of apolipoproteins residing on lipoproteins. In mammals, apoB exists in 2 forms: as apoB-100 representing the full length protein and as apoB-48, a truncated form corresponding to the N-terminal 48% sequence of apoB-100. Multiple investigations showed that the LDLR is capable to bind only lipid particles harboring apoB-100.
Furthermore, the second LDLR-ligand, apoE, was shown to exist in 3 isoforms, differing from each other by single amino acid substitutions. Several studies have provided evidence that the human LDLR displays a differential behavior in binding to the various apoE isoforms. While ApoE3 has the highest affinity for the receptor, apoE4 features a lower but modest affinity, whereas E2 displays only a weak binding capacity (Schneider, W.J., Kovanen, P.T.et al. 1981).

Since apoE need to be associated with lipids to confer high receptor binding activity apoE-containing particles were harvested from CHO cell lines stably secreting the recombinant isoforms. The resulting apoE particles have been characterized by other research groups also conducting studies with apoE (Stannard, A.K., Riddell, D.R. et al. 2001). Separation by gel electrophoresis revealed that the the apoE2-, E3- and E4-containing lipid particles have pre-α mobility similar to the majority of plasma HDL particles. Using non-denaturing gel electrophoresis, some differences between the isoforms can be denoted. As depicted in figure 3.2B, three main apoE particle populations were identified with hydrated diameters of 7.6, 8.0 and 10.4 nm. The 8 nm particle was prominent in apoE3 media, less abundant in apoE2 media, and absent in apoE4 media. The other particles were present in all three isoforms (Sacre, S.M., Stannard, A.K. et al. 2003). This difference in particle population confirms the effect of the distinct protein isoforms on the lipid association properties resulting in differently sized particles. To control the presence of apoE molecules, the particles were treated with detergent, heated to 95°C und further subjected to SDS-PAGE and Western Blot analysis (figure 3.2A).
The apolipoprotein E content of these particles was quantified by an ELISA kit and according to the obtained protein concentrations, the apoE particles were applied to several binding assays throughout this study.

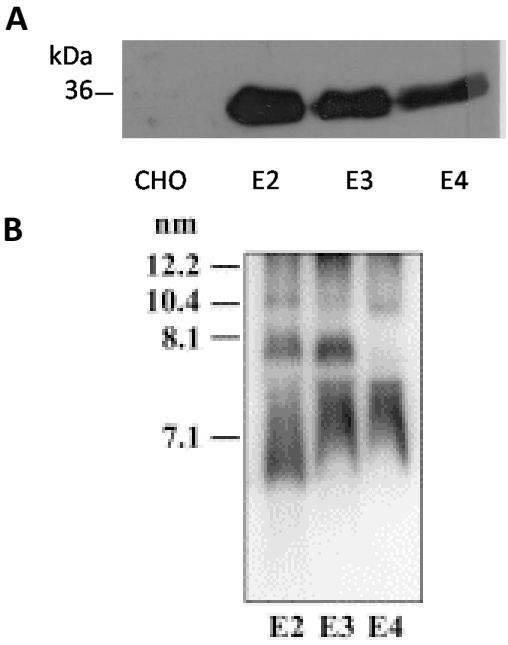

Figure 3.2 Characterization of apoE particles

A: ApoE particles secreted from CHO$^{apoE2/3/4}$ were separated by SDS-PAGE on 12% polyacrylamide gels under reducing conditions and analyzed by Western blot analysis using specific α-apoE antibodies. Equal amounts were used for each isoform quantified by enzyme-linked immunosorbent assay (ELISA).

B: Particle diameters from apoE2, -E3, -E4 particles derived from CHO cells were assessed by non-denaturing gel electrophoresis (NDGE) and immunoblotted for apoE as described by Sacre, S.M., Stannard, A.K et al. (2003).

Using a solid phase binding assay based on the principle of ELISAs, I investigated the binding characteristics of the LDL receptor to the apoE isoforms E2, E3 and E4. As a source of receptor, I cultivated mouse embryonic fibroblasts (M4 Δ806) expressing a truncated form of the human LDL receptor incapable of internalization, thus exhibiting an active ligand-binding domain. Total protein extracts were prepared, coated onto microtiter plates and incubated with increasing concentrations of apoE2-, E3- and E4-containing lipid particles. Using a monoclonal apoE antibody, it was possible to detect the amount of apoE captured on the plate through the specific interaction with the LDLR.

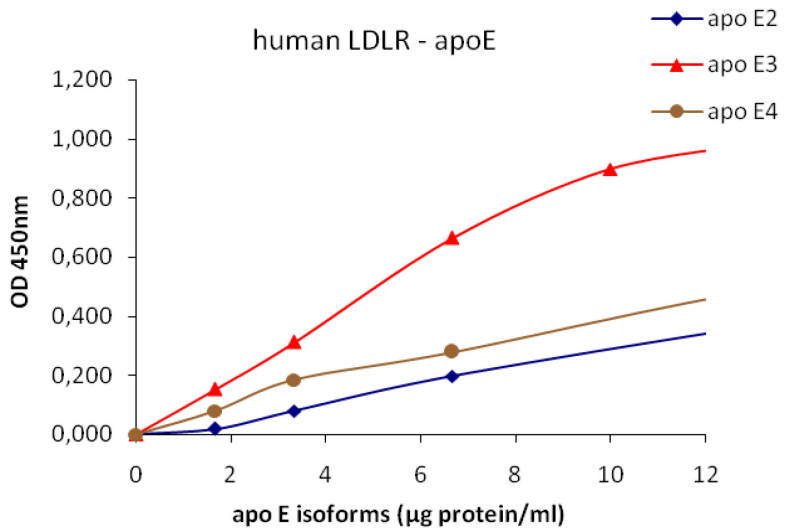

Figure 3.3 Saturation curve for the binding of apoE isoforms to the human LDLR

A solid phase assay was used to measure the interaction of human LDLR with apoE isoforms. Indicated concentrations of apoE2-, E3- and E4- containing lipid particles were added to wells previously coated with 45 µg M4 Δ806 cell extract. Bound apoE was determined by using anti-apoE antibody and visualization was performed as described in materials and methods. High affinity binding was calculated by subtracting non-specific binding from total binding. Each data point represents the average of duplicate determinations.

Figure 3.3 shows that apoE3 featured a high LDL receptor binding activity followed by apoE4, while the binding of apoE2-containing lipid particles was considerably lower. This pattern is in agreement with investigations by Weisgraber et al., where they reported a 50- to 100-times weaker binding potential for apoE2 (Weisgraber, K.H., Innerarity T.L. et al. 1982)

3.2 MOLECULAR CHARACTERIZATION OF ggLR8-APOE- INTERACTION

Very low-density lipoprotein receptors (VLDLR) show a very high degree of conservation among different species. Within mammals, i.e. man, mouse, rat and rabbit, there is a 95% identity between the corresponding receptor proteins (reviewed by Nimpf,J. and Schneider, W.J. 1998). The chicken VLDL receptor LR8 shares about 84% identical residues with the human VLDL receptor (Bujo, Hermann et al. 1994). Thus, the identity between human and chicken VLDL receptors is much greater than that between LDL and VLDL receptors of the chicken.
In contrast to the mammalian VLDLR, whose role in lipid metabolism is still not completely resolved, the function of LR8 in avian species is well established. It mediates a key step in the reproductive effort of the egg-laying hen. LR8 resides in the plasma membrane of the oocyte and is responsible for the deposition of the two yolk precursors vitellogenin and VLDL from the plasma into the growing oocyte. In addition, LR8 mediates the uptake of other transport proteins, such as transferrin, riboflavin-binding protein, retinol-binding protein, thiamin-binding protein, certain biotin-binding proteins, cobalamin-binding protein, and cholecalciferol (Nimpf, J., George, R. et al. 1988).
The receptor is specific for the apoB moiety of the VLDL particle, while apolipoprotein VLDL-II, a lipoprotein lipase inhibitor also present on VLDL particles from laying hens, was shown not to be involved in receptor binding (Nimpf, J., George, R. et al. 1988). Moreover, apolipoprotein E, an apolipoprotein, which is not produced in birds, but is regarded as the mammalian counterpart of VTG, was identified as an additional ligand for LR8 (Steyrer, E., Barber, D.L. et al. 1990).

Using the technique of direct ligand blot analysis, I investigated the binding properties between LR8 and the previously characterized apoE particles (figure 3.4). As a source of receptor I used membrane extracts of chicken ovarian follicles, which are enriched in the major oocyte receptor. After performing SDS-PAGE under non-reducing conditions, the nitrocellulose strips were incubated with apoE-containing particles. Following detection with specific apoE antibodies, receptor-ligand interactions could be detected for all three apoE isoforms (figure 3.4, lanes 3-5).
To detect LR8 in the ovarian follicle membrane extracts, antibodies against LR8 were used and visualized LR8 as a protein with a molecular weight of 95 kDa. Additional bands emerging above the 95 kDa protein represent receptor dimers or multimers favored under non-reducing conditions. Furthermore, when the follicle extract had been subjected to SDS-PAGE under reducing conditions by adding dithiotreitol as reducing agent, the binding of all three apoE isoforms was abolished. This demonstrates that the receptor binding activity of

LR8 for apoE requires the presence of intact disulfide bonds, as it is known for the interaction with LDL.

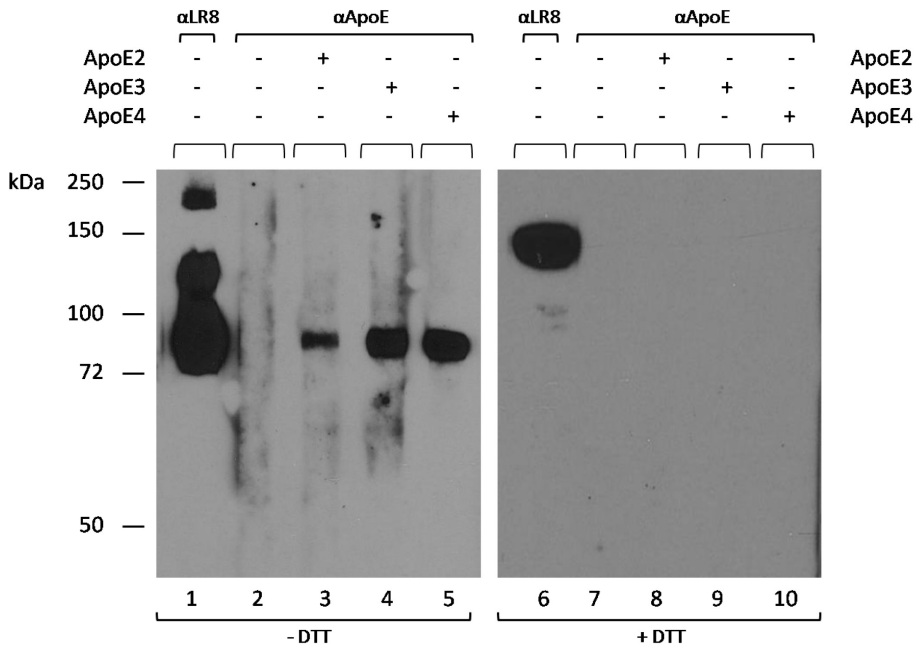

Figure 3.4 ApoE isoforms bind to chicken oocyte receptor LR8

A laying hen small white follicle membrane protein extract was prepared, and 25 µg extract per lane were subjected to non-reducing (-DTT, left) and reducing (+DTT, right) SDS-PAGE using a 7.5% PAA-gel, and separated proteins were blotted to nitrocellulose membrane. After the blocking of unspecific binding sites, 1 µg apoE2-, E3- and E4- particles harvested from $CHO^{apoE2/E3/E4}$ cells were added as ligands (lanes 3-5, 8-10). After washing, the bound apoE protein was detected using anti-apoE antibody. Anti-LR8 antibody (lanes 1, 6) was used to visualize LR8 in the ovarian follicle membrane extract. The bound primary antibodies were detected either with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG and visualized using the chemiluminescence detection method, as described in materials and methods. The positions of migration (kDa) of marker proteins are indicated.

The ability of human apoE to bind to chicken LR8 was further characterized using the ELISA-based solid phase binding assay. Microtiter plates were coated with follicle detergent extracts and incubated with the indicated concentrations of apoE-containing particles.
Comparable to the human LDLR, apoE3 displayed the highest binding capacity to chicken LR8. In mammals, ApoE2 is characterized by a low binding activity towards apoE- receptors. Interestingly, in the chicken system apoE4, and not apoE2, showed the least binding to LR8, while apoE2 had a lower binding potential than apoE3, which nonetheless was significantly higher than that for mammalian receptors (figure 3.5).

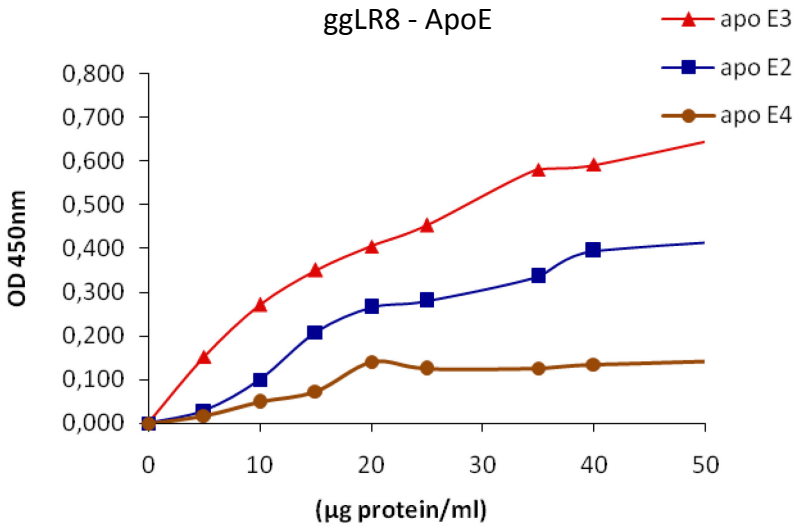

Figure 3.5 Saturation curve for the binding of apoE to the chicken oocyte receptor

Microtiter plates were coated with 25 µg small white follicle membrane extract, and after blocking with 2% BSA increasing concentrations of apoE2, E3 and E4 particles were added. Bound apoE was detected as described in materials and methods. High affinity binding was calculated by subtracting non-specific binding from total binding. Each data point represents the average of duplicate determinations.

The Receptor- associated protein (RAP) is another ligand known to bind to all members of the LDL receptor family. Under physiological conditions RAP is found within the endoplasmatic reticulum and plays an important role in the early processing of receptors by preventing the premature association with ligands on the one hand, and regulating the receptor transport to the cell surface on the other hand (Bu, G., Geuze, H.J. et al. 1995).
The potential of RAP to bind to the chicken VLDL receptor was evaluated by ligand blot analysis and is depicted in figure 3.6. Lane 8 represents the binding of His-RAP-myc to LR8 under non- reducing conditions. Lanes 2-4 show that His-RAP-myc very efficiently competes with all apoE isoforms for the binding sites on LR8. The addition of RAP as ligand completely abrogates the interaction of apoE2, E3 and E4. On the contrary, the binding of RAP itself was not altered by the presence of apoE (lane 5 apoE2, lane 6 apoE3, lane 7 apoE4), which demonstrates that RAP is a receptor ligand with very high affinity for LR8. Nevertheless, binding of RAP to LR8 was impaired when the binding analysis was performed under reducing conditions (data not shown).

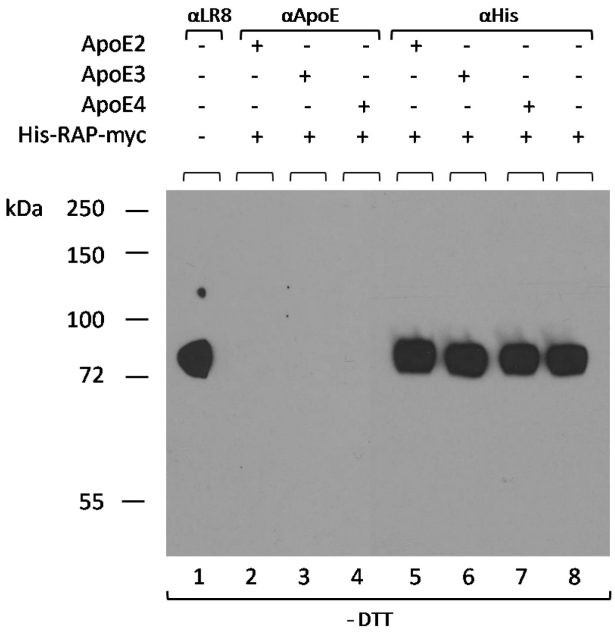

Figure 3.6 Receptor-associated protein (RAP) competes with apoE for receptor binding sites

25 µg small white follicle detergent extract per lane were subjected to SDS-PAGE under non-reducing conditions using 7.5% PAA-gels. Separated proteins were blotted to nitrocellulose and incubated with 1 µg apoE2-, E3- andE4- containing particles in the presence of 8µg His-RAP-myc. Detection was performed using anti-apoE antibody (lanes 2-4) and anti-His antibody (lanes 5-7). Lane 8 depicts the binding of His-RAP-myc to LR8 in the absence of competitor. Anti-LR8 antibody (lane 1) was used to visualize LR8 in the ovarian follicle membrane extract. The positions of migration (kDa) of marker proteins are indicated.

To further investigate the receptor- ligand interaction between LR8 and apoE, a competition assay was performed in order to determine whether apoE3 can be displaced from LR8 by its physiological ligand, VLDL. Blood of roosters was used for the preparation and isolation of plasma VLDL fractions. In order todemonstrate the protein constituents present on these particles, the isolated lipoprotein fractions were analyzed by SDS-PAGE under reducing conditions. Coomassie staining identified a protein band with an apparent molecular weight of approx. 500 kDa. Via Western Blot analysis, this band was identified as chicken apoB, the major protein constituent of VLDL and LDL particles. Consistent with the fact that ApoVLDL-II, the second major protein component of VLDL particles, is not produced in roosters and immature hens, this apolipoprotein was not present in the isolated fraction (Schneider, W.J., Carroll, R. et al. 1990).

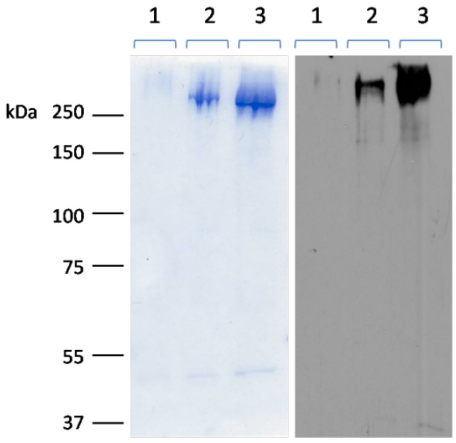

Figure 3.7 SDS-PAGE analysis of plasma VLDL

Plasma VLDL fractions were prepared as described in materials and methods. 1µg (lane 1), 2 µg (lane 2), and 3 µg (lane 3) protein were loaded and separated on 6% polyacrylamide gels under reducing conditions. The gel was either stained with Coomassie brilliant blue (left panel) or subjected to Western Blot analysis (right panel) using anti-ggapoB antibody. The positions of migration (kDa) of marker proteins are indicated.

Using these chicken lipoproteins as competitive ligands, I employed a direct binding assay incubating follicle detergent extracts with constant concentrations of apoE-containing lipid particles and increasing concentrations of VLDL. Since apoE3 is the isoform bearing the highest receptor affinity (see fig. 3.5), the competition assay was conducted with apoE3-containing particles. The results obtained from competition assays are shown in figure 3.8 and revealed the ability of VLDL particles to effectively displace apoE3 from the receptor binding site of LR8. This is an indication that both ligands may bind to closely spaced or identical receptor sites and when both ligands, apoE and VLDL, are present they compete for the binding to the receptor. Consequently, high concentrations of the one ligand (VLDL in this assay) lead to a reduction of binding of the second ligand (apoE3). As expected, the presence of rooster HDL in the incubation mixtures had no significant inhibitory effect on the receptor binding of apoE3, as the apoE-, apoE-free rooster HDL is known not to interact with LR8 (Steyrer, E., Barber, D.L. et al. 1990).

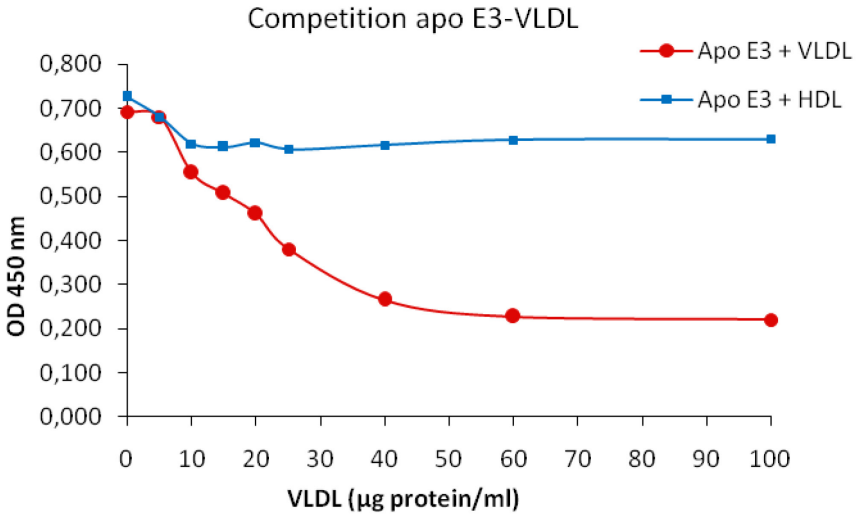

Figure 3.8 Competition of apoE3 and VLDL for the binding to ggLR8

The competitive assay was performed using an ELISA-based microtiter binding assay. 25 µg small white follicle membrane extract (100 µl) and apoE3-containing lipid particles (25 µg/ml protein) were incubated in the presence of increasing concentrations of plasma VLDL (filled circles) or HDL (filled squares). The amount of receptor-bound apoE3 was determined as described above.

3.3 MOLECULAR CHARACTERIZATION OF ggLDLR-APOE INTERACTION

Despite the structural similarity between the ggLDLR and LR8, their ligand-binding properties differ considerably. While LR8 binds apoB, apoE, and VTG, the ggLDLR binds solely to apoB-containing lipoproteins and prefers chicken LDL over VLDL as ligand (Hummel, S., Lynn, E.G. et al. 2003). This is interesting, since avian LDL and VLDL particles do not differ in the content of apolipoproteins that participate in receptor binding. The precise underlying mechanism for this binding behaviour remains to be investigated.
As a member of the LDLR family, the chicken LDL receptor constitutes the only LDL receptor that binds apoB but not apoE (Bujo, H., Hermann, M. et al. 1997). To confirm the inability of the chicken LDLR to bind to apoE I performed ELISA binding assays (figure 3.9).

Based on the finding that upon estrogen administration hepatic LDL receptor levels are significantly increased in roosters (Hummel, S., Lynn, E.G. et al. 2003) I prepared total protein extracts of livers from estrogen-treated roosters. To confirm the ligand-binding competence of the ggLDLR in the liver extract, the binding of physiological binding partners, namely VLDL and LDL, was tested. Figure 3.9 shows that the VLDL fraction was able to interact with the receptor, confirming both the presence and receptor binding activity of chicken LDLR. Additionally, the presence of ggLDLR in the liver extracts was shown by Western Blot analysis using a specific antibody against the ggLDLR (data not shown).

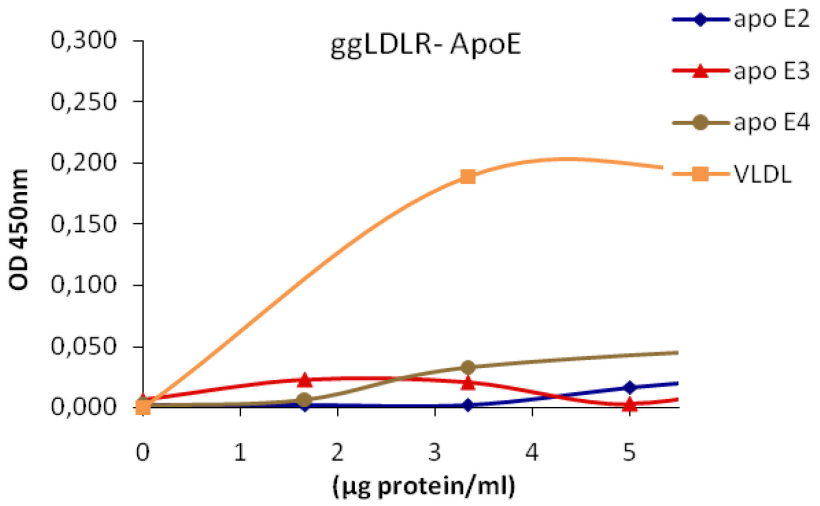

Figure 3.9 Binding of apoE-containing particles to ggLDLR

For the binding assay, 100 µg liver extract of estrogen-treated rooster was coated per well and incubated with the indicated concentrations of apo E2-, E3-, E4 -containing particles. Detection was performed according to materials and methods. Each data point represents the average of duplicate determinations.

3.4 MUTANGENESIS OF THE ggLDLR LIGAND-BINDING DOMAIN

The features of the human LDLR necessary for the recognition of apoE have been investigated extensively in the past. According to these studies, we especially focused on the 5th LA repeat in the ligand-binding region, which was repeatedly reported as absolutely necessary for apoE-binding (Russell, D.W., Brown, M.S. et al. 1989). The human receptor sequence harbors in LA repeat 5 a cluster of negatively charged residues consisting of the peptide ser-asp-glu-glu (SDEE), while the corresponding cluster in the chicken LDLR contains ser-asp-glu-asp (SDED) (figure 3.1). By replacing glutamic acid (E) with aspartic acid (D) we generated a receptor fragment with mutant ligand-binding domain. Furthermore, another cluster of acidic residues was taken into account. The sequence alignment of human LDLR and ggLR8 (figure 3.1) displays a perfect correlation in the first LA repeat including the cluster ser-asp-glu-ser (SDES). The chicken LDLR shows a difference in this sequence by containing ser-asp-glu-glu (SDEE) instead of ser-asp-glu-ser (SDES). Since the human LDLR binds apoE and the chicken LDLR does not, we altered the ggLDLR sequence according to the apoE-binding receptor. Finally, the 28 amino acid linker region of the ggLDLR was shortened to a length of 9 residues, since we hypothesized that the prolonged distance between LA repeats 4 and 5 may have an impact on the structure and function of the ligand-binding domain.

Cloning and mutagenesis of ggLDLR LA domain

The ligand-binding domain of the chicken LDL receptor ggLDLR LA1-7 has been cloned previously by Bajari et al. (Bajari, T.M., Strasser, V.et al. 2005). The fragment comprising the residues 16-327 of the extracellular receptor domain was cloned in the bacterial expression vector pMal2b providing the recombinant protein with an N-terminal maltose-binding protein (MBP) and a HIS- tag at the C-terminal end. The predicted molecular weight of the fusion protein is 75 kDa.

For site-directed mutagenesis, the vector pMAL containing LA repeats 1-7 of the ggLDLR was used as a template for the amplification reaction. In order to verify the presence and correct localization of point mutations and deletions, the plasmid DNA was subjected to sequencing. By aligning the obtained sequences with the wild type ligand-binding domain, I could confirm that all plasmids contained the correct inserts with the corresponding mutations. The receptor fragments were designated according to the localization of the introduced mutations. LA7m5 was generated by the substitution of aspartatic acid (D) to glutamatic acid (E) within the LA repeat 5. LA7del is characterized by the shortened linker region between LA 4 and LA5. LA7m constitutes a receptor mutant with a substitution of glutamic acid (E) by

serine (S) within LA repeat 1. LA7dbl harbours a double mutation, one being the same as in LA7m, the second mutation is the same as in LA7m5, where aspartatic acid (D) is replaced with glutamic acid (E) in repeat 5.

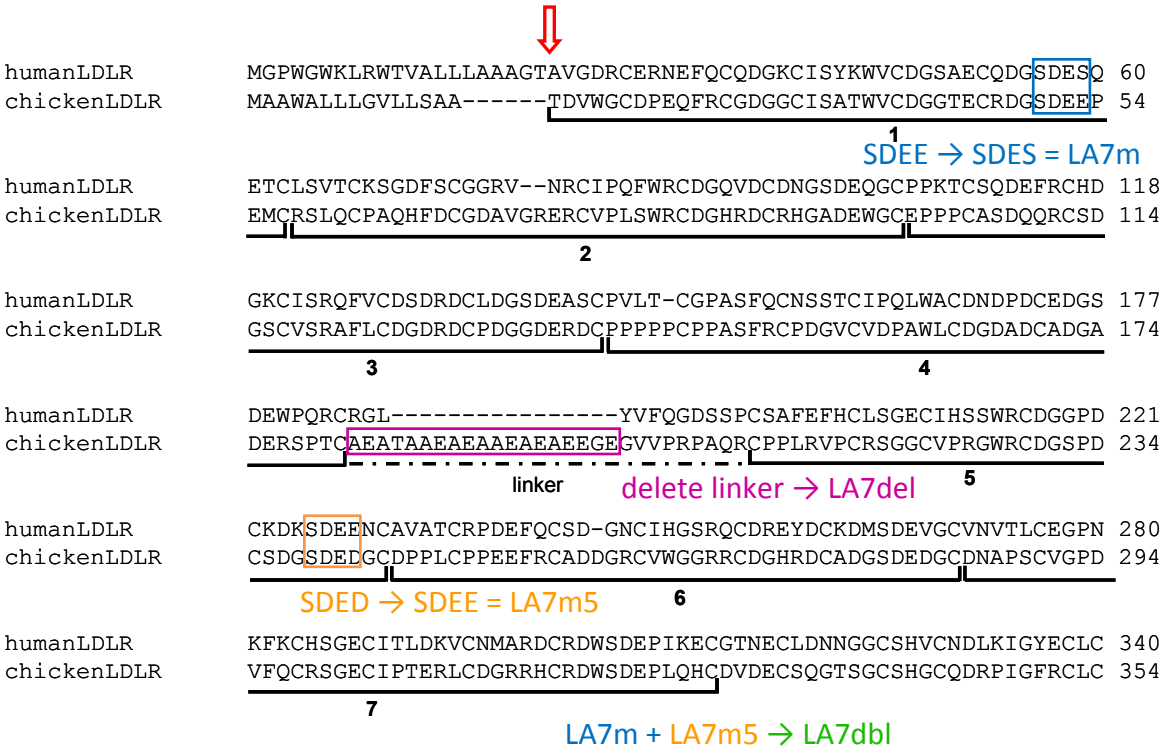

Figure 3.10 Protein sequences of human and ggLDLR LA repeats 1-7

Mutations generated by site-directed mutagenesis are indicated with boxes and the resulting protein designations (LA7m, LA7del, LA7m5, LA7dbl) are indicated in colours. The alignment starts with the methionine residue corresponding to the initiation codon. The signal peptide cleavage site is indicated with an arrow.

Expression and folding of mutant receptor fragments

Transformation into E. coli expression strains and protein expression were performed as described in materials and methods. Since the expressed receptor fragments contained a 6x His-tag on its C- terminus, a metal affinity chromatography based on the high affinity of His residues to Ni- ions was used in order to purify the recombinant proteins. Samples of the eluted fractions were analyzed by SDS-PAGE under reducing conditions and, to visualize proteins, subsequently stained with Coomassie blue. In addition to the receptor mutants described above, the wild type ligand-binding domain, which provided the basis for mutagenesis was also expressed and purified.

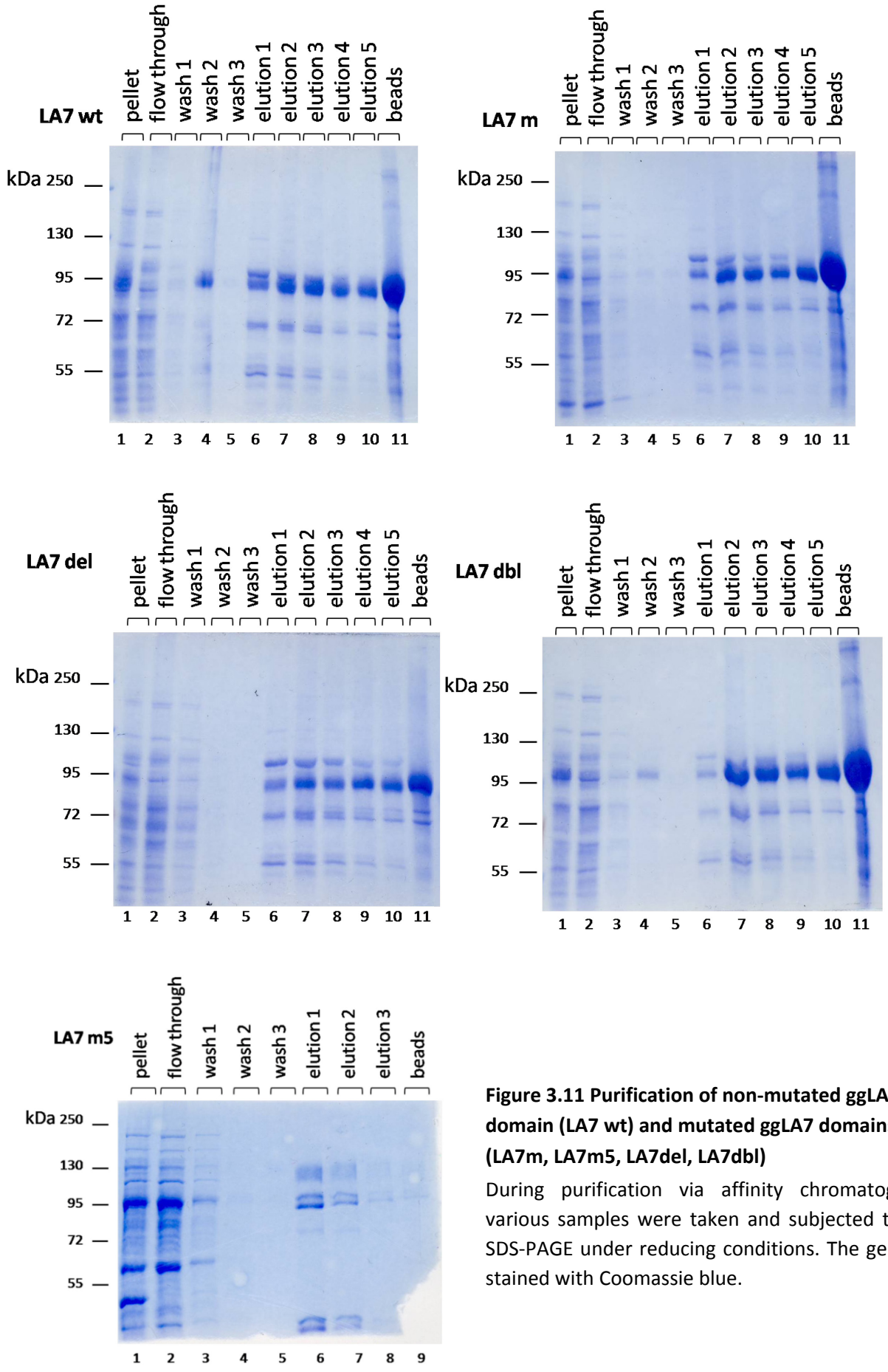

Figure 3.11 Purification of non-mutated ggLA7 domain (LA7 wt) and mutated ggLA7 domains (LA7m, LA7m5, LA7del, LA7dbl)

During purification via affinity chromatography, various samples were taken and subjected to 7.5% SDS-PAGE under reducing conditions. The gels were stained with Coomassie blue.

As depicted in figure 3.11, the expression level of LA7m5 turned out to be much lower than that of the other receptor fragments. Various atempts to optimize conditions during expression, e.g., increasing the imidazole concentration, reducing growth temperature, and increasing the concentration of antibiotics did not succeed. Consequently I excluded this receptor and proceeded with the remaining ones.

Since the receptor proteins were expressed in bacterial cells, which do not post-translationally modify proteins in the same manner as eukaryotes, protein expression was followed by a refolding procedure. Thereby, refolding was achieved by the assistance of Receptor-associated protein (RAP), the molecular chaperone for the LDLR family. For this purpose, I expressed and purified recombinant His-RAP-myc protein and immobilized a certain amount on CNBr- activated Sepharose beads. The process of protein coupling was verified by SDS-PAGE and Coomassie staining (figure 3.13).

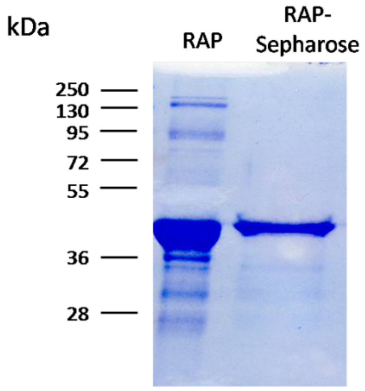

Figure 3.12 Immobilization of His-RAP-myc

Appropriate amounts of bacterially expressed and purified RAP were coupled to CNBr-activated Sepharose. To confirm the coupling efficiency, 25 µl beads were mixed with Laemmli buffer, heated to 95°C and subjected to 12% SDS-PAGE (lane 2). Additionally, 10 µg uncoupled, purified His-RAP-myc was loaded onto the gel (lane 1). Coomassie blue staining was used in order to visualize proteins.

To obtain receptors with a functional, correctly folded ligand-binding domain, the recombinant receptors were dialyzed in the presence of RAP-Sepharose under conditions that allow disulfide bond formation and Ca^{2+} incorporation. As correctly folded proteins were expected to remain bound to RAP, the receptor fragments were eluted from the resin. Eluted fractions were analyzed via SDS-PAGE to test for the purity and folding of the ggLDLR fragments (figure 3.14). Coomassie blue staining and Western Blot analysis revealed a significant difference in electrophoretic mobility between reduced and native proteins. After reduction with 25mM DTT, the obtained band had a size of ~ 90 kDa and was comparable with the band observed during purification. However, under non-reducing conditions the proteins migrated faster, representing a band at ~ 75 kDa. This significant difference in electrophoretic mobility is a hallmark for the folding of the cysteine-rich ligand-binding domain or repeats within it. Formation of disulfide bonds within the individual LA repeats leads to a more compact structural organization and consequently facilitates migration through the gel matrix. The observation that the various fragments displayed a distinct

mobility during electrophoresis allowed the conclusion that RAP assisted in the folding of LDL receptor fragments.

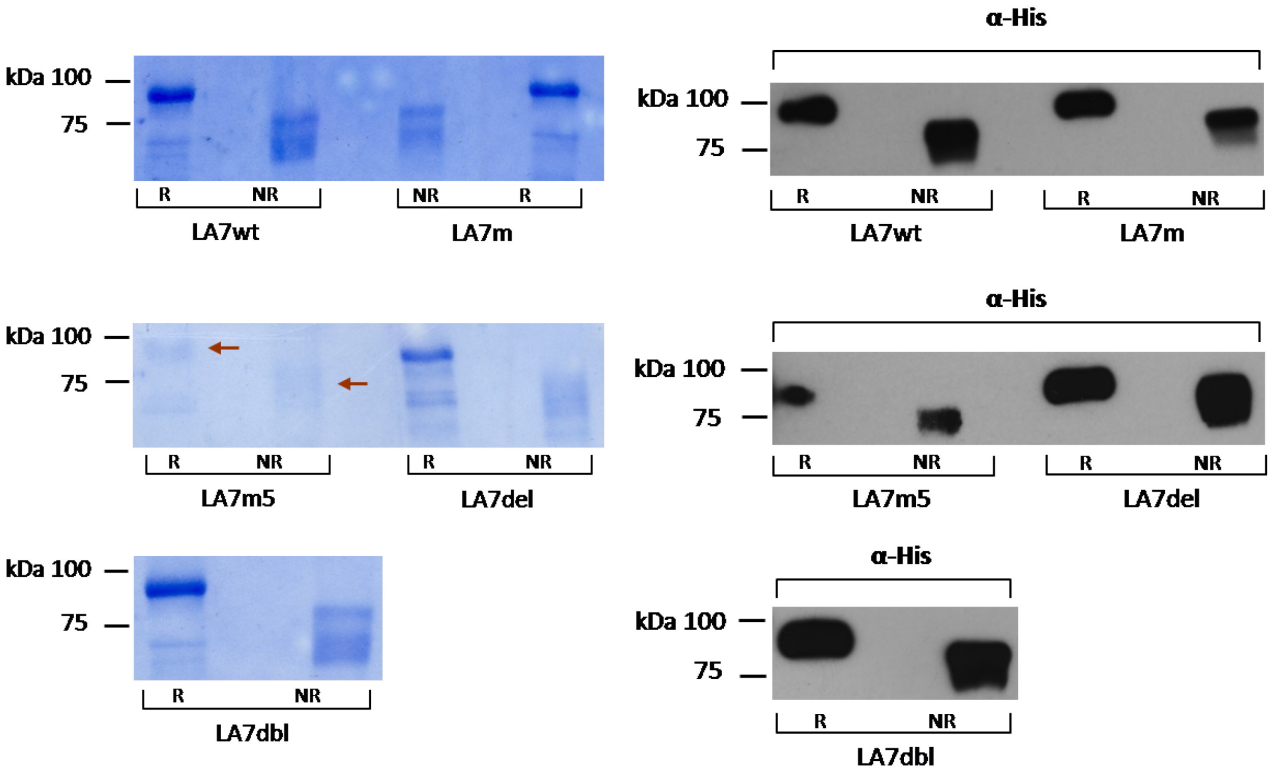

Figure 3.13 Comparison of receptor fragments under reducing and non-reducing conditions

2 µg of each ggLDL receptor protein was separated under reducing (R) and under non-reducing (NR) conditions on a 7.5% SDS polyacrylamide gel. Proteins were visualized by Coomassie blue staining (left anel) and by immunodetection using anti-His antibody followed by HRP-conjugated goat anti-mouse antibody and ECL detection (right panel).

In order evaluate the potential of various receptor fragments to bind RAP, an aliquot of the eluted fractions were incubated with recombinant, purified His-RAP-myc. Co-immunoprecipitations were performed using monoclonal anti-myc antibodies. The immunoprecipitates were washed, dissolved in Laemmli buffer and subjected to SDS-PAGE followed by autoradiography. The results shown in figure 3.15 demonstrate that all receptor fragments were capable to bind RAP. Thus, the result of the co-immunoprecipitation experiment suggests that the in-vitro folding procedure via binding to RAP acting as molecular chaperone indeed leads to active ligand-binding domains.

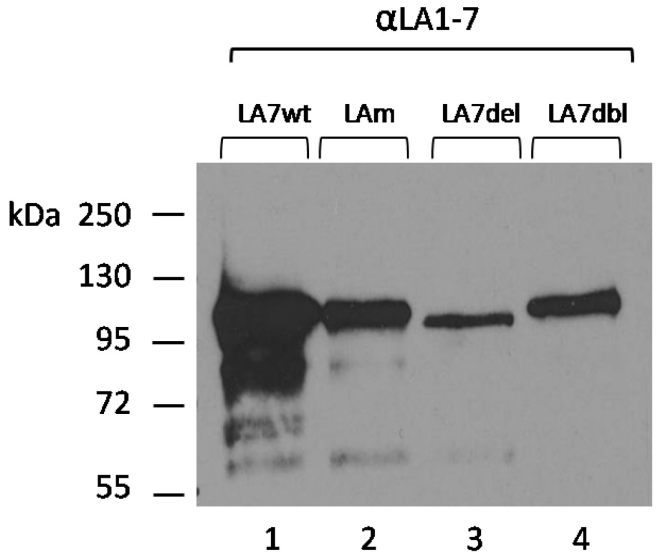

Figure 3.14 Co-Immunoprecipitation: RAP binds to refolded ggLDL receptor fragments

Receptor fragments (LA7wt, LA7m, LA7del, LA7dbl) obtained from the re-folding procedure (15 µg) were incubated with 15 µg purified His-RAP-myc. Co-immunoprecipitations were performed using monoclonal anti-myc antibodies and Protein-A Sepharose beads. After intensive washing of the beads, reducing Laemmli buffer was added and the mixtures were incubated for 10 min at 95°C. The supernatants were subjected to 7.5% SDS-PAGE and subsequent Western blot analysis using anti-ggLA1-7 antiserum. HRP-conjugated goat anti-rabbit antibody was used as secondary antibody, and visualization was performed using ECL.

Next I investigated, whether the introduction of mutations in the ligand-binding domain of the chicken LDLR had any impact on receptor function. For this purpose the binding of the two receptor ligands apoB and apoE was assessed by the ELISA-based solid phase binding assay. Isolated plasma VLDL derived from adult roosters were incubated in microtiter plates coated with equal amounts of purified, re-folded receptor fragments. The amount of bound VLDL particles was determined by the use of polyclonal antibodies directed against a fragment within chicken apoB-100, the major apolipoprotein component on these lipoproteins. Figure 3.16 clearly demonstrates that the unmodified ggLDLR ligand-binding domain (LA7wt) exhibits the highest binding of chicken VLDL. For LA7m a slightly lower binding could be observed. On the contrary, LA7del and LA7dbl were demonstrated to be dramatically impaired in binding to VLDL particles. Normalization of the obtained binding affinities to the binding of LA7wt revealed that LA7m retained a binding capacity of 82%, while the binding of LA7del and LA7dbl was diminished to approximately 20% for both.

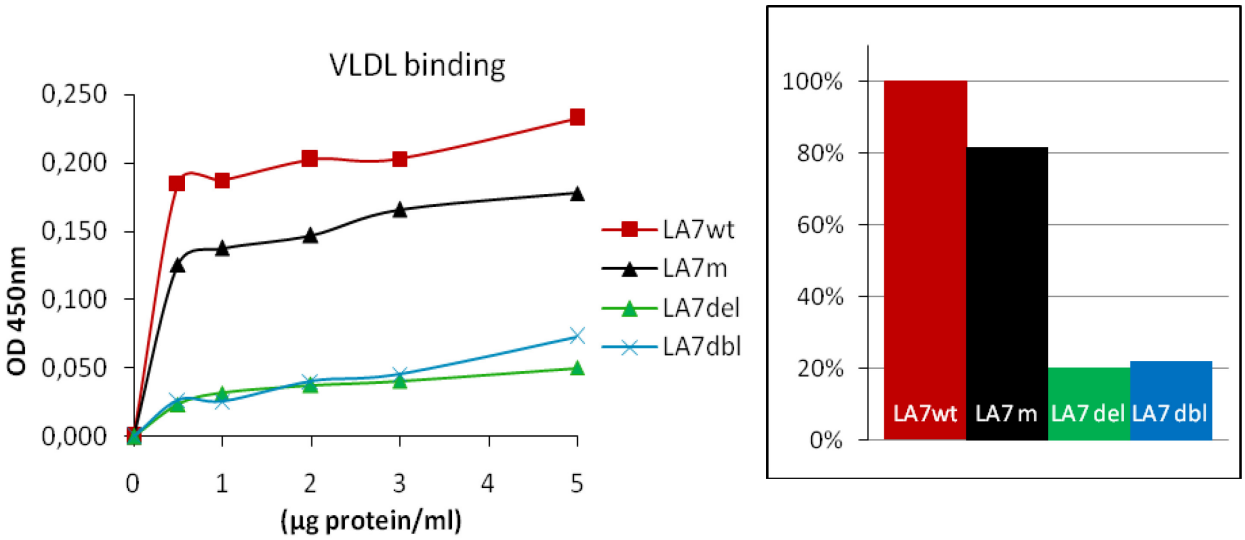

Figure 3.15 Binding of chicken VLDL to non-mutated ggLA domain (LA7 wt) and mutated ggLA domains (LA7m, LA7del, LA7dbl)

Left panel: Microtiter plates were coated with 2µg of the respective refolded receptor fragment and incubated with increasing concentrations of plasma VLDL. Captured VLDL-ApoB was determined using a polyclonal antibody against chicken apoB, followed by HRP-conjugated goat anti-rabbit antibody according to materials and methods. All data are the average of duplicate determinations. **Right panel:** The results from the ELISA binding assay are plotted as %-age of the binding of the wild type ggLDLR ligand-binding domain (LA7wt, 100%).

4. DISCUSSION

The biosynthesis of lipoprotein receptors belonging to the family of LDL receptor relatives (LR) is a complex, challenging process as the functionality of these molecules totally depends on the correctly folded structure of cysteine-rich domains. The ligand-binding domain consists of various numbers of highly conserved cysteine-rich repeats, whose conversion into a native, biologically active conformation includes the formation of disulfide bonds as well as the incorporation of Ca^{2+} ions. The efficient folding process requires the assistance of enzymes and molecular chaperones residing in the endoplasmatic reticulum (ER).
The role of RAP as molecular chaperone for members of the LDL receptor family is well established. RAP is considered to be an important determinant during the process of receptor folding. RAP binds to all LDLR family members and remains associated during receptor trafficking through the secretory pathway. However, RAP-assisted folding was shown to have a different importance in various cells and tissues. This indication was derived from RAP-knockout mice, in which the level of LDLR expression in the liver was unaffected, but was downregulated in the brain (Willnow, T.E., Rohlmann, A. et al. 1996; Veinbergs, I., Van Uden, E. et al. 2001). Furthermore, studies in the chicken revealed that the expression of RAP correlates with the expression of LR8 isoforms in chicken cells (Lindstedt, K.A., Mahon, M.G. et al. 1997). The level of RAP was shown to correlate positively with the splice variant LR8+, expressed in somatic cells (containing the O-linked sugar domain), while no apparent correlation to the LR8- variant (lacking the O-linked sugar domain), found on the surface of oocytes, could be determined. A likely explanation for the co-localization of LR8+ and RAP in somatic cells, e.g. granulosa cells, is that these cells produce other apolipoproteins and potential ligands of the LDL receptor family. RAP has been suggested to prevent the premature binding of these ligands during receptor biogenesis and trafficking to the cell surface. However, oocytes are not known to synthesize apolipoproteins that may act as ligand for LR8-. Consequently, LR8- does not seem to be dependent on RAP-assisted prevention of interactions with endogenous ligands. Nevertheless, this variant of LR8 is as well capable to interact with RAP as it is shown in figure 3.6, lane 8. RAP is believed to bind to the LA-binding repeats within the extracellular domain of the receptor, which is supported by the competitive displacement of ligands from LR8 by intracellular RAP (Hiesberger, T., Hermann, M. et al. 1995). This antagonistic feature of RAP was also observed in the current investigations, as the binding of apoE isoforms to LR8 was abolished upon incubation with RAP (figure 3.6, lanes 2-4).

In vitro studies of receptors belonging to the LDLR family require the maintenance of the same disulfide bonded structures as produced by the cellular synthetic machinery, a difficult aspect of working with the cysteine-rich domains of LRs. Simmons et al. published a protocol for the refolding of a functionally active ligand-binding domain of the human LDLR produced in bacterial cells (Simmons, T., Newhouse, Y.M. et al. 1997). Taking into account that correct folding requires various critical factors, such as the presence of Ca^{2+}, a relatively high pH value (> 8.0) and the addition of a thiol exchange system, they converted the recombinant, purified receptors into a folded conformation. To enrich the proteins that are active in ligand recognition, they used LDL affinity chromatography. However, the recovery of correctly

folded receptor was reported to be low (Marlovits, T.C., Abrahamsberg, C.et al. 1998). In other studies, immobilized receptor-associated protein (RAP) was successfully used to promote and increase the folding efficiency of recombinant VLDL receptors (Ronacher, B., Marlovits, T.C. et al. 2000).
Here, I applied RAP-coupled Sepharose in the re-folding process of chicken LDLR fragments. Bacterially expressed receptor ligand-binding domains were incubated in the presence of RAP-Sepharose under conditions that allow the formation of disulfide bonds and the incorporation of Ca^{2+}. To investigate the correct folding and functionality of the receptor fragments, solid phase binding assays were performed using apoB-harboring VLDL particles, which are well characterized as ligands for the ggLDLR under physiological conditions. The receptor fragment consisting of the ligand-binding domain of the chicken LDLR was capable to bind to apoB-containing plasma lipoproteins (figure 3.16). Thus, the refolding procedure into biologically active receptor fragments was concluded to have been successful.

Besides apoB, apoE constitutes the second major ligand for the mammalian LDLR. Notably, apoE does not bind to the LDLR with high affinity unless it is associated with lipids. A key characteristic of the human LDLR is that it differentially recognizes apoE-containing lipidated particles depending on which apoE-isoform is present. The three apoE isoforms E2, E3, and E4 differ from each other by single amino acid substitutions that were characterized to have a great impact on structure, function, and lipid profiles in humans. ApoE3 shows the highest receptor binding activity, followed by apoE4 with modest binding, while E2 displays a significantly decreased ability to bind to human LDLR. The data obtained from solid phase binding assays (figure 3.3) reflect the previously observed binding behavior of human LDLR to the distinct apoE-isoforms.

Studies in the model organism chicken have revealed apolipoprotein E as a mammalian counterpart to the chicken yolk precursor vitellogenin. Although not existing in the avian species, apoE was shown to bind to the chicken oocyte receptor LR8 (Steyrer, E., Barber, D.L. et al. 1990). Conversely, vitellogenin, which is absent in humans can bind to certain members of the mammalian LDL receptor gene family, namely the LDLR and LRP1 (Stifani, S., Barber, D.L. et al. 1991). Comparative analyses revealed homologous segments between VTG and apoE that are necessary to mediate receptor binding (Steyrer, E., Barber, D.L. et al. 1990). Moreover, both receptors bind apolipoprotein B-100-containing lipoproteins, whereas the avian receptor even binds to VLDL and LDL of mammalian origin, albeit with lower affinity (George, R., Barber, D.L. et al. 1987).
Besides the similar lipoprotein specificities, several findings emphasize additional similarities between the two receptors from different species. Immunological relationship was established as polyclonal antibodies directed against the purified bovine LDLR cross-react with both the human LDLR and the chicken oocyte receptor LR8 (George, R., Barber, D.L. et al. 1987). Apart from the different molecular weight, the mammalian LDLR is more akin to the chicken LR8 than to its functional homologue, the chicken LDLR. This receptor is involved in the regulation of cholesterol homeostasis in somatic cells analogous to the mammalian LDLR but in turn this receptor binds to apoB- but not to apoE-containing lipoproteins.

In my diploma thesis I now applied ELISA- and co-immunoprecipitation methodology to confirm previous observations that chicken LR8 has the ability to interact with apoE. However, unlike the human LDLR LR8 discriminates less against the isoforms apoE2 (figure 3.5). Competitive binding assays with plasma VLDL demonstrated that apoE3 competed with plasma VLDL for the binding to LR8. This observation indicates that apoE and apoB bind to closely spaced or even identical sites within the receptor.
Solid phase binding assays using chicken tissue extracts as a source of chicken LDLR showed the inability of this receptor to interact with apoE-containing ligands (figure 3.9). Based on prior mutational analysis I selected potential binding-reactive sites within the ligand-binding domain of the chicken LDLR, and applied site-directed mutagenesis with the primary objective to delineate defined sequence motifs that contribute to the binding of apoE-containing ligands, and possibly to create receptor fragments that have gained apoE-binding capacity.
The different mutated ggLDLR ligand-binding domains were expressed, re-folded in the presence of RAP and subjected to ELISA-based binding. According to the differential binding characteristics of the different mutated ggLDLR ligand-binding domains, it can be reasoned that the first LA repeat seems not to be involved in the binding of VLDL via apoB. On the contrary, the alteration of the cluster from SDED to SDES in LA5 had a significant impact on the binding behaviour by reducing VLDL binding to 20% relative to wild type receptor binding. These findings are in agreement with multiple previous investigations suggesting that LA repeat 1 does not participate in apoB binding, while the other repeats are critical for the interaction with this ligand.
The ligand-binding domain of the ggLDLR is built up by seven adjacent LA-repeats that are separated from each other by short linker regions of 4-5 residues with exception of the LA4-5 module pair, which is linked by a much longer region (28 residues). Shortening the linker region between LA repeat 4 and 5 from 28 to 9 residues (the length of the linker in the human LDLR) lowers VLDL binding approximately to the same degree (22% normalized to wt receptor binding). In general, the linker regions are thought to confer flexibility and to contribute to the ability to bind a diverse range of protein- and lipoprotein ligands. The observed reduction of binding upon shortening the repeat-spanning region between LA4 and 5 implies that it seems necessary to keep a certain distance between these two repeats in order to confer high binding affinity for apoB-containing ligands.
Unfortunately, initial investigations examining the binding activity of the purified, refolded receptor fragments towards apoE-containing particles did not lead to reproducible results (not shown), possibly due to the difficulty in obtaining sufficient amounts of active receptor. Thus, to obtain a better basis for future experiments, an efficient alternative for receptor protein expression would be cells other than bacteria that allow posttranslational modifications as well as protein folding in vivo.

A further point concerns the specific biophysical properties of apoE. Until recently it was assumed that apoE must be associated with lipids in order to confer high receptor binding affinity. Recently, the interaction of apoE with two other members of the human LDL receptor family, LRP1 and VLDL receptor, were investigated (Ruiz, J., Kouiavskaia, D.et al. 2005). It was reported that both receptors bound apoE, but unlike the LDLR they do not

discriminate between the apoE isoforms. However, the interaction of apoE with the VLDLR displayed asignificant difference in that it recognizes all isoforms also in a lipid-free state. This finding proposes that the binding of apoE in the lipid-bound versus lipid-free state may be of physiological significance and may relate to distinct functions of apoE-binding receptors. In general, despite the ever increasing knowledge about the multiple roles of apolipoprotein E, both in the plasma and central nervous system, extensive further studies will be required to completely understand the complexity of the functions of apoE and ApoE-receptors.

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8. ABBREVIATIONS

α	alpha, anti
A	adenine, alanine
Å	Ångström
ACAT	Acyl-CoA:cholesterol acyltransferase
AD	Alzheimer's disease
Amp	ampicillin
Apo	apolipoprotein
APS	ammonium persulfate
bp	base pair
BSA	bovine serum albumin
C	cytosine or Celsius
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CE	cholesterolester
CM	chylomicrons
CNBr	cyanogen bromide
C-terminus	carboxy-terminus
Da	Dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	desoxynucleotidetriphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme- linked immunosorbent assay
ER	endoplasmatic reticulum
FCS	fetal calf serum
FH	familial hypercholesterolemia
fwd	forward
g	gram or gravity
G	guanine
gg	gallus gallus

h	hour
HDL	high-density lipoprotein
His	histidine
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	horseraddish peroxidase
hs	homo sapiens
HSPG	heparan sulfate proteoglycane
IDL	intermediate-density lipoprotein
IgG	immunoglobulin G
IP	immunoprecipitation
IPTG	isopropyl β-D thiogalactoside
kb	kilo bases
KBr	potassium bromide
kDa	kilo dalton
l	liter
LA	type A ligand-binding domains
LB	luria broth
LCAT	lecithin:cholesterol-acyltransferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LH	laying hen
LPL	lipoprotein lipase
LR8	low-density lipoprotein receptor related protein with 8 ligand-binding domains
LRP	low-density lipoprotein receptor related protein
µg	microgram
µl	microliter
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MWCO	molecular weight cut-off
Ni-NTA	nickel nitriloacetic acid
nm	nanometer
nt	nucleotides
N-terminal	amino-terminal

o/n	over night
OD	optical density
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PSCK9	proprotein convertase subtilisin-like kexin type 9
R/O	restricted ovulator
RAP	receptor associated protein
rev	reverse
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sec	second
SRE	sterol responsive element
SREBP	sterol responsive element binding protein
T	thymine
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TBS	tris-buffered-saline
TEMED	N,N,N´,N´tetramethylethylendiamine
TG	triglyceride
TMB	3,3′,5,5′-Tetramethylbenzidine
U	unit
UV	ultraviolet
V	volt
VLDL	very low-density lipoprotein
VLDLR	very low-density lipoprotein receptor
VTG	vitellogenin

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08/2008 – 08/2009	**Medical University of Vienna/MFPL** Diploma thesis at the Department of Medical Biochemistry, Dr. Bohrgasse 9, 1030 Vienna Title: "Mutational analysis of Ligand- LDL Receptor Interactions"
10/2004 -08/2008	**University of Vienna** Undergraduate Studies in Microbiology and Genetics, University of Vienna, Austria
2001/2004	**Vienna General Hospital AKH** „Akademie für den medizinisch-technischen Laboratoriumsdienst", AKH Vienna, Lazarettgasse 14, 1090 Vienna, Austria Diploma thesis: "Analysis of additional drug use of opioid-addicted pregnant women under substitution treatment with Methadon or Buprenorphin."
09/1993 – 06/2001	**Bundesrealgymnasium (BRG) Rohrbach, Austria** Main focus on foreign languages and natural sciences

EMPLOYMENT

Since 02/2006 Vienna General Hospital AKH
Clinical Department of Blood Group Serology and Transfusion Medicine, Division of Blood Group Serology
Biomedical technician (20h/week)
Main tasks: Blood typing via serological and molecular biological methods, Serological cross-matching and preparation of blood preservations

10/2004-01/2006 Vienna General Hospital AKH
AKH Vienna, Department of Gynaecology, Division of Medical Genetics
Biomedical technician (20h/week)
Main tasks: prae- and postnatal cytogenetic analysis: Cultivation of white blood cells, amniotic fluid- and trophoblast cells, Preparation and study of G-banded karyotypes, Molecular cytogenetics (FISH)

ADDITIONAL INFORMATION

Language skills
German native language
English fluent oral & written
French good knowledge
Italian basic knowledge

Computer Skills
Good knowledge with the user interface Windows XP
MS Office (Word, Excel, Powerpoint)
Adobe Photoshop
Online biological databases (PubMed, Ensemble, Blast)