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Identification of a New Binding Partner of
Chicken Apolipoprotein A-V

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ABSTRACT

Triglycerides, cholesterol, and phospholipids are essential components of all known biological systems. They function as energy source and starting point for many metabolic pathways. Therefore, lipid and lipoprotein metabolism are highly regulated processes, which ensure normal embryonic development and maintenance of systemic homeostasis in higher organisms. Lipoproteins function as transport vehicles for water-insoluble nutrients and other essential compounds, such as cholesterol and triglycerides, from their sites of assembly or synthesis via the circulation to peripheral tissues. There, a class of important biological molecules, the cell surface-exposed receptors, which mainly belong to the family of LDL receptor proteins, mediate the uptake of these lipoproteins. In the present study, I particularly focused on the interaction of certain of these receptors with the apolipoprotein moiety of lipoproteins. This interaction is one key step in the underlying functional mechanism by which lipoproteins and the corresponding important nutrients reach the tissues where they are needed. To gain better insights into the apolipoprotein-receptor interactions and their impact on lipid metabolism, these studies were performed in the chicken, a well-established and powerful model organism for lipid and lipoprotein research.

One member of the apolipoprotein family with lipoprotein receptor-binding ability was found to be apolipoprotein A-V (apoA-V), discovered in man and mouse in 2001. ApoA-V is a regulator of plasma triglyceride levels, as initially demonstrated using transgenic and gene knockout technologies in the mouse. Many functions have been proposed for this apolipoprotein, but the exact mechanism of how it alters triglyceride homeostasis remains to be clarified. However, human apoA-V was shown to possess the capacity to bind to the LDL receptor-related protein (LRP1), the mosaic receptor LR11, which is also a member of the LDL receptor family, and sortilin, a member of the Vps10p receptor family. The ability of apoA-V to bind to cell surface receptors was also demonstrated in the chicken system. For instance, the major oocyte plasma membrane receptor LR8 binds and internalizes chicken apoA-V (ggapoA-V) into the yolk compartment of the germ cell. Since apoA-V was shown to be a constituent of VLDL and HDL plasma lipoprotein fractions, this process appears to be crucial for the efficient clearance of these particles from the circulation. To gain more insights into the biology of ggapoA-V concerning its receptor-binding ability, several chicken tissues were scanned in order to identify additional interaction partners of ggapoA-V.

Using ligand blot analysis, the peripheral membrane protein cubilin, a receptor for various well-studied ligands in mammals, but not yet characterized in chicken, was identified as binding partner of ggapoA-V. The yolk sac, which constitutes an important extraembryonic structure

supplying the growing embryo with nutrients, was shown to be a primary site of cubilin expression. The endodermal endothelial cells of the yolk sac, which face the yolk compartment, have been reported to express a specific set of cell surface receptors possessing high endocytic activity. However, detailed molecular mechanisms underlying this transport process are largely unknown. Therefore, the identification of a new receptor involved in endocytic processes as a binding partner of ggapoA-V sheds new light on the delivery of nutrients at the feto-maternal interface.

In concert with the facts that (i) ggapoA-V is bound and subsequently endocytosed by LR8 into the growing oocyte, (ii) ggapoA-V is present in the yolk compartment of the germ cell, (iii) the protein can also be detected in the yolk sac, and (iv) ggapoA-V is bound by cubilin most probably present at the surface of endodermal endothelial cells of the yolk sac, the steps constituting the transport pathway of ggapoA-V from the maternal liver to the circulation of the developing embryo have now been defined.

ZUSAMMENFASSUNG

Triglyzeride, Cholesterin und Phospholipide sind wichtige Bestandteile aller lebenden Organismen. Diese Substanzen dienen als Energiequelle und Ausgangsmaterial für eine große Anzahl an Stoffwechselvorgängen. Um zu gewährleisten, dass mit dem Lipidstoffwechsel verbundene Prozesse wie Embryonalentwicklung, Steroidhormonbiosynthese und Energiehomöostase problemlos ablaufen können, bedarf es einer komplexen und strikten Regulation dieser Vorgänge.
Die Hauptaufgabe von Lipoproteinen besteht im Transport von wasserunlöslichen Stoffen wie Triglyzeriden und Cholesterin aus der Blutbahn in Bereiche des Körpers, an denen sie benötigt werden. Viele Gewebe, die auf die Aufnahme von Lipiden angewiesen sind, produzieren eine hoch konservierte Klasse von Lipoproteinrezeptoren, nämlich jene der Familie der LDL-Rezeptor Proteine. Die meisten dieser Rezeptoren weisen eine hohe Endozytose-Kapazität auf, mit der die Aufnahme der Lipoproteinpartikel erst ermöglicht wird.
In der vorliegenden Studie wurde die Interaktion und Bindungsaffinität der Apolipoproteinkomponente der Lipoproteine mit an der Zelloberfläche lokalisierten Rezeptoren genauer untersucht. Als Modellorganismus für die hier durchgeführten Experimente wurde das Huhn (*Gallus gallus*), ein beliebter Modellorganismus zur Erforschung des Lipidstoffwechsels, herangezogen.

Ein Mitglied der Apolipoproteine, die Rezeptorbindungsaktivität aufweisen, ist das erst im Jahr 2001 charakterisierte Apolipoprotein A-V (apoA-V). Im Tiermodell konnte mit apoA-V knock-out und transgenen Mäusen gezeigt werden, dass apoA-V großen Einfluss auf den Plasmatriglyzeridspiegel hat. Funktionelle Studien zeigten ein breites Wirkungsspektrum des Proteins auf, jedoch konnte der exakte Mechanismus mit dem apoA-V den Triglyzeridspiegel beeinflusst noch nicht im Detail aufgeklärt werden.
Eine interessante Eigenschaft dieses Apolipoproteins ist seine hohe Bindungsaffinität an die Ligandenbindungsdomäne von Rezeptoren. Im Menschen wurden bis jetzt zwei Rezeptoren der LDL-Rezeptor Familie, LRP1 und LR11, und ein Protein der Vps10p-Rezeptor Familie, Sortilin, als Bindungspartner von apoA-V identifiziert. Diese Rezeptorbindungseigenschaft von apoA-V konnte auch im Huhn bestätigt werden. Der homologe Rezeptor zum menschlichen VLDL Rezeptor, LR8, den Hühner an der Oberfläche ihrer Oozyten exprimieren, bindet das ggapoA-V Protein mit hoher Affinität. Weiters konnte gezeigt werden, dass dieser Rezeptor ggapoA-V in den Oozyten aufnimmt. Da apoA-V ein Bestandteil von VLDL und HDL Lipoproteinfraktionen ist, kann die Rezeptorbindung dieses Proteins als wichtig für das Entfernen von Lipoproteinen aus dem Blutkreislauf und der Aufnahme dieser Partikel in verschiedenste Gewebe angesehen werden. Im Huhn scheint dieser Mechanismus die Aufnahme von Lipoproteinen in den

wachsenden Oozyten zu begünstigen. Um mehr über die Rezeptorbindungsaktivität von ggapoA-V zu erfahren, wollten wir neue Bindungspartner von diesem Apolipoprotein ausfindig machen.

Im Dottersack, einer extraembryonalen Struktur des Hühnerembryos die den wachsenden Embryo mit Nährstoffen versorgt, konnte ein neuer Interaktionspartner von ggapoA-V detektiert werden. Das isolierte Protein konnte mittels Massenspektroskopie als Cubilin identifiziert werden. Cubilin, ein im Huhn bis jetzt noch nicht charakterisiertes Protein, ist ein peripheres Membranprotein, das häufig an der Oberfläche von Epithelzellen anzufinden ist. Dieses Glykoprotein bindet ein großes Spektrum an Liganden und besitzt eine gut charakterisierte endozytotische Aktivität. Die Entdeckung, dass Cubilin ein Protein des Dottersackes des Huhnes ist, ergänzt die Daten von früheren Studien. Diese zeigten, dass der Dottersack ein spezifisches Set von Rezptoren mit Endozytoseeigenschaften synthetisiert. Da die Transportprozesse, die essenziell für die Ernährung des heranwachsenden Embryos sind, noch nicht im Detail verstanden werden, trägt die Identifizierung von Cubilin als Bindungspartner von ggapoA-V im Dottersack des Huhnes zur Verbesserung des Verständnisses dieser Mechanismen bei.

Gemeinsam mit den schon bekannten Details, dass (i) ggapoa-V vom Oozytenrezeptor LR8 gebunden und in die Keimzelle aufgenommen wird, (ii) ggapoA-V im Dotter der Eizelle detektiert werden konnte, (iii) das Protein auch im extraembryonalen Dottersack anwesend ist und (iv) ggapoA-V von Cubilin gebunden wird, sind nun alle Abschnitte des Transportweges von ggapoA-V von seinem Syntheseort in der mütterlichen Leber bis hin zu den Strukturen des heranwachsenden Embryos bekannt.

1.INTRODUCTION

1.1. Structure and Function of Lipoproteins

Lipoproteins function as transport vehicles for water insoluble nutrients and other essential compounds, such as cholesterol and triglycerides, from their sites of assembly or synthesis through the circulation to peripheral tissues (Elkin 1997). The general structure of these large lipid-protein complexes is widely conserved across species, pointing to their functional importance.

The inner core of lipoproteins consists of hydrophobic triglycerides and cholesterol esters, which are surrounded by a layer of polar lipids including free cholesterol and phospholipids, as well as apolipoproteins (Figure 1.1). These molecules, by exposing their hydrophilic domains to the surrounding milieu, ensure the transport capacity of lipoproteins.

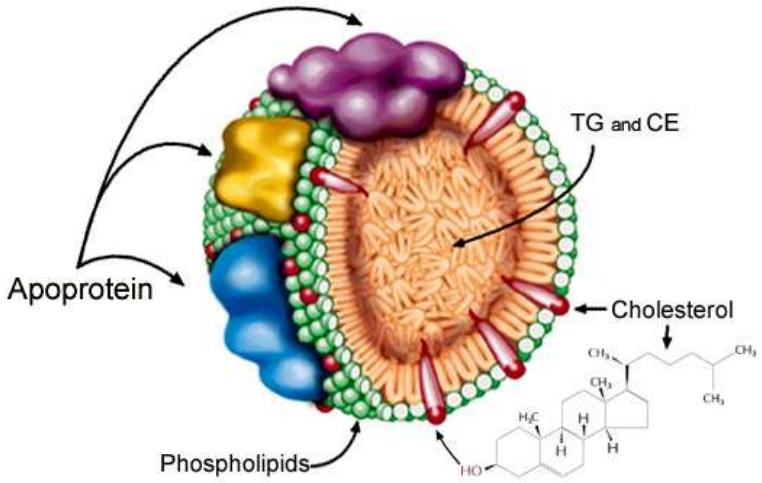

Figure 1.1. Schematic view of a lipoprotein particle. TG, triglycerides; CE, cholesterol esters (Picture from: www.peprotech.com)

1.2. Chicken Lipoproteins

Analogous to the situation in mammals, lipoprotein metabolism in the chicken presents an essential pathway for the delivery of lipids to specific tissues. The transported components are mainly required as energy source, for storage, and for the biosynthesis of membranes and steroid hormones. In addition, close similarities exist when comparing lipoprotein profiles of male or immature female chickens to that in mammals (Elkin 1997).

In contrast to these similarities, the differences in the lipid transport system of mature, egg-laying birds to that of the mammalian system are the decisive factor for making these animals perfect model organisms to study estrogen-induced hyperlipidemia, diet-induced

hypercholesterolemia and the molecular mechanisms underlying lipoprotein synthesis (Chapman 1980).

In general, lipoproteins are divided into different classes according to their size, density, and the presence of specific apoproteins on their surface. Table 1 shows a comparison of the lipid content between chicken and human lipoproteins.

Lipoprotein composition	**Porto / Chylomicrons**		**VLDL**		**LDL**		**HDL**	
	LH	**Human**	**LH**	**Human**	**LH**	**Human**	**LH**	**Human**
Density (g/cm^3)	<0.94		0.94 – 1.006		1.006 – 1.063		1.063 – 1.210	
Size (nm)	~150	~600	27 - 54	25 - 75	~23.4	20 - 25	17	7 - 12
Triglycerides	~90	~86	61.3	~55	11 - 41	7 - 11	~8	6 - 7
Cholesteryl esters	n.d.	2 - 4	2.9	15 - 23	7 - 35	47 - 51	8	24 - 45
Unesterified cholesterol	n.d.	1 - 3	5.4	4 - 9	8 - 13	10 - 12	1.5	6 - 8

Table 1.1. Comparison between chicken and human lipoproteins.

LH = Laying hen, VLDL = very-low -density lipoprotein, LDL = low-density lipoprotein, HDL = high-density lipoprotein. Triglycerides, cholesteryl esters, unesterified cholesterol = % of total dry weight of isolated lipoprotein fractions. Values for human lipoprotein particles are from Dennis E. Vance: Biochemistry of Lipids, Lipoproteins and Membranes, 5th Edition, laying hen lipoprotein composition was taken from (Chapman 1980).

1.2.1. Portomicrons

Exogenous lipids are taken up intestinally and packaged into large, triglyceride-rich particles called portomicrons. These portomicrons share functional and structural features with the mammalian chylomicrons concerning chemical composition and size of theses particles. In contrast to their mammalian counterparts, portomicrons are secreted directly into the portal vein, consistent with the fact that the lymphatic system is only poorly developed in chickens. However, when chicken lipoprotein fractions were separated with different techniques in several studies (Chapman 1980) a separate portomicron entity could not be identified (Steinmetz and Schneider 1999). One reason for why portomicrons have not been isolated until now could be that they are immediately and efficiently endocytosed by the avian hepatic lipoprotein receptors such as low-density lipoprotein receptor related protein 1 (LRP1) (Schneider 1995). Moreover, in contrast to mammals, intestinally synthesized portomicrons also contain apoB-100, as

chickens lack apoB-48, which is the major apolipoprotein component of chylomicrons. However, to learn more about this species of lipoproteins, further studies will be necessary.

1.2.2. Very-low-density lipoprotein (VLDL)

Avian VLDL is the major carrier of hepatically derived triglycerides. The VLDL particle is exclusively synthesized in and secreted by the liver and provides peripheral tissues, essentially the laying hen's growing oocytes, with lipids and other nutrients.

The major apolipoprotein present on VLDL particles is apoB-100, which is also synthesized by the liver and is a key regulator of VLDL synthesis and secretion (Fisher and Ginsberg 2002). A dramatic estrogen-induced increase of VLDL synthesis and secretion occurs in female chickens at the onset of egg production. Concentrations reach 1000-2000 mg VLDL-triglycerides / 100ml serum in mature laying hens, with the majority transported to the growing oocytes (Chapman 1980).

1.2.3. Vitellogenin

Vitellogenin is a lipophosphoglycoprotein exclusively synthesized by the liver and only present in egg laying birds (Chapman 1980). Like the VLDL particle, the plasma concentrations of vitellogenin increase dramatically via estrogen-induced hepatic synthesis.

Vitellogenin, together with VLDL, represents the major portion of the egg's yolk. These macromolecules bind to and are endocytosed into the growing oocyte by the chicken homolog of the mammalian VLDL receptor (VLDLR), termed LR8. Upon receptor-mediated endocytosis, the protein moieties of these lipoproteins are proteolytically degraded, thereby generating a characteristic set of polypeptides (Retzek, Steyrer et al. 1992). The catalyst for this intraoocytic processing of vitellogenin (and also for apoB) was found to be the lysosomal aspartyl protease Cathepsin D, which could be purified from oocytic yolk and preovulatory follicles (Retzek, Steyrer et al. 1992). The major proteolytic fragments of vitellogenin, lipovitellins and phosphitin, localize to membrane-enclosed endosomes in the yolk compartment termed yolk spheres, which reach a diameter of 140µm (Schneider, Osanger et al. 1998). Thus, the initial uptake, and the subsequent postendocytic processing of vitellogenin in the oocyte's yolk compartment is proposed to play an important role in the reproductive effort of the hen (Retzek, Steyrer et al. 1992).

1.2.4. Low-density lipoprotein (LDL)

A small amount of the VLDL fraction circulating through the bloodstream of chickens is lipolyzed to LDL via the action of lipoprotein lipase (LPL) bound to the luminal surface of endothelial cells

(Elkin 1997). Beside the fact that the chicken is an organism with a predominating VLDL lipoprotein fraction, LDL levels are in general rather low. Nevertheless, an avian LDL receptor (LDLR) orthologue was characterized in 2003 by Hummel et al, showing that this receptor prefers LDL over VLDL as ligand (Hummel, Lynn et al. 2003). The chicken LDLR shows the same structural features as its mammalian counterpart (Hummel, Lynn et al. 2003) demonstrating that the protein has been conserved in evolution. Differences can be seen when comparing avian and human LDLR expression levels, being rather low in the avian species (Schneider 2007). Furthermore, the delivery of LDL-derived cholesterol seems to be exclusively relevant in steroidogenic tissues, such as the adrenal and the ovary, which are also the main expression sites of the avian LDL receptor (Schneider 2007).

1.2.5. High-density lipoprotein (HDL)

HDL represents, besides VLDL, the main class of lipoprotein particles that are synthesized in and secreted by the liver and possibly certain peripheral tissues (Hermier 1997). The major apolipoprotein present on the HDL particle, apoA-I, is also synthesized almost exclusively by the liver. One characteristic feature of the HDL fraction is that it can be subdivided into several subclasses containing HDL1, HDL2, and HDL3, where HDL1 is the largest and least dense particle. The nascent, discoidal HDL fraction, which is secreted by the liver into the circulation contains about 1-4 copies of apoA-I. ApoA-I, located on the surface of the HDL particle, recovers cholesterol from the periphery to incorporate it into HDL. The enzyme lecithin:cholesterol acyltransferase (LCAT), which is also bound to the HDL particle, converts this cholesterol into insoluble cholesteryl-esters, which form the core of the growing HDL particle. Finally, these lipid-enriched HDL particles (mainly HDL1) become selectively internalized by the liver, thereby acting as the main mediators of the reverse cholesterol transport. Furthermore, the HDL particles can provide steroidogenic tissues like adrenals or the ovary with cholesterol.

In contrast to avian VLDL, the synthesis of apoA-I and HDL particles appears not to be induced by estrogen in the chicken. Rather, there is evidence that the apoA-I expression is reduced at the onset of egg laying (Schneider 1995). Nevertheless, it was shown that a significant amount of HDL particles are present in the egg's yolk (Vieira, Vieira et al. 1995). Furthermore, due to the fact that a specific oocyte plasma membrane receptor for the uptake of HDL particles could not be detected, an alternative process such as bulk phase uptake via a general endocytic activity of the oocyte plasma membrane has been proposed (Schneider 1995; Vieira, Vieira et al. 1995).

1.3. Apolipoproteins of the Chicken

Apolipoproteins present the protein moiety of lipoproteins. Each lipoprotein class is endowed with a specific set of apolipoproteins. These proteins are localized on the surface of the lipoprotein particles and are responsible for the very distinct features of each lipoprotein class. Table 2 shows the basic properties of chicken apolipoproteins characterized so far and their association with different lipoprotein classes.

Apolipoprotein	Chromosomal location	Molecular weight (kDa)	Primary source	Lipoprotein association	Function
ApoA-I	24	28	Liver, Intestine, Kidney	HDL	Reverse chol. transport, LCAT activator
ApoA-IV	24	38	Liver, Intestine	Not known	Lipid binding, LCAT activation
ApoA-V	24	40	Liver, Intestine	VLDL, lipid free, possibly HDL	Binds LR8, LRP380, TG modulation
ApoB-100	3	~500	Liver, Intestine, Kidney	VLDL+IDL, LDL, "light" HDL (kidney)	Controls VLDL secretion, LDLR + LR8 ligand
ApoC-III	24	9	Liver, Kidney, Fat	Not known	Lipase modulator
ApoVLDL-II	1	15 (homodimer)	Liver	VLDL	Inhibition of LPL

Table1.2. Chicken apolipoproteins.

1.3.1. Apolipoprotein A-I

Apolipoprotein A-I, whose gene is a member of the *APOA1/C3/A4/A5* gene cluster, is the major apolipoprotein of the HDL particle and well described in mammals as well as in chickens. The galline, mature apoA-I protein comprises 240 amino acids and has a molecular weight of about 28 kDa (Rajavashisth, Dawson et al. 1987). Although the protein shows only 48% sequence identity with mammalian apoA-I, the two proteins share structural and functional similarities. Both proteins are enriched in alpha helical structures at their C-terminus, a hallmark of many known apolipoproteins. Furthermore, it was shown that chicken apoA-I is capable in mobilizing cellular cholesterol, which subsequently is available for the esterification via LCAT, present on HDL particles. In agreement with the fact that chicken apoA-I has two potential lipid binding sites (residues 43-63 at its C-terminus, and residues 189-240 at its N-terminus), the protein has the ability to transform phospholipid vesicles into discoidal bilayer structures in vitro (Kiss, Ryan et al. 2001).

Analysis of the expression patterns of chicken apoA-I by Northern blotting revealed the presence of apoA-I mRNA in the liver, intestine, brain, adrenals, kidney, heart and muscle (Rajavashisth, Dawson et al. 1987). This expression pattern is very different to that of human

apoA-I, which is only expressed in the liver and intestine. Interestingly, the mRNA distribution of chicken apoA-I resembles that of mammalian apoE (Rajavashisth, Dawson et al. 1987).
In contrast to many other chicken apolipoproteins, a decrease of apoA-I expression was found when a chicken hepatoma cell line was treated with estrogen in cell culture experiments (Hermann, Foisner et al. 2003).
Furthermore, chicken granulosa cells (GC), which surround the growing oocyte, produce apoA-I. This was shown in (Hermann, Lindstedt et al. 1998), where cultured GC's secreted apoA-I containing particles of high density (< 1.2 g/cm^3) into the medium. These apoA-I containing particles might support the rapidly growing germ cell by the transfer of lipids directly to the plasma membrane of the oocyte. Another possible mechanism of these apoA-I containing particles is the delivery of cholesteryl esters to the steroid-producing cells of the theca layer (Hermann, Lindstedt et al. 1998).

1.3.2. Apolipoprotein A-IV

As another product of the *APOA1/C3/A4/A5* gene cluster, apoA-IV is one of the best characterized chicken apolipoproteins. The gene is located on chicken chromosome 24 and the apoA-IV gene is transcribed in the same direction as those specifying apoA-I and apoA-V. (Steinmetz and Schneider 1999). The mature chicken apoA-IV protein contains 347 amino acids, has a molecular weight of about 38 kDa and lacks cysteine residues. The primary expression sites of the avian apoA-IV gene are liver and intestine, as revealed by Northern Blot analysis (Steinmetz and Schneider 1999). The protein contains alpha helical structures, has lipid binding affinity and is capable of activating LCAT in vitro (Steinmetz and Schneider 1999). Although the human apoA-IV protein shows about 57% sequence identity with its avian counterpart, structural and also functional features seem to be different. Chicken apoA-IV is very hydrophobic and also is more acidic than its mammalian orthologue. The chicken apoA-IV protein is truncated at position 346 and thus lacks the mammalian C-terminal EQQQ motif. In 2000, Weinberg et al. showed with Fourier transform plots and Circular dichroism spectroscopy that chicken apoA-IV structurally and biophysically seems to be more related to human apoA-I than to human apoA-IV (Weinberg, Cook et al. 2003). They suggested that apoA-IV originates from a duplication of the apoA-I gene as a result of evolutionary changes in the genomic structure.
Only little is known about the association of chicken apoA-IV with lipoproteins. Its mammalian orthologue is found to be an apoprotein of chylomicrons and HDL with a high ability to activate LCAT. Due to its expression in the liver, one function of avian apoA-IV could be that it associates with portomicrons and facilitates the uptake of these particles into the liver. In addition, apoA-IV as a constituent of HDL is thought to be involved in the mobilization of cholesterol from peripheral cells. Therefore, apoA-IV could be important in providing the

growing oocyte with cholesterol or in mediating the reverse cholesterol transport back to the liver (Steinmetz and Schneider 1999).

1.3.3. Apolipoprotein B

Apolipoprotein B (apoB) is unique among the plasma apolipoproteins because of its extremely high overall hydrophobicity, high molecular weight (~500 kDa) and inability to transfer between lipoproteins of different classes (Kane 1983). In humans, apoB occurs in two forms, apoB-100 and apoB-48. The larger apoB-100 consits of 4536 amino acids, whereas apoB-48, arising by cotranscriptional mRNA editing, corresponds to the N-terminal 48% of apoB-100 (Chen, Habib et al. 1987). In humans, apoB-100 is predominantly synthesized in the liver, whereas apoB-48 is mainly expressed in the intestine (Glickman, Rogers et al. 1986).

In contrast to that, in the chicken the apoB-100 mRNA is unedited. Therefore, these animals produce apoB-100 only. The chicken apoB-100 mRNA has approximately the same size as the mammalian apoB transcript (~14kb), present at high levels in the liver and intestine. Unlike in mammals, the chicken apoB-100 mRNA is also found in the kidney. The hepatic mRNA levels increase rapidly following treatment with estrogen.

As the major protein component of VLDL, apoB-100 is important for the structural and functional integrity of the lipoprotein particle. For example, apoB, together with the microsomal triglyceride transfer protein (MTP) is essential for the assembly and secretion of VLDL particles. MTP functions in transferring lipids to apoB during its translation. Furthermore, MTP translocates triglycerides into the endoplasmatic reticulum (ER), where apoB-free lipid droplets form. These droplets associate with the nascent apoB-containing particles to form mature VLDL lipoproteins (Davidson and Shelness 2000). The major portion of hepatically synthesized VLDL is taken up by the major oocyte receptor LR8 into the growing germ cell (Bujo, Hermann et al. 1994). ApoB-100, present on these VLDL particles, mediates the receptor binding of VLDL (Nimpf, George et al. 1988) leading to subsequent endocytosis. Like vitellogenin, apoB-100 undergoes partial proteolytic degradation after the uptake into the growing oocyte, mediated by the protease Cathepsin D (Retzek, Steyrer et al. 1992). This proteolysis leads to a specific pattern of apoB-fragments in the oocyte's yolk. However, this degradation was shown not to affect the recognition of yolk VLDL by LR8 (Nimpf, Radosavljevic et al. 1989), which is also expressed on the surface of endodermal endothelial cells (EEC's) of the embryonic yolk sac (Hermann, Mahon et al. 2000). Therefore it is proposed that VLDL particles from the yolk are delivered to the EEC layer of the yolk sac by an endocytic process. Following uptake, the yolk VLDL particles are degraded in the EEC's and most probably used for subsequent reassembly and/or resynthesis of embryonic VLDL particles. That reassembly of VLDL occurs in the EEC layer is supported by (i) the presence of apoB mRNA and the MTP protein in the yolk sac (Hermann, Mahon et al. 2000), and (ii) the absence of apoVLDL-II from VLDL in the embryonic circulation.

The apoB production in chicken kidney was measured to be approximately 30% of that of the liver (Tarugi, Ballarini et al. 1998). Whereas the hepatic apoB is only present on VLDL and LDL lipoprotein particles, renal apoB was shown to be associated with VLDL, LDL and also with HDL fractions (Tarugi, Ballarini et al. 1998). Unlike in the liver, estrogen treatment has no effect on the synthesis of apoB in the kidney, nor does it induce the expression of the hepatic VLDL-associated lipoprotein lipase inhibitor apoVLDL-II (Kirchgessner, Heinzmann et al. 1987; Lazier, Wiktorowicz et al. 1994).
However, whether renal synthesis of apoB-containing lipoproteins contributes to the regulation of plasma lipoprotein levels in the chicken remains to be elucidated.

1.3.4. Apolipoprotein C-III

Only very little is known about apolipoprotein C-III (apoC-III) in the chicken. As the product of another member of the *APOA1/C3/A4/A5* gene cluster, apoC-III comprises 92 amino acids, including a 21 amino acid signal peptide. The protein has a calculated molecular weight of 9 kDa and shares 32% sequence identity with human apoC-III. The expression of chicken apoC-III is mainly restricted to the liver, with low amounts of apoC-III mRNA also detected in the kidney and fat as shown by Northern blot analysis (Nikolay 2006).
Detailed studies in the mammalian system revealed that plasma apoCIII levels are positively correlated with plasma triacylglycerol concentrations and that the protein is a direct or indirect inhibitor of LPL. To gain better insights into the biology of chicken apoC-III, further studies have to be conducted.

1.3.5. Apolipoprotein VLDL-II

In the laying hen, the second major protein component of the VLDL particle besides apoB is apolipoprotein VLDL-II (apoVLDL-II). The laying hen's serum contains about 1.5 mg/ml VLDL protein, whereof approximately 40-60% corresponds to apoVLDL-II. Therefore, the serum concentration of apoVLDL-II ranges from 600 to 900 µg/ml in the laying hen (Schneider, Carroll et al. 1990). Located on chicken chromosome 1, the gene for apoVLDL-II encodes a 9.5 kDa protein which associates via a single cysteine residue into a disulfide-bonded homodimer. Moreover, apoVLDL-II is exclusively present on VLDL particles of laying hens, but is not found in roosters or immature hens (Schneider, Carroll et al. 1990).
With the onset of egg laying, massive amounts of VLDL particles are transported from the liver to the growing oocyte. Interestingly, it was found that these particles remain unaffected during the transport through the circulation, since no appreciable hydrolysis of triglycerides occurs. The presence of active LPL on the surface of capillary endothelial cells, and in addition, the synthesis of LPL by the granulosa cell layer of the chicken follicle, make the fact that the lipid

composition of yolk VLDL particles resemble that of the VLDL fraction present in the circulation an even more astounding event.

This protection of the VLDL particles against lipolysis on their way from the liver to the growing oocyte was found to be mediated by apoVLDL-II, which is a potent inhibitor of LPL (Schneider, Carroll et al. 1990). Therefore, the inhibition of LPL in the periphery leads to an accumulation of triglycerides in the female germ cell.

The mature form of chicken apoVLDL-II present on VLDL particles is a homodimer. In contrast, apoVLDL-II from the Japanese quail (*Coturnix coturnix*), where the cysteine at residue 75 is replaced by a tryptophan, is associated with VLDL as a monomer (MacLachlan, Steyrer et al. 1996). Nevertheless, dimerization of the protein is not required for exerting its inhibitory effect since both avian proteins markedly inhibit the activity of LPL (MacLachlan, Steyrer et al. 1996).

1.3.6. Mammalian Apolipoprotein A-V

Identification and genomic organization

The gene encoding apolipoprotein A-V (apoA-V) was first identified in 2001 by Pennacchio et al (Pennacchio, Olivier et al. 2001). They looked for so far unidentified evolutionary conserved sequences in the well studied *APOA1/C3/A4* gene cluster on human chromosome 11q23 by comparing about 200 kilo base pairs (kbp) of mouse and orthologous human DNA (Pennacchio, Olivier et al. 2001). A well conserved sequence stretch, located ~30 kilobases (kb) proximal to the *APOA1/C3/A4* gene cluster (Figure 1.2), was identified as a four exons containing ~1100 base pair (bp) open reading frame. This human genomic locus encodes a 366 amino acid protein including an amino terminal signal peptide with 71% sequence identity to its mouse counterpart. Furthermore, it was shown that murine apoA-V shares the greatest sequence homology with apoA-IV (24% identical and 49% similar). Northern blot analysis from several human and mouse tissues using *APOA5* cDNA probes revealed two transcripts about 1.3 and 1.9 kb, predominantely present in the liver of both species (Pennacchio, Olivier et al. 2001).

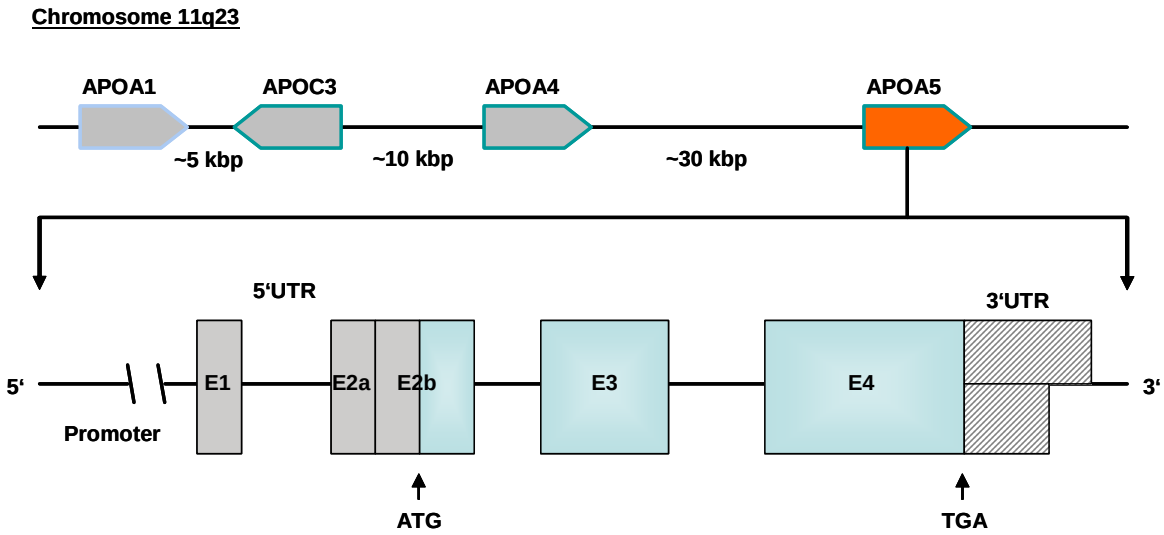

Figure 1.2. ApoA-V is a member of the *APOA1/C3/A4* gene cluster on human chromosome 11. The gene is composed of four exons (E1, E2a, E2b, E3, E4), a 5' untranslated region (5'UTR, grey boxes) and an alternative 3'UTR. The protein coding exons (E2b, E3, E4) are marked in blue. ATG and TGA represent the start and stop codons, respectively.

Biochemical and structural properties of apoA-V

ApoA-V, identified as a family member of the exchangeable apolipoproteins, shares common structural motifs with this group of proteins. Human apoA-V is nearly insoluble at neutral pH and lacks common sites for glycosylation (Beckstead, Oda et al. 2003). The amino acid composition of apoA-V is similar to that found in other apolipoproteins, whereas far-UV circular dichroism (CD) studies of apoA-V revealed that the protein is composed of 32% alpha helix, 33% beta sheet, 16% beta turn and 18% random coil in the lipid-free form (Beckstead, Oda et al. 2003). Thus, unlike most other apolipoproteins, apoA-V displays significant amounts of beta structure. Furthermore, apoA-V binds to dimyristoylphosphatidylcholine (DMPC) vesicles, thereby showing solubility at neutral pH and an increase in alpha helical structure up to ~50%.

At least 3 alpha helices were shown to be located in the N-terminal region (amino acids 1-146) of the apoA-V protein (Wong, Beckstead et al. 2008), whereas the C-terminal part of the protein was reported to possess strong lipid binding activity (Beckstead, Wong et al. 2007).

APOA5 gene regulation

In general, the plasma levels of apoA-V are low. Van der Vliet et al (van der Vliet, Sammels et al. 2001) reported concentrations about 1µg/ml in rodents' plasma, whereas Ishihara et al (Ishihara, Kujiraoka et al. 2005) and O'Brien et al (O'Brien, Alborn et al. 2005) observed plasma levels of 0.1–0.4µg/ml in humans. Thus, plasma levels of apoC-III and apoA-I are approximately 6– and 50–fold higher than those of apoA-V, respectively (Hahne, Krempler et al. 2008).

APOA5 gene expression is regulated by several transcription factors. The peroxisome proliferator activated receptor alpha (PPARA), the hepatocyte nuclear factor 4 alpha (HNF4A), and the upstream regulatory factor 1 (USF1) contribute to an upregulation of apoA-V mRNA levels (Prieur, Coste et al. 2003; Nowak, Helleboid-Chapman et al. 2005; Prieur, Schaap et al. 2005). Moreover, apoA-V mRNA levels were elevated in rats after partial hepatectomy (van der Vliet, Sammels et al. 2001). In contrast, liver X receptor alpha (LXRA) and insulin (by phosphorylation of USF1) were shown to down-regulate *APOA5* transcription (Nowak, Helleboid-Chapman et al. 2005; Jakel, Nowak et al. 2006). In addition, by simulating a condition of acute inflammation in a mouse model, a significant degradation of apoA-V mRNA was observed in response to the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) (Becker, Schomburg et al. 2006).

Physiological role of apoA-V

ApoA-V is a regulator of plasma triglyceride (TG) levels. This was initially demonstrated using mouse transgenic and gene knockout technologies. Mice overexpressing a human *APOA5* transgene showed decreased plasma TG levels of about 70% compared to their control littermates. In contrast, apoA-V knockout mice showed a fourfold increase of plasma TG's (Pennacchio, Olivier et al. 2001). These data are in sharp contrast to those observed for apoC-III knockout and transgenic mice, indicating that these two proteins have antagonizing effects on plasma TG levels. Also some single nucleotide polymorphisms (SNP's) in the *APOA5* gene show a strong correlation with plasma TG levels in humans.

Furthermore, apoA-V associates with lipoprotein particles. The protein is present in VLDL, HDL and chylomicrons, but not in the LDL fraction of human lipoproteins (O'Brien, Alborn et al. 2005). Interestingly, in the apoA-V knockout mouse, the synthesis of VLDL particles was increased. Thus, it was proposed that apoA-V may act as an inhibitor of VLDL–triglyceride production (O'Brien, Alborn et al. 2005).

In a study of Merkel et al in 2005 (Merkel, Loeffler et al. 2005) it was found that LPL, bound to heparan sulfate proteoglycans (HSPG's), was activated by apoA-V, but the exact underlying molecular mechanism remains to be elucidated. Moreover, apoA-V was reported to associate with lipid droplets in a rat hepatoma cell line (Shu, Ryan et al. 2008). These findings, together with the fact that partial hepatectomy in rats increases apoA-V transcription (van der Vliet, Sammels et al. 2001) as well as induces lipid droplet formation (Turro, Ingelmo-Torres et al. 2006) indicate new functional properties of apoA-V.

Receptor binding

Recent reports have demonstrated a high capacity of apoA-V to bind various cell surface receptors. In experiments using surface plasmon resonance (SPR) it was shown that lipid free as well as DMPC–complexed apoA-V interacts with LRP1, the mosaic type-1 receptor

LR11/SorLA, and sortilin (Nilsson, Lookene et al. 2007; Nilsson, Christensen et al. 2008). Cell experiments showed that the binding of apoA-V to LR11 and sortilin resulted in rapid internalization of the protein into intracellular compartments. Moreover, apoA-V was able to enhance the binding of chylomicrons to LRP1-covered sensor chips (Nilsson, Lookene et al. 2007). The binding affinity of apoA-V to these receptors was decreased in the presence of heparin.

Together, these findings show that apoA-V binds to receptors possessing ligand binding domains present in proteins of the LDLR family (LRP1, LR11/SorLA) and to receptors of the Vps10p family (Sortilin).

In addition to the receptor binding ability of apoA-V, (Gin, Beigneux et al. 2007) reported that DMPC disks containing apoA-V bound to the glycosylphosphatidylinositol-anchored high-density lipoprotein–binding protein 1 (GPIHBP1), which is found exclusively on the luminal surface of endothelial cells (Gin, Beigneux et al. 2007).

1.3.7. Chicken Apolipoprotein A-V

The gene encoding chicken apolipoprotein A-V (ggapoA-V) is located on chicken chromosome 24 in a gene cluster that also contains *APOA1* and *APOA4* (Dichlberger, Cogburn et al. 2007). Like its human and mouse counterpart, ggapoA-V is synthesized as a precursor protein including an amino terminal signal peptide. This precursor protein comprises 351 amino acids and shows a high degree of conservation from chicken to mammals (42% identities between the chicken and human protein and 39% between chicken and murine apoA-V (Dichlberger, Cogburn et al. 2007)).

The mature protein comprises 328 residues and has a molecular mass of about 40 kDa. Using Western blot analysis, the ggapoA-V protein could be detectecd in chicken plasma, where it was found in the VLDL and in the d > 1.21 (HDL) fractions. The protein is also present in several tissues of the chicken, including the liver, small intestine, kidney, brain, and ovarian follicles (Dichlberger, Cogburn et al. 2007).

As its mammalian counterpart, ggapoA-V shows high affinity binding properties to cell surface receptors, as demonstrated for the main chicken oocyte receptor LR8 (Dichlberger, Cogburn et al. 2007). Interaction of ggapoA-V with LR8 is proposed to play an important role in the delivery of TG-rich lipoproteins to the growing oocytes of the chicken. In addition, ggapoA-V binds to another, chicken specific, member of the LDLR family termed LRP380 (Dichlberger A., unpublished data). This large protein is also proposed to be an oocyte specific, membrane associated receptor.

1.4. Binding Partners of Apolipoprotein A-V

The high capacity of apoA-V to bind endocytic receptors was shown in several studies using various methods. Until now, four proteins have been identified which show high-affinity interaction with this apolipoprotein, all of them acting as receptors with high endocytic activity. Figure 1.3 shows the overall structural composition of the binding partners of apoA-V.

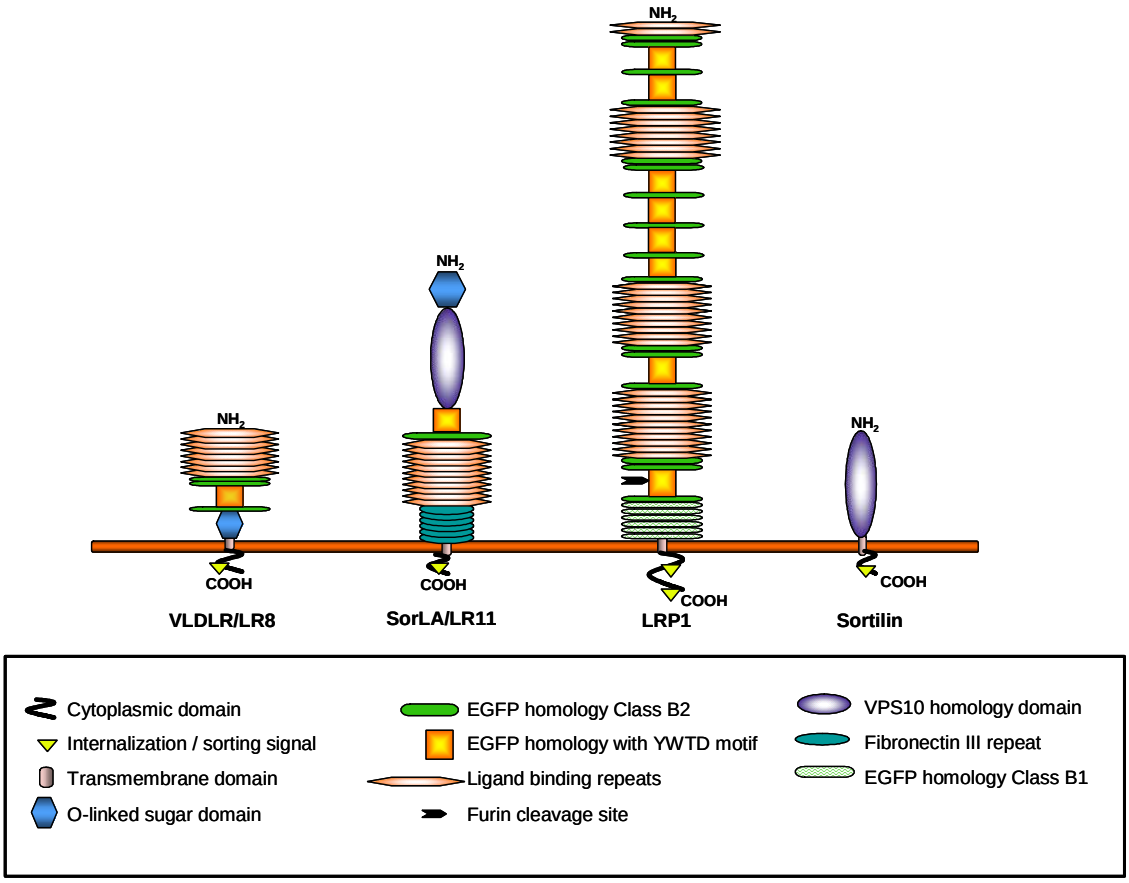

Figure 1.3. Thus far identified binding partners of apoA-V. ApoA-V was shown to interact with LR8, LR11 and LRP1, which are members of the LDLR family, and with sortilin, a member of the Vps10p receptor family.

1.4.1. LR8

A 95 kDa chicken plasma membrane receptor, predominantly expressed by the chicken oocyte, was initially identified as the receptor for the major components of yolk, VLDL and VTG (Stifani, Barber et al. 1990). Molecular characterization of this protein revealed a close structural relationship to the LDLR. Therefore, this protein was identified as a member of the LDLR supergene family, characterized by the presence of preserved structural domains. In order of appearance from the carboxy terminus, these structural domains of the receptor are (i) a cytoplasmic tail of about 50 amino acids containing the internalization sequence Asn-Pro-Xaa-Tyr (NPXY) which is essential for the targeting of the receptor into clathrin-coated vesicles; (ii) a short transmembrane stretch consisting of ~ 20 hydrophobic amino acids; (iii) the O-linked sugar domain, enriched in O-glycosylated serine and threonine residues; (iv) the epidermal growth factor (EGF) precursor homology domain comprising about 400 amino acids. This domain is composed of three repeats with high homology to EGF and a six-bladed beta propeller, with each of these six stretches carrying the consensus sequence motif Tyr-Trp-Thr-Asp (YWTD); (v) the ligand binding domain, exposed at the amino terminus of the protein which consists of approximately 40 amino acid residues, is highly enriched in cysteine residues and negatively charged amino acids. Chicken LR8 contains eight such ligand binding repeats (LA-repeats), in contrast to the seven LA-repeats of the LDLR (Schneider 2007). Eight LA-repeats are also a hallmark for the group of the mammalian VLDL receptors (VLDLR). Therefore, chicken LR8 represents the functional homolog to the mammalian VLDLR.

The gene for LR8 is located on the chicken sex chromosome Z (Bujo, Hermann et al. 1994) and the sequence shows 84% identity to its human counterpart. Chicken LR8, which shows high binding affinity for its ligands apoB present on VLDL particles (Nimpf, Radosavljevic et al. 1989) and the lipovitellin domain of VTG (Stifani, Barber et al. 1990), is essential for oocyte growth and development. The functional importance of the receptor is demonstrated by a non-laying chicken strain (Restricted Ovulator = R/O) carrying a single point mutation in the LR8 gene. Because VLDL-associated triglycerides are not deposited into the oocyte, these chickens develop severe hyperlipidemia and atherosclerotic lesions.

The predominant expression of LR8 in the chicken oocyte resembles that of the mammalian VLDLR, which also appears to be expressed in ovarian tissues (Webb, Patel et al. 1994). Besides its ligands apoB and VTG, LR8 was shown to bind the 39 kDa receptor-associated protein (RAP). RAP acts as a chaperone in the endoplasmic reticulum and binds to the ligand binding domains of all members of the LDLR family in intracellular compartments. This binding is crucial for the correct folding of the receptor proteins and prevents premature intracellular binding of their ligands (Stifani, Barber et al. 1990).

Furthermore, like all VLDLRs, LR8 exists in two different forms, LR8- and LR8+, generated by differential splicing (Hermann, Mahon et al. 2000). LR8-, which lacks the O-linked sugar domain

is predominantly expressed by the chicken oocyte. In contrast, LR8+ is the major form expressed by somatic cells (Hermann, Mahon et al. 2000), although at very low levels compared to those of LR8- in the oocytes.

1.4.2. Sortilin

Sortilin is a glycoprotein of approximately 100 kDa first isolated from human brain extracts by using RAP affinity chromatography (Petersen, Nielsen et al. 1997). The gene for human sortilin maps to chromosome 1p and encodes an 833-amino acid type I receptor. Sortilin was found to be expressed in several tissues including the brain, placenta, skeletal muscle, heart, spinal cord and testis (Mazella 2001). The protein shows no homology to the LDL receptor family members. Therefore, sortilin represents the first RAP binding partner not related to the LDL receptor family.

Furthermore, sortilin is the receptor for the neuropeptide neurotensin, which exerts various functions in mammals. In addition to its functional importance in the brain, neurotensin induces hypotension, decreases gastric acid secretion, and activates lipid digestion in the periphery (Mazella 2001). Sortilin was also identified as a major component of glucose transporter (Glut)-4 containing vesicles in rat adipocytes (Lin, Pilch et al. 1997).

The characteristic structural motif of sortilin is a cysteine-rich domain, homologous to a domain in the yeast vacuolar protein sorting 10 protein (Vps10p), which represents the extracellular or luminal part of the protein (Mazella 2001). In yeast, the protein is a sorting receptor for carboxypeptidase Y, located mainly in the trans-Golgi network (TGN). Figure 1.4 shows a comparison between the yeast sorting receptor Vps10p and human sortilin.

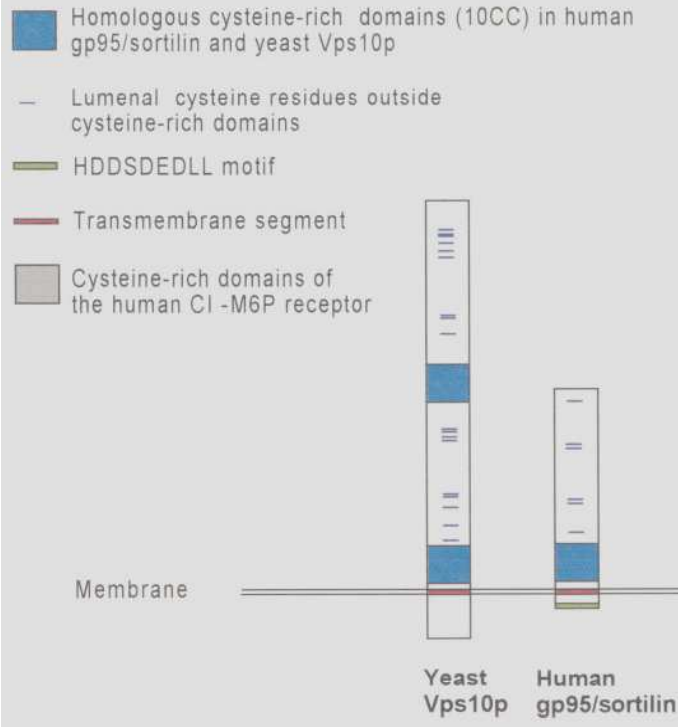

Figure 1.4. Comparison of the yeast sorting receptor Vps10p and human sortilin. Sortilin is composed of one Vps10p domain which shapes the luminal/ extracellular part of the protein. The most conserved stretch of the Vps10p domain, highly enriched in cysteine residues, is illustrated in blue. Moreover, sortilin contains 6 additional cysteines (lines), which are located in the extracellular region of the protein (Figure adapted and modified from (Petersen, Nielsen et al. 1997)).

The Vps10p domain is a ~ 700-amino acid module that functions as a protein interaction motif (Willnow, Petersen et al. 2008). The yeast Vps10p protein contains two such domains with an overall identity between them of 20%, whereas sortilin harbours only one, in agreement with the fact that the luminal/extracellular domain of human sortilin consists of exactly 726 amino acids (Petersen, Nielsen et al. 1997; Willnow, Petersen et al. 2008). Alignment of sortilin with the yeast Vps10 protein revealed a stretch of 140 amino acids with high homology between the two proteins. This region, located in the C-terminus of sortilin, contains 10 cysteine residues. Interestingly, these cysteines show an almost identical spacing in sortilin and Vps10p (Petersen, Nielsen et al. 1997). The structural homology between these “10 cysteine consensus” (10CC) regions most probably defines the core structural motif of the Vps10p domain (Petersen, Nielsen et al. 1997). Furthermore, the Vps10p domain of sortilin contains 6 additional cysteine residues, located more amino terminally when compared to the 140 amino acid core module (Figure 1.4).

The intracellular, C-terminal domain of sortilin shows sequence stretches with homologies to mannose-6-phosophate (M6P) receptors. At the very C-terminal tail, a nine-residue sequence (HDDSDEDLL) is found which is also present in the extreme C-terminus of the M6P receptors (Petersen et al). This peptide contains a casein kinase II phosphorylation site and an adjacent dileucin motif, both mediators for the formation of clathrin-coated vesicles and further TGN sorting (Petersen, Nielsen et al. 1997). In addition, the cytoplasmic tail of sortilin contains two

more partly overlapping sorting signals. The sequence YSVL (consensus motif YXXZ) mediates rapid internalization of some membrane receptors, including proteins of the M6P receptor family. The other motif (FLVHRY), located in close proximity to the membrane-spanning domain and partly overlapping with the YSVL motif, constitutes another internalization/sorting signal. Again, this sequence stretch with the overall motif (F/Y)XXXX(F/Y) is also found in M6P receptors and in members of the LDLR family (Petersen, Nielsen et al. 1997).

Sortilin is synthesized as an inactive precursor protein, which is converted to its active form by the proprotein convertase furin in late Golgi compartments. The furin cleavage site (R^{-4} - Xaa^{-3} - R/K^{-2} - R^{-1} - X^{+1}) precedes the Vps10p domain at the protein's amino terminus. Only after the maturation step, the receptor is able to bind its ligands, as is the case for the insulin receptor and LRP receptors (Ullrich, Gray et al. 1986).

Once translocated to the plasma membrane, it has been proposed that sortilin serves as a scavenger receptor for lipoprotein lipase (LPL) and/or neurotensin. Following endocytosis, these proteins are degraded in lysosomal compartments of the cell (Mazella 2001).

The numerous ligands already identified for this protein, its endocytic activity and the presence of structural motifs also found in other transmembrane receptors, provide evidence that sortilin presents a multifunctional protein involved in various cellular processes.

1.4.3. LR11 / SorLA

LR11 (LDLR related protein with 11 ligand binding domains, also termed SorLA), a highly conserved, 250 kDa type 1 receptor, belongs to the family of the LDLR related proteins (Jacobsen, Madsen et al. 2001). LR11 is a very complex seven-domain mosaic protein, which shows several interesting properties. The receptor shows > 80% sequence identity among mammals and birds, and its expression, in contrast to that of the LDLR, is not regulated by estrogen. Furthermore, LR11s are present in high amounts in the brain and have structural components which are not present in other members of the LDLR family. From its amino terminus towards its carboxy terminus, the extracellular or luminal part of the receptor is made up of a Vps10p domain which resembles that of sortilin, a cluster of five F/YWTD / beta propeller modules, an EGF-like module, a cluster of 11 LA repeats and a domain of six fibronectin type III repeats. The structural differences between sortilin and LR11 (SorLA) are shown in Figure 1.5.

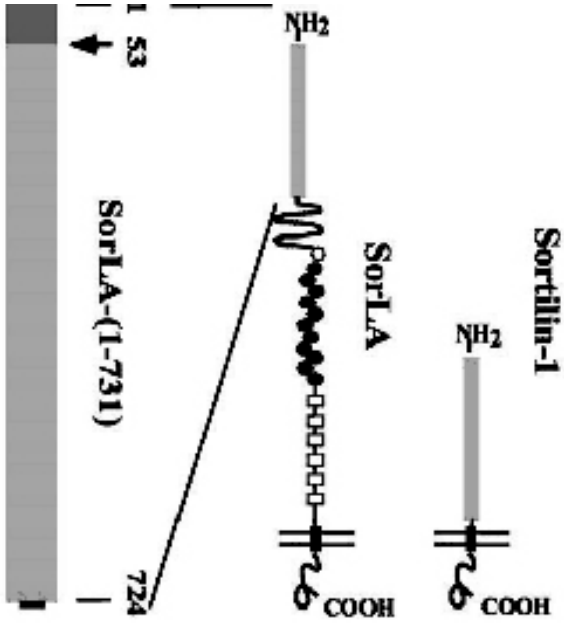

Figure 1.5. Schematic representation of LR11 (SorLA) and Sortilin. The extracellular domain of LR11 is composed of a Vps10p domain (grey), five (F/Y)WTD / beta propeller domains, an EGF-like module (open circle), 11 LA repeats (filled circles), and 6 fibronectin type III repeats (squares). In contrast, the Vps10p domain covers the whole extracellular part of sortilin. LR11, like sortilin, contains a furin cleavage site close to the amino terminus and preceding the Vps10p domain. The cleavage of the propeptide in the TGN converts the receptor into its active conformation (Figure adapted from (Jacobsen, Madsen et al. 2001)).

LR11 harbours a single transmembrane domain and a cytoplasmic tail which contains the signal sequence (F/Y)XXXX(F/Y) and an acidic cluster (residues 2162-2170), characteristic for proteins with endocytic and / or sorting activity (Jacobsen, Madsen et al. 2001). Furthermore, Jacobsen et al (Jacobsen, Madsen et al. 2001) showed, using recombinantly expressed human LR11, that this receptor, like sortilin, is synthesized as an inactive proreceptor. After cleavage of the propeptide by furin in the TGN, the protein achieves its active conformation. The luminal Vps10p domain, as well as the domain containing LA-repeats, show effective ligand binding properties. Using surface plasmon resonance studies, (Jacobsen, Madsen et al. 2001) showed binding of RAP, LPL, neurotensin, and apoE to the luminal domains of human LR11.

The site of highest LR11 expression was found to be the brain. This holds true for humans, rodents and also for avian species. In addition, to a smaller extent, mRNA of chicken LR11 could be detected in the adrenal gland and testis (Morwald, Yamazaki et al. 1997).

In addition to the role of LR11 as an endocytic receptor, the fact that a large amount of the protein localizes to perinuclear compartments led to the proposal that LR11 can also function as a sorting receptor (Jacobsen, Madsen et al. 2001).

1.4.4. LRP1

The low density lipoprotein receptor related protein 1 (LRP1) is one of the largest members of the LDLR family with an overall size of about 600 kDa. Its name is due to the fact that this unusually large membrane protein is composed exclusively of structural elements also present in the LDLR. In contrast to the seven ligand binding domains of the LDLR, LRP1 contains 31

LA-repeats clustered from the amino terminus into groups of 2, 8, 10, and 11 modules, respectively (Nimpf, Stifani et al. 1994). Furthermore, the receptor contains 22 EGF precursor homology domains (B1 and B2) and eight beta propeller structures carrying the YWTD motif (Figure 1.3). The receptor contains a single transmembrane segment. The intracellular stretch of about 100 amino acids contains two signals important for receptor endocytosis (Davis, van Driel et al. 1987). LRP1, like Sortilin and LR11, gets post-translationally modified by the protease furin, resulting in the generation of a 85 kDa membrane spanning protein and a 515 kDa extracellular domain that remain non-covalently associated (Herz, Kowal et al. 1990).

LRP1 is a multifunctional receptor involved in endocytic and signalling processes. To date it is known that LRP1 binds and subsequently endocytoses about 40 ligands, underlining the functional importance of this receptor. However, one of the most prominent ligands for LRP1 is alpha-2 macroglobulin (α-2M) (Strickland, Ashcom et al. 1990), a plasma protein that functions in inactivating proteinases present in the circulation. Therefore the receptor has also been termed LRP1/α-2M receptor. Mammalian LRP1 was shown to be the receptor which mediates the uptake and clearance of apoE-containing chylomicron remnants by the liver (Beisiegel, Weber et al. 1989). Furthermore, RAP was shown to be a ligand with chaperone function for LRP1.

Essential signalling functions for LRP1 were described in the vessel wall, where it controls the proliferation of vascular smooth muscle cells, thereby protecting from atherosclerosis (Boucher, Gotthardt et al. 2003). LRP1 is also involved in signalling processes in the brain and in the lung (Boucher, Gotthardt et al. 2003).

Chicken LRP1 was first described by Nimpf et al in 1994 (Nimpf and Schneider 1994), where an overall identity of 83% was observed between the protein sequence of chicken and its mammalian counterpart, with the intracellular domains being completely identical. Also the overall structures of human and chicken LRP1 are very similar. For instance, all cysteine residues present in the protein show perfect alignment between human and chicken. For the avian extracellular protein domain, Ca^{2+}, α-2 macroglobulin, and vitellogenin were identified as binding partners (Nimpf, Stifani et al. 1994). Interestingly, the laying hen expresses two different α-2M's. One is the classical, circulating α-2M with homology to the mammalian protein. The other one, termed ovostatin, also has high sequence similarity to mammalian and chicken α-2M (Nimpf, Stifani et al. 1994). In contrast, ovostatin is deposited into the egg white and is suggested to be the avian homologue to mammalian pregnancy zone proteins (Devriendt, Van den Berghe et al. 1991), which were also shown to bind LRP1 (Gliemann, Moestrup et al. 1986). Pregnancy zone proteins are expressed at high levels in the human trophoblast, which forms the outer layer of the blastocyst (Gliemann, Moestrup et al. 1986).

These findings suggest that LRP1 presents another important receptor involved in yolk deposition into the growing oocytes of egg laying species.

1.5. Aims of the Thesis

Because apolipoprotein A-V (apoA-V) attracts more and more attention as a potent regulator of plasma triglyceride levels, the general aim of my thesis was to learn more about the biology of this apolipoprotein. In particular, I focused on the property of apoA-V to interact with cell surface receptors. Therefore, the elucidation of new binding partners of apoA-V in the chicken was a major objective of my work. Fortunately, it was possible to detect new interaction partners of ggapoA-V in the chicken embryonic yolk sac by ligand blot analysis. One of these proteins was identified as chicken cubilin, a protein involved in the endocytosis of various ligands. This interesting finding raised a number of questions and provided the basis for new ideas and experiments.

2. MATERIALS and METHODS

2.1.Chemicals and Enzymes

Chemicals used for the production of buffers, solutions, and media were obtained from: Amersham Biosciences, Amresco, Applichem, Bio-Rad, Calbiochem, Fluka, Gerbu, Merck, Pierce, Roche, Roth, Sigma Aldrich, Star Lab and Zymed. The following polymerases were used: DyNAzyme EXT DNA Polymerase from FINNZYMES, SUPERScript RNase H^- Reverse Transcriptase II from Invitrogen, Pfu Ultra II Fusion HS DNA Polymerase from Stratagene and High Fidelity Polymerase from Fermentas. Other DNA-modifying enzymes used were T4-DNA ligase and Shrimp Alkaline Phosphatase (SAP) from Roche. All restriction enzymes were purchased from Roche and Fermentas Life Sciences.

2.2. Bacterial Strains and Vector Systems

Strain	One Shot TOP10 Chemically Competent *E. coli*
Genotype	F⁻*mcr*AΔ(*mrr-hsd*RMS*mcr*BC)ϕ80*lac*ZΔM15Δ*lac*X74*Deo*R*rec*A1 *ara*D139Δ(*araleu*)7697*gal*U*gal*K*rps*L(Str^R)*end*A1*nup*G
Reference/ Source	Invitrogen

Table 2.1. Bacterial strain used for transformations.

Strain	One Shot BL21 Star(DE3) Chemically Competent Cells
Genotype	*F^- ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm rne131 (DE3)*
Reference/ Source	Invitrogen

Table 2.2. Bacterial strain used for recombinant protein expression.

Vector	pBluescript II SK(+/-) phagemid
Size	2,961 basepair
Genotype	*f1(+/-) origin, ColE1 origin, LacI, LacZ, MCS, Ampicillin*
Reference/ Source	Stratagene

Table 2.3. Vector system used for cloning.

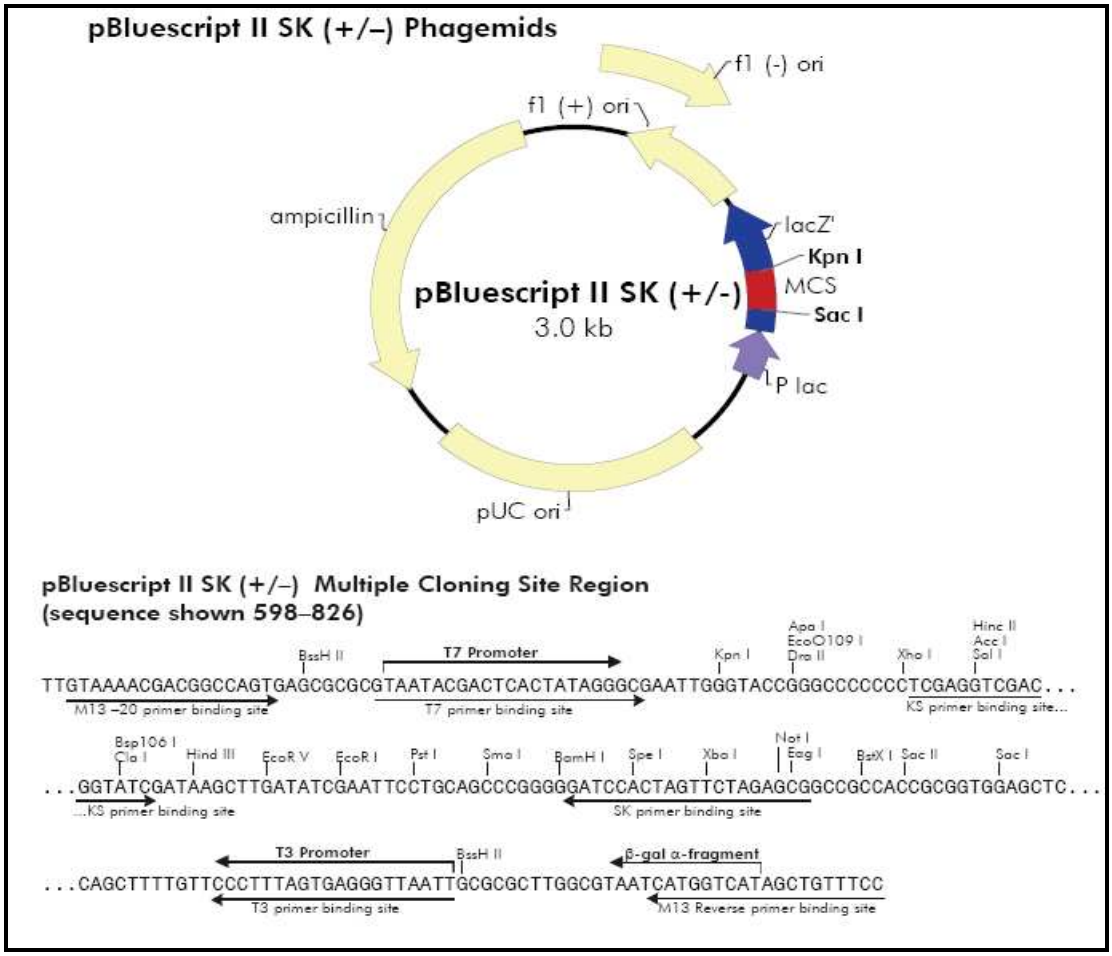

Figure 2.1. Genetic map of pBluescript II SK (+).

Vector	pCR®2.1-TOPO®
Size	3,931 basepairs
Genotype	*LacZα fragment, M13 reverse priming site, MCS, T7 promoter/priming site, M13 forward priming site, f1 origin, kanamycin resistance, ampicillin resistance, pUC origin*
Reference / Source	Invitrogen, TA cloning kit

Table 2.4. T/A cloning vector.

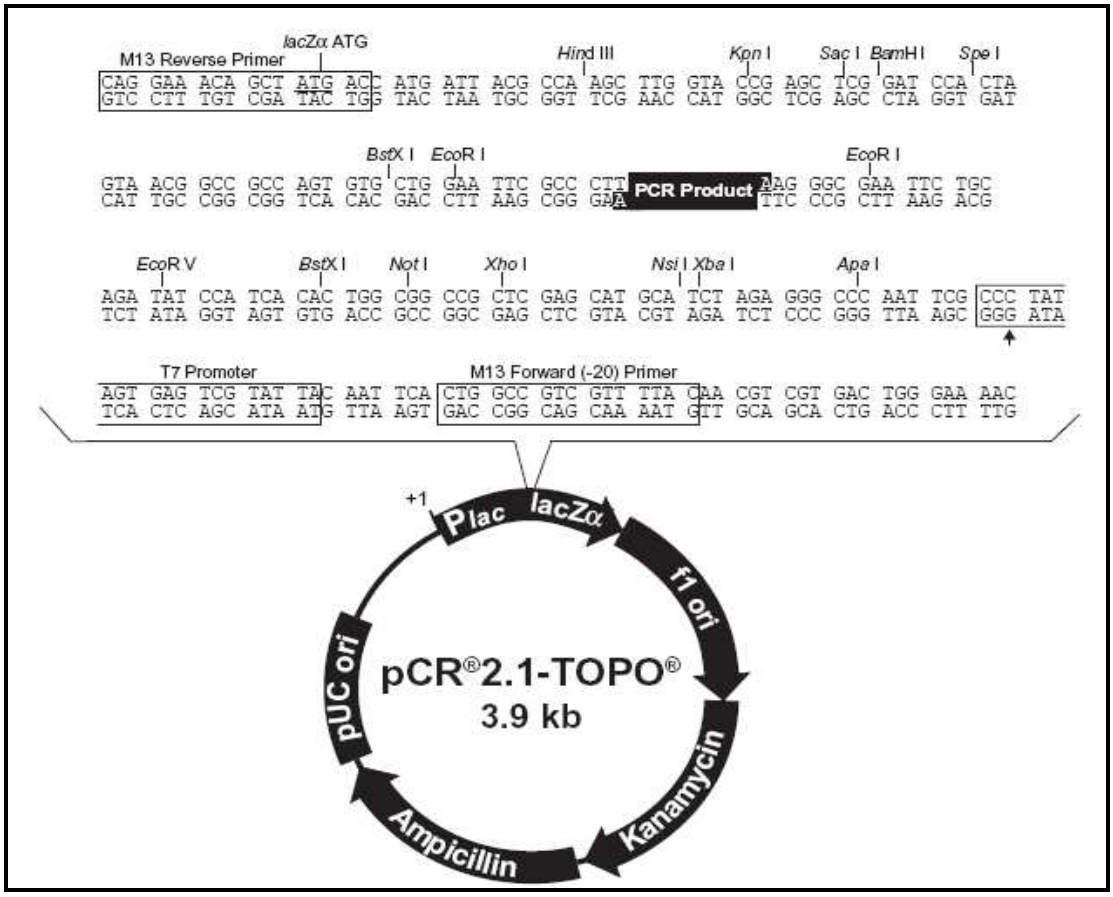

Figure 2.2. Genetic map of T/A cloning vector pCR 2.1-TOPO.

Vector	pET25b(+)
Size	3,931 basepairs
Genotype	*T7 promoter, T7 transcription start, pelB coding sequence, Multiple cloning sites (NcoI – XhoI), HSV Tag, His Tag, T7 terminator, lacI, pBR322 origin, bla, f1 origin*
Reference / Source	Invitrogen, TA cloning kit

Table 2.5. pET-25b(+) expression vector.

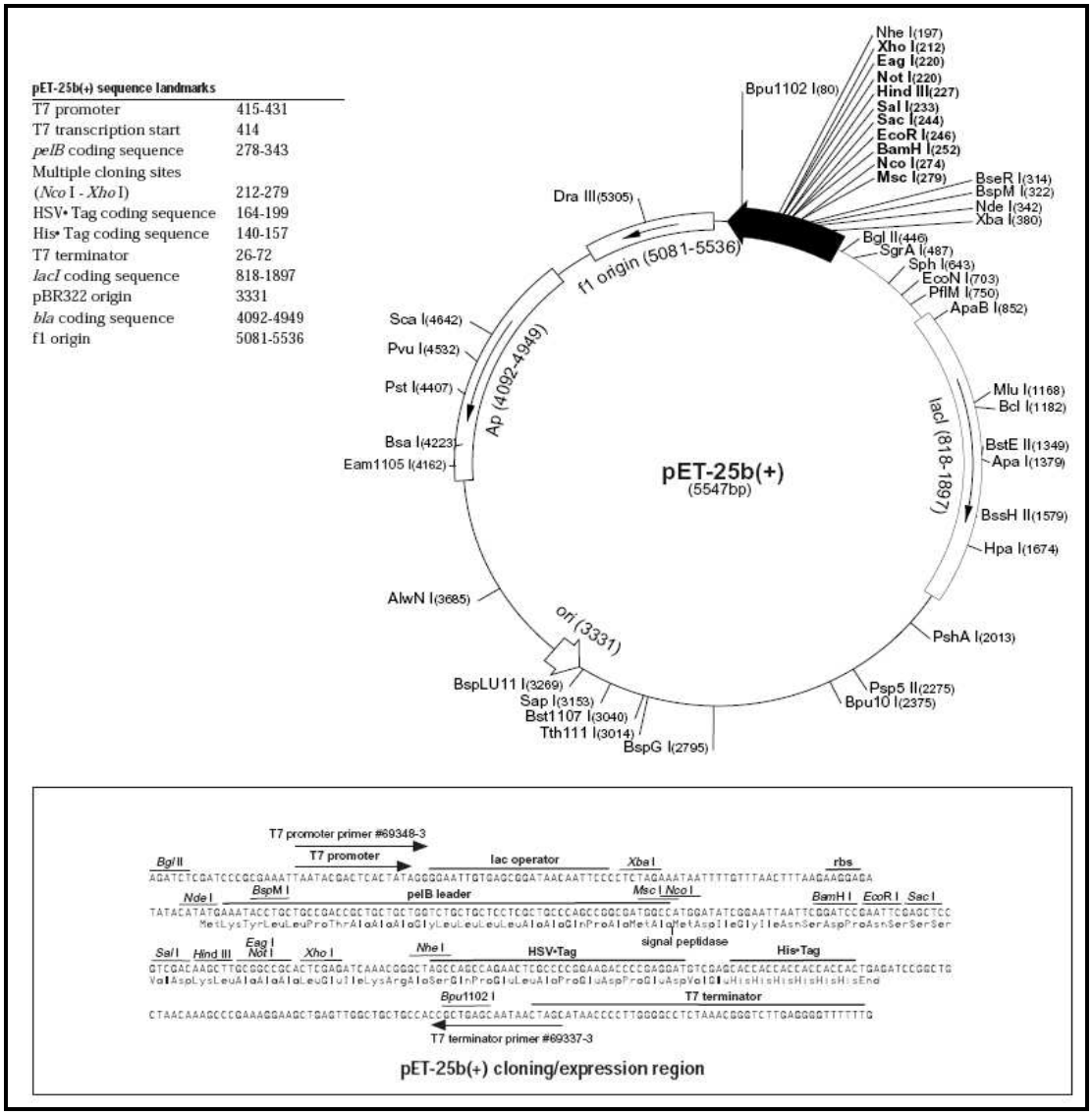

Figure 2.3. Genetic map of pET-25b(+) expression vector.

2.3. Animals

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.

2.4. Oligonucleotide Primers

The designed oligonucleotides were synthesized by MWG-Biotech AG.

pBluescript II SK(+/-)

Name	Sequence
T3	5‘-AAT TAA CCC TCA CTA AAG GG-3‘
T7	5‘-TAA TAC GAC TCA CTA TAG GG-3‘

Table 2.6. Specific primers used for sequencing PCR.

T/A cloning vector

Name	Sequence
M13fwd	5‘-GTA AAA CGA CGG CCA G-3‘
M13rev	5‘-CAG GAA ACA GCT ATG AC-3‘

Table 2.7. Specific primers used for sequencing PCR.

pET-25b(+) vector

Name	Sequence
T7 promoter primer	5’-TAA TAC GAC TCA CTA TAG G-3’
T7 terminator primer	5’-GCT AGT TAT TGC TCA GCG G-3’

Table 2.8. Specific primers used for sequencing PCR.

Primers used for cloning of the Cubilin fragment for antibody production

Name	Sequence
Ab_fwd_NcoI	5’-AAC CAT GGT CTA TGA TGA ACA GCC T-3’
Ab_rev_EcoRI	5’-TTG AAT TCG GTT TCC TCT GCA GAG T-3’

Table 2.9. Chicken Cubilin sequence-specific primers.

2.5. Molecular Biological Methods: DNA

2.5.1. cDNA Synthesis

For the synthesis of cDNA, SuperScript II Rnase H^- Reverse Transcriptase from Invitrogen was used. This enzyme synthesizes first strand cDNA and is able to generate cDNA up to 12.3 kb.
Total RNA was isolated as described in 2.6.1. and the following components were mixed together in a nuclease free microcentrifuge tube:

1µl oligo (dT) primers
3-5µg total RNA
1µl 10mM dNTP mix (10mM each dATP, dGTP, dCTP and dTTP)
Sterile distilled water up to 12µl

The mixture was heated to 65°C for 5min and afterwards quick chilled on ice. The contents of the tube was collected by brief centrifugation and the following components were added to the mixture:

4µl 5x First-Strand Buffer
2µl 0.1M DTT

The contents of the tube was mixed gently and incubated at 42°C for 2min. 1µl of SuperScript II RT (200 units) was added to each tube and mixed gently by pipetting up and down. An incubation step of 50min at 42°C followed. To inactivate the reaction, the tubes were heated at 70°C for 15min.
The cDNA was now used as template for amplification in PCR.

2.5.2. Polymerase Chain Reaction (PCR)

To amplify specific DNA fragments from previously synthesized cDNA, the Thermocycler T3000 from Biometra was used. PCRs were carried out with reaction volumes of 25 and 50µl. The polymerases used are listed in 2.1. Following components of a PCR reaction were mixed on ice in a nuclease free microcentrifuge tube:

50µl reaction volume
1µl cDNA
5µl 10x buffer (polymerase specific)
1µl forward + reverse primers (25pM / µl)
1µl dNTP mix (10mM)
1µl polymerase (Dynazyme or High Fidelity)
40µl ultrapure H_2O

25µl reaction volume

0.5µl cDNA

2.5µl 10x buffer (polymerase specific)

0.5µl forward +reverse primers (25pM / µl)

0,5µl dNTP mix (10mM)

0.5µl polymerase (Pfu)

20µl ultrapure H_2O

An optimized PCR program for the used primers and the length of the amplified fragment was chosen.

		program
Initial denaturation		10 min at 94℃
Denaturation	40 cycles	30sec at 94℃
Annealing		30sec at 55℃
Extension		30sec at 72℃
Last extension		10 min at 72℃

Table 2.10. Program used for PCR.

All PCR products were analysed by agarose gel electrophoresis.

2.5.3. DNA Gel Electrophoresis

Separation of DNA fragments was performed by gel electrophoresis through 1 % (w/v) agarose gels with a constant voltage of 100 V for 30 min. DNA samples were mixed with 5x DNA loading buffer containing Bromophenol blue or Xylene Cyanol FF serving as front marker and glycerol to increase the density of the sample, ensuring that DNA can be evenly loaded into the sample well. 1x TAE was used as electrophoresis buffer. For size determination of DNA fragments, the 1 Kb Plus DNA Ladder from Invitrogen was used. The agarose gel contained the fluorescent intercalating dye ethidium bromide to visualize the DNA fragments under ultraviolet light (366 nm).

50x TAE, pH 8.0
2 M Tris/HCl
1 M acetic acid
0.1 M EDTA

5x DNA loading buffer
50 % Glycerin
0.1 M EDTA 50x TAE Bromophenol blue or Xylen Cyanol FF

Ethidium bromide stock solution
10 mg/ml in H_2Odd

StarPure AGAROSE from StarLab

2.5.4. DNA Gel Extraction

PCR products or restriction enzyme digested DNA fragments were extracted and purified from an agarose gel using the QIAquick Gel Extraction Kit Protocol from Quiagen. This protocol is used to extract and purify DNA of 70bp to 10kb from standard or low-melt agarose gels in TAE or TBE buffer. All centrifugation steps were carried out in a table centrifuge at the highest setting (13.000rpm).

The desired DNA bands were excised from the gel using a sterile, sharp scalpel and transferred into a microcentrifuge tube. To one volume of gel, 3 volumes of buffer QG were added and the mixture was incubated for 10min at 50^{o}C until the gel slice had completely dissolved. To help dissolve the gel, the samples were vortexed every 2-3min during the incubation. After the gel slice had completely dissolved, 1 gel volume of isopropanol was added and mixed. To bind DNA to a column, a QIAquick spin column was placed into a provided 2ml collection tube. The sample was loaded onto the column and centrifuged for 1min to bind DNA. The flow through was discarded and an additional centrifugation step was carried out with 0.5ml of buffer QG into the same collection tube. The flow through was discarded and the membrane was washed by adding 0.75ml PE, let the column stand for 5min and centrifugation for 1min After discarding the flow through, the QIAquick column was centrifuged for an additional 1min. The QIAquick column was placed into a clean 1.5ml microcentrifuge tube. To elute DNA, 30µl of elution buffer EB were applied directly to the centre of the membrane and let stand for 1min. Subsequently, the column was centrifuged for 1min and the purified DNA was used for further applications.

2.5.5. Vector Dephosphorylation and Precipitation

The vector pET-25b(+) was digested with the appropriate restriction enzymes. For ligation of DNA fragments into pET-25(b+) and pBluescript, these vectors were dephosphorylated (pET + pBluescript) and precipitated (pET-25b+).

Dephosphorylation of pET-25b(+) and pBluescript
20µl restriction reaction
4µl 10x SAP buffer
3µl SAP
13µl ddH_2O
40µl total volume

The mixture was incubated 15min at 37°C, dephosphorylation was inactivated for 20min at 65°C.

Precipitation of pET-25b(+)
40µl dephosph. reaction
27µl 1M NaAc
54µl Isopropanol
14µl ddH_2O

The vector was vortexed and precipitated for at least 30min at –20°C. The vector was centrifuged for 30min at 4°C and 13.000rpm and the pellet was washed with 1ml 70% Ethanol. After an additional centrifugation step for 10min at 4°C and 13.000rpm, the supernatant was discarded and the vector pellet was dried for 5min at 37°C.

2.5.6. DNA Ligation

Ligation of a vector with the DNA of interest was carried out using the TOPO vector from the TOPO TA Cloning Kit (Invitrogen) or pET-25b(+) and pBluescript vector systems. Desired DNA fragments were extracted and purified from agarose gels as described in 2.5.4. Vectors and the DNA of interest were cut with the appropriate restriction enzymes. The following components were mixed on ice in sterile microcentrifuge tubes:

Ligation using the TOPO vector system
4µl fresh PCR product
1µl Salt Solution
1µl TOPO vector

The ligation was mixed gently and incubated for 5min at RT. Tubes were placed on ice before proceeding to One Shot Chemical Transformation.

Ligation using pET-25(b+) and pBluescript vector systems
Dry pET-25b(+) vector pellet / 1µl pBluescript
17µl pure PCR product
2µl 10x Ligation buffer
1µl T4 DNA Ligase

Components were mixed well and the ligation reaction was incubated o.n. at 18°C.

2.5.7. Transformation of competent E. coli

One aliquot (50µl/tube) of One Shot TOP10 Chemically Competent *E. coli* or One Shot BL21 Star(DE3) Chemically Competent Cells was defrosted for 5min on ice. 2 to 10µl of the ligation reaction were directly pipetted into the vial of competent cells and mixed by tapping gently. The reaction was incubated on ice for 30min. The cells were heatshocked for 30sec at 42℃ and chilled on ice for 2min. 250µl of provided S.O.C. medium were added and the cells were incubated for 1hr at 37℃ by shaking for regeneration. 50 and 150µl of the transformation mixture were plated onto LB-Amp, LB-Amp/X-Gal or LB-Kan/X-Gal plates and incubated o. n. at 37℃. White colonies were checked for containing the correct plasmid by mini-preparation of the plasmid DNA, followed by restriction enzyme digestion.

2.5.8. Mini Preparation of Plasmid DNA

Plasmid DNA was isolated using the Fast Plasmid Mini kit from Eppendorf. The kit provides a fast, non-organic method for isolating high purity plasmid DNA from 1.5 to 3ml of *E.coli* bacterial cultures. The Fast Plasmid Mini technology uses a single solution for cell resuspension, lysis and DNA trapping.

The Complete Lysis Solution is stored at 4^{o}C for all applications. All centrifugation steps were performed in a table centrifuge at RT and 13.000rpm.

1.5ml of *E.coli* o.n. culture transformed with a specific plasmid was transferred in a provided 2ml microcentrifuge tube and pelleted by centrifugation for 1min. The supernatant was decanted and 400µl of ice cold Complete Lysis Solution were added. The cells were resuspended by constant vortexing for 30sec at the highest setting. Afterwards the lysate was incubated for 3min at RT. The lysate was transferred to a Spin Column Assembly and centrifuged for 1min. 400µl Diluted Wash Buffer were added to the Spin Column and the column was again centrifuged for 1min. The filtrate was discarded and the Spin Column was placed back into the waste tube. Another centrifugation step was done to dry the Spin Column. The Column was transferred into a provided Collection Tube and 50µl of Elution Buffer were added directly to the center of the Spin Column membrane. To elute plasmid DNA, the Column was centrifuged for 1min. The eluted DNA was immediately used for downstream applications or stored at –20^{o}C until usage.

2.5.9. Midi Preparation of Plasmid DNA

For the midi-preparation of plasmid DNA the QIAfilter Plasmid Midi Kit from QIAGEN® was used. All centrifugation steps were carried out in Sorvall GSA and HB-6 rotors. 25ml of LB medium containing the appropriate antibiotic was inoculated with a single colony or with 100µl of mini - o.n. culture and incubated o. n. at 37℃ by vigorous shaking. The bacterial cells were harvested by centrifugation at 6000x g for 15min at 4^{o}C. The supernatant was discarded and

the cell pellet was resuspended in 4ml of buffer P1 until no cell clumps remained. After adding 4ml of buffer P2 and mixing thoroughly by vigorously inverting the sealed tube 4-6 times, the lysate was incubated at RT for 5min. During the incubation periode, the QIAfilter Cartridge was prepared and 4ml chilled buffer P3 were added to the lysate. After mixing by inverting 4-6 times, the lysate was poured into the barrel of the QIAfilter Cartridge. The mixture was incubated at RT for 10min. During the incubation periode, the QIAGEN-tip 100 was equilibrated by applying 4ml of buffer QBT, and the column was allowed to empty by gravity flow. The cap was removed from the QIAfilter Cartridge outlet nozzle and the plunger was gently inserted into the QIAfilter Midi Cartridge. Afterwards, the lysate was filtered into the previously equilibrated QIAGEN-tip. The lysate was allowed to enter the resin by gravity flow and the QIAGEN tip was washed subsequently with 2x 10ml of buffer QC. The DNA could be eluted with 5ml of buffer QF into a sterile 15ml Falcon tube. 3.5ml of isopropanol were added to precipitate the eluted DNA. After mixing, the sample was immediately centrifuged at 15.000x g for 30min at 4^{o}C. The supernatant was carefully decanted and the DNA pellet was washed with 2ml of room temperature 70% ethanol. After an additional centrifugation step at 15.000x g for 10min the supernatant was carefully decanted. The DNA pellet was air-dried for 5-10min at RT and finally the DNA was redissolved in a suitable amount of ddH_2O. DNA concentration was determined using the Nano Drop from PeqLab with a wavelength of 260nm.
The purified plasmid DNA was immediately used for downstream applications or stored at -20^{o}C until usage.

Buffer P1 (resuspension buffer)
50 mM Tris-Cl, 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-Cl, pH 8.5
15 % isopropanol (v/v)

2.5.10. Restriction Enzyme (RE) Digestion

The following components were mixed and incubated for 90min at 37^{o}C in a sterile microcentrifuge tube:

5µl Mini Prep / 5µg Midi Prep
2µl specific RE buffer
0.5µl RE
ddH_2O up to a final volume of 20µl

The appropriate restriction buffer was chosen in agreement with the recommendations of the manufacturer. The degree of digestion was checked by agarose gel electrophoresis.

2.6. Molecular Biological Methods: RNA

2.6.1. Isolation of total RNA

Total RNA was isolated from tissues using the NucleoSpin RNA II Kit from Genxpress (Machery Nagel). About 30 to 40mg of freshly or frozen tissue were weighed into a sterile Eppendorf tube containing 350µl buffer RA1 and 3.5µl ß-Mercaptoethanol. Tissues were homogenized using a Potter and filtered through a NucleoSpin Filter Column (violet ring) to reduce viscosity. The mixture was applied onto the column and centrifuged for 1min at 11.000x g in a 2ml Collection tube. The NucleoSpin Filter Column was discarded and 350µl 70% Ethanol were added to the lysate by pipetting up and down. For each preparation, one NucleoSpin RNA II Column (light blue ring) was placed into a new 2ml Collection tube. The lysate was pipetted up and down 2-3 times and loaded to the column. The sample was centrifuged for 30sec at 11.000x g and the Column was placed into a new collection tube. For desalting the membrane, 350µl MDB (Membrane Desalting Buffer) were loaded and centrifuged for 1min at 11.000x g to dry the membrane. To remove contaminating DNA, a DNase reaction mixture was prepared in a sterile microcentrifuge tube. For each isolation, 10µl rDNase was added to 90µl Reaction buffer for rDNase and mixed by flicking the tube. This mixture was added directly to the center of the silica membrane of the column and incubated at RT for 15min. To wash and dry the silica membrane, 200µl buffer RA2 were added to the NucleoSpin RNA II column and centrifuged for 30sec at 11.000x g. The column was now transferred to a new collection tube. A second wash step was done with 600µl RA3 followed by a centrifugation step for 30sec at 11.000x g. The last washing step was performed by adding 250µl RA3 to the NuceloSpin RNA II column. The column was centrifuged for additional 2min at 11.000x g to dry the membrane completely. Afterwards the column was placed into a nuclease-free Collection tube. For elution of highly pure RNA, 60µl of RNase free Water (supplied) was added directly to the center of the membrane and centrifuged for 1min at 11.000x g. RNA concentration was measured with the NanoDrop from PeqLab and the RNA´s were stored at –80^{o}C until usage.

2.7. Molecular Biological Methods: Protein

2.7.1. Preparation of Triton X-100 Total Protein Extracts

Freshly or frozen obtained chicken tissues were placed in ice-cold homogenization buffer (4 ml/g wet weight) and homogenized with an Ultra-Turrax T25 homogenizer 3 times for 20 seconds each. The homogenates were centrifuged for 10 min at 620x g and 4^{o}C. The supernatant was transferred to a fresh tube and 1/20 of 20% Triton X-100 was added. The mixture was than incubated for 30 minutes at 4^{o}C and finally centrifuged at 60.000 rpm for 1 hour at 4^{o}C. The supernatant was pooled and the protein concentration was determined by the method of Bradford using the BioRad protein assay (BIORAD).

Homogenization Buffer:

20 mM Hepes pH 7.4

300 mMSucrose

150 mM NaCl

Proteinase inhibitor cocktail tablettes (ROCHE)

2.7.2. Preparation of Triton X-100 Membrane Protein Extracts

Buffer A (4 ml/g net weight) was added to the tissue samples, followed by homogenization using an Ultra-Turrax T25 homogenizer, 3 times for 20 seconds each. If necessary, homogenization was extended until the homogenization was complete. The samples were centrifuged at 2000x g for 10 minutes at 4^{o}C in a SS34 rotor and the supernatant was poured over four layers of cheesecloth into a fresh beaker on ice. The filtrate was centrifuged using an Ultra-centrifuge at 50.000 rpm for one hour at 4^{o}C. The supernatant was discarded and the pellets were washed as follows: They were resuspended in 3ml buffer A by aspirating through an 18gge needle and then flushed through a 22gge needle. The suspensions were combined in a fresh beaker on ice and aliquots were centrifuged at 50.000 rpm for 1 hour at 4^{o}C. The supernatant was discarded and the pellets were resuspended in 5ml buffer B, flushing again through an 18gge needle and then through a 22gge needle. Afterwards 4% of the total volume of 4M NaCl was added and the samples were sonicated for 30 seconds at setting 6. Finally, 1/10 of 10% Triton X-100 was added to the samples and centrifuged for 1 hour at 4^{o}C and 50.000 rpm. The supernatant was aliquoted and stored at -80^{o}C until usage. Protein concentration was determined using the method of Bradford.

Buffer A
20 mM Tris/HCl
1 mM $CaCl_2$
150 mM NaCl
Proteinaseinhibitor

Buffer B
250mM Tris/Maleat, pH 6
2mM $CaCl_2$
Proteinaseinhibitor

2.7.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Extracted and recombinant proteins were separated on 10%, 12% and 4.5-18% gradient gels with a thickness of 1 mm using the BioRad Mini gel system.

Components (1gel)	Stacking gel 4%	Separating gel 6%	Separating gel 10%	Separating gel 12%	Gradient gel 18%	Gradient gel 4,5%
H_2Odd	1525µl	2650µl	2025µl	1675µl	435µl	1920µl
1.5 M Tris-HCl pH 8.8	-	1250µl	1250µl	1250µl	810µl	810µl
0.5 M Tris-HCl pH 6.8	625µl	-	-	-	-	-
30% polyacrylamide	325µl	1000µl	1650µl	2325µl	1980µl	495µl
10% SDS	25µl	50µl	50µl	50µl	31,5µl	32,25µl
10% APS	12.5µl	50µl	25µl	25µl	25µl	25µl
TEMED	2.5µl	4µl	2.5µl	5µl	5µl	5µl

Table 2.11. Ingredients of a protein gel.

The components of the separating gel were mixed and poured into the previously cleaned gel unit and overlayed with a small quantity of isopropanol. The gel was allowed to polymerize for about 15min. The components of the stacking gel were combined and poured onto the separating gel after complete removal of the isopropanol. The combs were inserted and the stacking gel was allowed to polymerize for at least 15min. After removal of the combs, the slots were rinsed with ddH_2O and the gel was put into the buffer chamber filled with electrophoresis buffer. The protein samples were mixed with Laemmli buffer (reducing or non-reducing; as a reducing agent, Dithiothreitol (DTT) was added up to a final concentration of 25mM) and incubated for 5min at 95^{o}C (only samples under reducing conditions). The gel was run at 80V until the samples reached the separating gel and then shifted to a constant voltage of 180V until the Bromphenol Blue front reached the bottom of the gel. As a protein marker, Unstained Precision Plus Protein Standard (Bio Rad) served as size markers. For every protein gel, 8µl

marker was applied. The gels were used for Coomassie blue and Silver Stainings or for Western and Ligand Blot analysis as described below.

30% Polyacrylamide
29.2% acrylamide
0.8% N,N´-methylenebisacrylamide

4x Laemmli buffer
8ml 78% glycerine
6ml 20% SDS
0.4ml 1M Tris-HCl pH 7.4 - 7.5
ddH_2O to a final volume of 20ml
+25mM Dithiothreitol (DTT) for reducing conditions

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

2.7.4. Western Blot Analysis

Semi Dry Blotting

Transfer of proteins from SDS-gels to nitrocellulose membranes (Hybond^{TM}-C Extra for optimized protein transfer, from Amersham Biosciences) was performed in a BioRad semi-dry blotting unit. The blot was prepared as followed: three Whatman papers soaked in 1x transfer buffer were applied to the blotting unit, followed by the nitrocellulose membrane, the gel and finally three more soaked Whatman papers. To avoid air bubbles, which decrease the quality of protein transfer, a falcon tube was rolled over the blot. The blot was covered with the lid of the blotting unit. Blotting was performed for 90min at 200mA for one gel, at 400mA for two gels and so on. To check the transfer efficiency, the membrane was stained with Ponceau S. Blotted proteins and the standards were visualized by destaining with ddH_2O. The bands in the protein standard lane were marked with a pencil.

Detection

Enhanced Chemiluminescence (ECL) was used to visualize proteins on immunoblots. ECL is based on enzyme-catalyzed production of light where horse raddish peroxidase conjugated to the secondary antibody catalyzes the oxidation of luminol in the presence of H_2O_2. The membrane was blocked for 1 hr at room temperature (RT) with 5% non-fat dry milk in 1x TBST. After removing the blocking solution, the primary antibody diluted in 5% milk in 1x TBST was added. Dilutions of the antibody were dependent on its affinity. The blot was incubated for at least 1 hr at RT or overnight (o.n.) at 4^{o}C with gentle agitation. Then the membrane was washed 3 times each for 10min with 1x TBST, followed by the addition of an appropriate amount of secondary, HRP conjugated antibody in 5% milk 1x TBST. After 1 hr of incubation at RT with

gentle agitaion, the solution was discarded and the membrane was washed another 3 times with 1x TBST for 10min each. Finally, the membrane was incubated in a 1:1 mixture of ECL solution I and II (Pierce) for three minutes and put into an exposure cassette. Films (Kodak) were exposed in the dark room, depending on the intensity of the signal and background.

1x Transferbuffer
25mM Tris/HCl
192mM glycine

1xPBS
137mM NaCl
0.27mM KCl
10mM Na_2HPO_4
1.7mM KH_2PO_4

1x TBST
25mM Tris/HCl pH 7.4
140mM NaCl
2.5mM KCl
0.1% Tween 20

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

2.7.5. Ligand Blot Analysis

Blotting of proteins to the Nitrocellulose membrane and subsequent blocking of the membrane was performed according to Western Blots. The blocking buffer used in ligand blot experiments was 1x TBST + 2mM $CaCl_2$ + 5% non-fat dry milk.

After blocking of the nitrocellulose membrane for at least 1 hr at RT, a specific ligand was added to the incubation solution to allow for binding to proteins immobilized on the membrane in the presence of $CaCl_2$. Ligand incubation was performed o.n. at 4^{o}C under gentle agitation. After removal of the ligand solution, the membrane was washed 3 times for 20min with 1x TBST + 2mM $CaCl_2$. After washing, a ligand-specific antibody was diluted in 1x TBST + 2mM $CaCl_2$ + 5% non fat dry milk and applied to the membrane. The blot was incubated for at least 1 hr at RT with gentle agitation. Subsequently the membrane was washed three times each for 10min with 1x TBST + 2mM $CaCl_2$, followed by the addition of an appropriate amount of secondary, HRP conjugated antibody in 1x TBST + 2mM $CaCl_2$ + 5% milk. After 1 hr of incubation at RT under gentle agitation, the solution was discarded and the membrane was washed another three times with 1x TBST + 2mM $CaCl_2$ for 10min each. Finally, the membrane was incubated in a 1:1 mixture of ECL solution I and II (Pierce) for three minutes and put into an exposure cassette. Films (Kodak) were exposed in the dark room, depending on the intensity of the signal and background.

2.7.6. Reverse Ligand Blot

Transfer of proteins from SDS-gels to nitrocellulose membranes was performed as described above (2.7.4. Western Blot Analysis). Ligands, normally used for forward blotting were transferred to the nitrocellulose membrane and the specific protein bands were cut from the membrane. Single membrane pieces were incubated o.n. at 4^{o}C in Eppendorf-tubes under gentle agitation with protein extracts containing potential binding partners for the protein blotted to the nitrocellulose membrane. After removal of the protein extract, membrane pieces were washed 3 times for 5min with 1x TBST + 2mM $CaCl_2$ on an Eppendorf thermomixer 5436 at maximum speed at RT. Afterwards, each membrane piece was transferred into a separate, fresh tube. Elution of proteins bound to the nitrocellulose membrane was achieved by the addition of 100µl of 0,1M Citric acid, pH 2.5 to the membrane piece and shaking of the mixture for 2min on the thermomixer at maximum speed. To concentrate the amount of eluted proteins, membrane pieces treated under the same conditions were pooled. Elution was accomplished with an appropriate amount of Citric acid. The eluate was now loaded to SDS-PAA gels and subsequently used for Coomassie blue – and/or Silver staining for the detection of eluted proteins.

2.7.7. Protein detection using Coomassie Blue staining

After gel electrophoresis, the gel was incubated for 1 hr in Coomassie brilliant blue solution. After removal of the Coomassie solution, the gel was incubated in Destain solution for 1-2 hours until protein bands became visible.

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

Destain
10% Acetic acid
30% Methanol in ddH_2O

2.7.8. Silver Stain

Silver staining of proteins in gels was performed using the SilverSNAP Stain Kit II from Pierce. All steps for the silver staining of proteins in Polyacrylamide Gels were performed in a single clean staining tray with constant gentle shaking. Fixing, Ethanol and Stop solutions were prepared in advance. Other solutions were prepared immediately before use.

After gel electrophoresis, the gel was washed 2 times for 5min with ddH_2O. For fixation, the gel was incubated in fixing solution 2 times for 15min consisting of 30% ethanol and 10% acetic acid in ddH_2O. After fixation, the gel was washed with two changes of 10% ethanol in ddH_2O and another two times for 5min each in ddH_2O. For sensitizing, the gel was incubated for exactly 1min in Sensitizer Working Solution by mixing 1 part SilverSNAP Sensitizer with 500 parts ddH_2O. Then the gel was washed with two changes of ddH_2O for 1min each. For the staining reaction, Stain Working Solution was prepared by mixing 1 part SilverSNAP Enhancer with 50 parts SilverSNAP Stain. The gel was incubated in Staining Solution for 30min and subsequently washed with two changes of ddH_2O for 20sec each. The Developer Working Solution was prepared by mixing 1 part SilverSNAP Enhancer with 50 parts Silver SNAP Developer and the gel was incubated in Developer Working Solution until protein bands appear (2-3 minutes). When the desired band intensity was reached, the Developer Working Solution was replaced with prepared Stop Solution (5% acetic acid in ddH_2O). After a brief washing step, the Stop Solution was replaced and incubated for additional 10min. For storage, the gels were soaked in 5% Glycerin in ddH_2O for 2-3 hours or o.n., and afterwards dried using the vacuum gel drier at 65^{o}C.

Blum Silver Staining for Mass Spectroscopy

For the delivery of protein samples for Mass Spectroscopy, the Silver Stain protocol of Blum (Blum, H., Beier, H., and Gross, H.J. 1987 Electrophoresis 8, 93-99) was used.

The gel was fixed in 40% Ethanol / 10% Acetic acid in ddH_2O for 1 hr. Afterwards the gel was washed 2 times for 20min in 30% Ethanol / ddH_2O and 1 time for 20min in ddH_2O. Sensitzing was done for 1min in Sensitizer Solution. Now the gel was washed with three changes of ddH_2O for 20 seconds each. Silver staining was performed in Sliver Solution for 20min at 4^{o}C followed by washing with three changes of ddH_2O. Development was carried out in Developer Solution until protein bands appeared (3-5min). The gel was washed with ddH_2O for 20sec and the staining reaction was stopped by incubating the gel in Stop Solution for 5min. After washing 3 times for 10min with ddH_2O, the gels were stored in ddH_2O containing 1% acetic acid. Protein bands were now cut from the gel in a cell culture lamina and sent to the MFPL Mass Spectroscopy facility.

Sensitizer Solution
0.02% Sodioum thiosulfate in ddH_2O

Silver Solution
0.1% Silver nitrate in ddH_2O

Developer Solution
3% Sodium carbonate
0.05% Formaldehyde in ddH_2O

Stop Solution
5% Acetic acid in ddH_2O

2.7.9. Expression and Purification of Recombinant Proteins

A small amount of an *E.coli* glycerol stock, transformed with the pET-25b(+) expresssion vector containing the gene of interest cloned into the multiple cloning site of the vector, was inoculated into 6ml of LB-Amp medium and incubated o.n. at 37^oC under vigorous shaking. The o.n. culture was inoculated into 500ml of LB-Amp medium in a sterile flask and incubated at 37^oC on the shaker (145rpm). Cells were grown to an optical density (OD 600) of 0.6 and protein expression was induced by adding Isopropyl ß-D-1- thiogalactopyranoside (IPTG) to a final volume of 1mM. Protein expression was performed for 3 hours at 37^oC on the shaker (145rpm). Afterwards the cells were harvested by centrifugation for 15min at 4^oC and 3500 rpm in a Sorvall centrifuge using a GS3 rotor. The cell pellet was resuspended in 10ml of Lysis buffer with proteinase inhibitor (ROCHE). The cell suspension was sonicated 8 times for 30sec at 4^oC, and the lysed cells were centrifuged for 15min at 4000rpm and 4^oC using a Heraeus table centrifuge. The supernatant was poured into a fresh 50ml Falcon tube and filled up to a final volume of 50ml with Lysis buffer / proteinase inhibitor (ROCHE). To this mixture, 1ml of Ni-NTA Sepharose beads was added and rotated o.n. at 4^oC. Beads were harvested by centrifugation for 5min at 3500rpm and 4^oC and the supernatant was removed. To remove contaminations from the beads they were washed 4 times with 25ml of Wash buffer. After each washing step, the beads were harvested by centrifugation for 5min at 3500rpm and 4^oC. Proteins (expressed with an C-terminal His-Tag) bound to the Ni-NTA beads were eluted 2 times in 1ml of Lysis buffer / proteinase inhibitor (ROCHE) by rotating the beads for 15min at 4^oC at the highest setting. The beads were again harvested by centrifugation and the supernatant was collected. To remove Imidazol from the collected samples, the solution was dialyzed for 1 hr against 1x TBS + 125mM Imidazol, 1 hr against 1x TBS + 60mM Imidazol and o.n. against 1x TBS at 4^oC using the SnakeSkin Pleated Dialysis Tubing (7,000 MWCO) from Pierce. After dialysis, the protein concentration was determined using the method of Bradford. Pure protein samples were stored at -80^oC until usage.

Lysis buffer
50mM Tris/HCl pH 8.0
500mM NaCl
1% Trition X-100
10% Glycerol
20mM Imidazol

Wash buffer
50mM Tris/HCl pH 8.0
500mM NaCl
0,15% Tween
10% Glycerol
1% Triton
30mM Imidazol

Elution buffer
50mM Tris/HCl pH8.0
500mM NaCl
250mM Imidazol

2.7.10. Co-Immunoprecipitation

Co-immunoprecipitation was carried out to precipitate a protein complex of two proteins.
A protein extract was diluted 1:10 in 1x TBST/2mM $CaCl_2$ and mixed with a recombinant protein source, proposed to be a binding partner for proteins present in the extract, for 2 hours on a rotator at 4^{o}C under gentle agitation. Following this pre-incubation, an antiserum, specific for the recombinant protein, was added in appropriate amounts.
Samples were incubated for at least 1 hr at 4^{o}C on a rotator under gentle agitation. After this incubation period, the Protein A Sepharose beads (usually 50-70µl wet volume) were added to the mixtures. Samples were incubated overnight at 4^{o}C under gentle agitation as mentioned above. The next day, the samples were centrifuged for 3min at 2000 rpm and 4^{o}C. The supernatant was discarded and the beads were washed three times with 1ml 1x TBST + 2mM $CaCl_2$ + proteinase inhibitor (ROCHE). In between the washing steps, the beads were centrifuged for 3min at 2000 rpm and 4^{o}C to remove the supernatant. After the final washing-step, the supernatant was discarded and an appropriate amount of 4x Laemmli buffer + 25mM DTT were added to the protein A beads. Afterwards, samples were incubated 5min at 95^{o}C to separate the proteins from the beads. Samples were centrifuged and the supernatant was applied to SDS-PAGE. The immunoprecipitated proteins could now be analyzed (Coomassie gel, Silver stain, or Western blot).

2.7.11. Affinity Chromatography

Coupling a ligand to cyanogen bromide (CnBr) activated Sepharose 4B (GE Healthcare):

All buffers used were filtered using a glass filter funnel from Millipore (Filter: Type HA / 0.45µm) to remove undissolved material. 3mg of ligand for coupling to the beads was dialyzed o.n. against coupling buffer for the removal of chemicals which would impair the coupling reaction.

1g of CNBr Sepharose beads were swollen in 1mM HCl by rotating the beads at RT for 30min. To remove additives, the beads were washed with 100ml of 1mM HCl on a glass filter funnel. The beads were activated by adding and immediate sucking of 20ml coupling buffer. The activated beads were transferred into a 50ml Falcon tube already containing the ligand solution (~20ml) + proteinase inhibitor (ROCHE). For the binding of the ligand to the beads, the protein-Sepharose mixture was rotated for 2 hours at RT. Using the glass filter funnel, the liquid was sucked and the beads were washed with 150ml of coupling buffer. To block remaining binding sites on the Sepharose beads, and to avoid unspecific binding, the beads were incubated with 30ml of 1M Ethanolamin / HCl, pH 8.3 and rotated for 2 hours at RT. The Ethanolamin was removed and the beads were washed with 60ml of buffer A and B alternately according to the following scheme:

- 2 times buffer A, 2 times buffer B
- 4 times buffer A and buffer B alternately

Finally, the beads were washed with Storage buffer + proteinase inhibitor and resuspended in the same buffer in a 50ml Falcon tube. The coupled beads were stored at 4^{o}C in Storage buffer until usage.

Coupling buffer pH 8.5
0.1M $NaHCO_3$
0.5M NaCl

Buffer A pH 8.0
0.1M Boric acid
1M NaCl

Buffer B pH 4.0
0.1M NaAc
1M NaCl

Storage buffer pH 7.4
25mM Tris/HCl
0.01% EDTA
0.02% NaN_3

Equilibration and packaging of the column

Packing and equilibration of the column as well as affinity chromatography (AC) were done at 4°C. All buffers and materials were cooled to 4°C before use. The column used was an Econo-Pac Column from Bio-Rad with a total volume of ~12ml and an inner diameter of 0,8cm.

The column was first equilibrated in 100ml AC buffer. Afterwards the diluted protein extract was loaded onto the column and the extract was allowed to circulate for 2 hours through the column. Before loading the extract onto the column and after the incubation period, samples were taken from the protein extract for analysis. The column was washed with 400ml AC buffer for the removal of unspecific proteins present in the column. After the washing step, an aliquot of beads was removed.

Affinity chromatography buffer (AC buffer) pH 7.4
20mM Tris /HCl
0.2% NP40 (Detergent)
2mM $CaCl_2$
150mM NaCl

Elution

The elution step of the AC column was done with buffers in consecutive steps. All elution fractions were collected in Eppendorf tubes for further analysis on Coomassie and Silver stain gels.

Elution was carried out 2 times with 1ml of 25mM EDTA + proteinase inhibitor and afterwards 2 times with 1ml of 4M NaCl + proteinase inhibitor. The column was washed with 20ml of AC buffer whereas the first four ml of this washing step were also collected in tubes.

Regeneration of the ligand-coupled beads

The column was washed with 20ml of buffer A and buffer B two times alternately.

Storage of the column

The beads were stored in Storage buffer + proteinase inhibitor at 4°C.

2.7.12. Antibody Generation

Antiserum against chicken Cubilin was raised against a 96-amino acid fragment located in the very N-terminal region of the protein, which represents the only structurally unique motif of the protein. This fragment was cloned, expressed, and purified according to the methods described above.

For the first injection, 300µg of antigen-mix was mixed with GEBRO complete adjuvant and injected intradermally to an adult New Zealand White rabbit. Boosters were performed with the

antigen in complete GEBRO adjuvant 3 and 6 weeks after the first injection with 300µg of antigen mix. Blood samples were taken after 8 weeks, incubated at RT for 1 hr and afterwards stored o.n. to let coagulation proceed. After stirring the coagulated blood samples with a metal stirrer, the samples were centrifuged at 4000 rpm for 15 min. The supernatant representing the antiserum was aliquoted and stored at –20℃. The obtained serum was tested by Western blot analyses. Preimmune serum from the same rabbit served as control.

2.7.13. Utilized Antibodies and Antisera

Protein	Specific antibody	Dilution
ggApoA-V	Rabbit anti-ggApoA-V antiserum	(1:2000)
hApoA-V	Rabbit anti-hApoA-V antiserum	(1:1000)
hApoA-V-His	Mouse anti-His antibody	(1:1000)
ggCubilin	Rabbit anti-ggCubilin antiserum	(1:2000)
ggLRP 380	Rabbit anti-LRP 380 antibody	(1:1000)
ggLRP2	Rabbit anti-LRP2 antibody	(1:1000)
ggLRP1	Rabbit anti-LRP1 antibody	(1:1000)
ggLR8	Rabbit anti-LR8 antibody	(conc. 10µg/µl)
ggLR8B	Rabbit anti-LR8B antibody	(1:1000)
ggLR11	Rabbit anti-LR11 antibody	(1:1000)
GST-RAP	Mouse anti-GST antibody	(1:2000)
Rabbit IgG	Goat anti-rabbit IgG	(1:50 000)
Mouse IgG	Goat anti-mouse IgG	(1:1500)

Table 2.12. Used antibodies and antisera.

3. RESULTS

Generally, the aim of my diploma thesis was to expand our work on the biology of apoA-V in our model organism, the chicken. In more detail, I focused on the interesting property of apoA-V to bind and interact with certain cell surface receptors in its lipid-associated and lipid-free form, which was shown by in vitro studies using SPR and ligand blotting (Dichlberger, Cogburn et al. 2007; Nilsson, Christensen et al. 2008). Many studies in the past have shown that apoA-V circulates in the plasma in a lipid-bound form on the surface of Chylomicrons, VLDL, and HDL lipoproteins. There, apoA-V mediates the clearance of these particles from the circulation by various mechanisms. However, the physiology of lipoprotein-associated apoA-V is not completely understood. In addition, evidence exists that apoA-V is also present in a lipid-free form in the plasma. To gain better insights into the functional properties of apoA-V as a modulator of triglyceride levels, we wanted to identify new binding partners of ggapoA-V.

3.1. Search for New Interaction Partners of ggapoA-V

As indicated in the Introduction, several studies reported the high affinity of apoA-V to bind to and interact with certain cell surface receptors. Human apoA-V was shown to bind to the LDLR related proteins LRP and LR11, and to a protein belonging to the family of Vps10p-domain receptors termed sortilin (Nilsson, Lookene et al. 2007; Nilsson, Christensen et al. 2008). Furthermore, in our lab it was shown that ggapoA-V binds to the chicken VLDLR homologue LR8 (Dichlberger, Cogburn et al. 2007), located on the surface of the chicken oocyte. This interaction was studied by using ligand blot analysis, a well established method used in our lab. Because this receptor-ligand interaction can be demonstrated very reproducibly with the applied ligand blot methodology, the binding of ggapoA-V to LR8 served as a consistent positive control during my experiments (Figure 3.1).

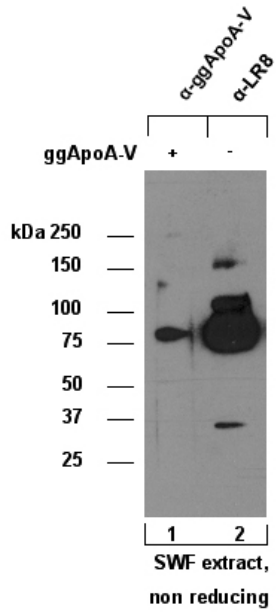

Figure 3.1. Chicken apoA-V binds to the major oocyte receptor LR8. A laying hen (LH) small white follicle (SWF) membrane protein extract was prepared, and 100µg extract per lane were subjected to non-reducing, 4.5%-12% gradient gel SDS-PAGE and blotted to nitrocellulose membrane. After blocking of unspecific binding sites with blocking buffer, ggapoA-V (150µg crude bacterial protein extract) was added as a ligand (lane 1). After extensive washing, the bound ggapoA-V protein was detected using α-ggapoA-V antiserum. To lane 2, a specific α-LR8 antibody was applied to verify the presence of the receptor. As a secondary antibody, an HRP-conjugated α-rabbit antibody was used. Visualization was performed using ECL.

To gain more insights into the biology of ggapoA-V concerning its receptor-binding ability, ligand blot analyses were expanded using other chicken tissues as a potential source for new interaction partners of ggapoA-V. The chicken brain, chicken adrenals, and the embryonic yolk sac, tissues known to utilize and/or metabolize lipids and lipoproteins, were selected for binding studies by ligand blots. No interaction partners of ggapoA-V could be identified when adrenal protein extracts were used as a source for possible receptors. Furthermore, ligand blotting was applied to chicken brain protein extracts. Generally, the brain is an interesting tissue for interaction studies of apoA-V with proteins expressed there. Two receptors, LR11 and sortilin, for which it is known that their major site of expression is the brain, were shown to be binding partners for hapoA-V (Nilsson, Lookene et al. 2007; Nilsson, Christensen et al. 2008). Furthermore, LR8B, a member of the LDLR family with eight ligand-binding domains is known to be specifically expressed in the brain (Stockinger, Hengstschlager-Ottnad et al. 1998).
Nevertheless, ligand blot analysis did not unambiguously reveal an interaction partner of ggapoA-V in the chicken brain (Figure 3.2.); possibly, binding to LR11 and LR8B could be seen (lanes 1,3).

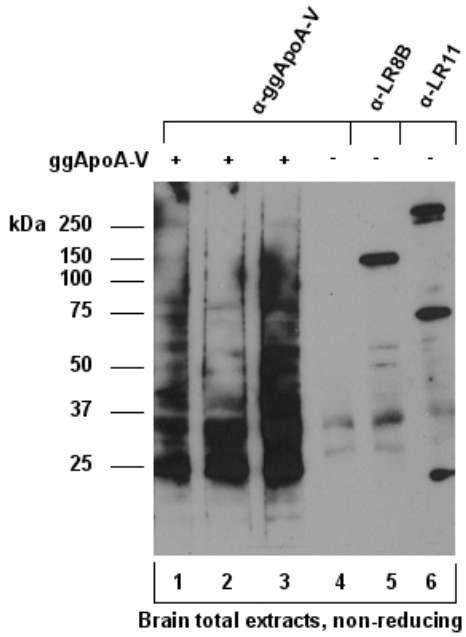

Figure 3.2. Ligand blot analysis with ggapoA-V on chicken laying hen brain extracts under non-reducing conditions. Brain total extracts (200µg/lane) were subjected to SDS-PAGE on 4.5 – 12% gradient gels and afterwards blotted to a nitrocellulose membrane. After blocking, the membrane strips were incubated with ggapoA-V crude protein extracts (lanes 1-3). After extensive washing, bound apoA-V was detected using ggapoA-V antibody (lanes 1-4). As a control for the quality of the brain protein extract, receptors present in the chicken brain (LR11, LR8B) were detected using specific antibodies (lanes 5 and 6). Bound primary antibodies were detected using an HRP-conjugated α-rabbit antibody. Visualization of the blot was done using ECL.

Several ligand blots with crude ggapoA-V as well as with purified ggapoA-V as ligand could not clearly confirm the binding of the protein to LR11. Nevertheless, faint bands can be seen for ggapoA-V interacting with a protein at 250 kDa (lanes 1 and 3), which is also the size of LR11 (lane 6). Another band, representing ggapoA-V bound to the nitrocellulose membrane, appears at ~80 kDa (lane 2), co-migrating with the second immunoreactive protein recognized by the α-LR11 antibody (lane 6). The high background seen in lanes 1-3 likely was the result of long exposure times.

Because no clearly identifiable interaction partner of ggapoA-V could be observed in the brain, the yolk sac (YS), an extraembryonic structure essential for chicken embryonic development, was tested. Chicken yolk sac, isolated at day 10 of chicken embryonic development, was used for further ligand blot experiments. A yolk sac membrane protein extract was prepared to avoid contaminations with yolk and other components that are in close contact with the two cell layers, which form this sac-like structure.

When ggapoA-V was used as a ligand on yolk sac membrane extracts, two interaction partners were observed (Figure 3.3, lane 1). One ggapoA-V binding protein had a size of approximately 110 kDa, the other one migrated with a molecular mass of more than 250 kDa. When no ligand was added to the membrane, none of these proteins were detected (lane 2). This confirms that the presence of apoA-V is essential to detect these proteins. To exclude the possibility that the larger binding partner of ggapoA-V presents chicken LRP2, which is known to be present in the yolk sac, lane 3 was probed with an α-LRP2 antibody. A clear difference in the molecular mass of the ggapoA-V binding partner (large band in lane 2) and LRP2 (lane 3) was observed.

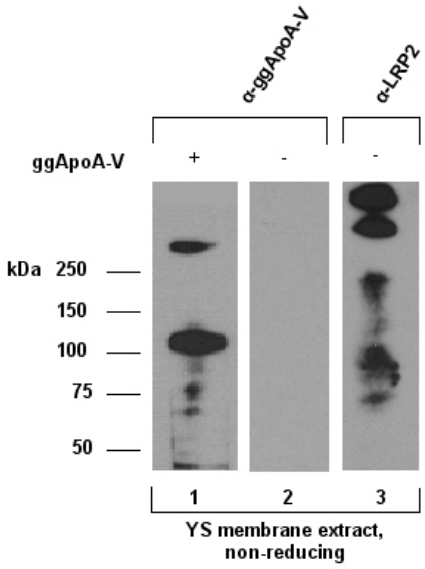

Figure 3.3. Ligand blot analysis with ggapoA-V on chicken yolk sac membrane extracts. 150µg of freshly prepared chicken yolk sac membrane extract per lane was subjected to 4.5-12% gradient gel SDS-PAGE under non-reducing conditions and subsequently blotted to nitrocellulose membrane. The membrane was cut into single strips, and 150µg ggapoA-V crude protein extract was added to the membrane representing the ligand (lane 1). After extensive washing, the bound ligand was detected with an α-ggapoA-V antibody. No ligand was added to lane 2, and lane 3 was probed with an α-LRP2 antibody. HRP-conjugated goat α-rabbit antibody was used as secondary antibody, and visualization of the blot was performed using ECL.

The interaction partners of ggapoA-V in the yolk sac could be consistently detected in several different ligand blots exclusively under non-reducing conditions (no binding can be detected under reducing conditions, data not shown). The bands visualized on the ligand blot (Figure 3.6, left panel) represents high affinity binding of ggapoA-V to protein(s) present in the chicken yolk sac.

Having discovered new binding proteins of ggapoA-V in the yolk sac, the question came up of how to identify and characterize these proteins. In the first attempts, I performed Co-

immunoprecipitations (Co-IP) using protein A-Sepharose beads. For the binding of ggapoA-V to its receptors, the ligand was incubated with diluted yolk sac membrane extract for 2 hours. Then, α-ggapoA-V antiserum was added to the mixture to capture any complexes of apoA-V with interacting proteins. The mixture was rotated over night in the presence of Protein A – beads. After washing, the bound proteins were eluted from the beads and analyzed on a Coomassie-stained gel (Figure 3.4, right panel).

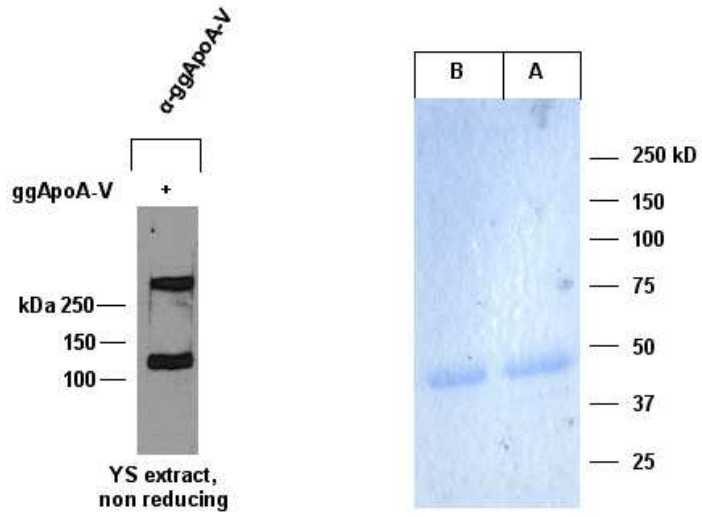

Figure 3.4. Left panel: Ligand blot under non-reducing conditions showing ggapoA-V binding to proteins present in the chicken yolk sac. Yolk sac protein extract was subjected to 4.5-12% SDS-PAGE and subsequently blotted to nitrocellulose membrane. As a ligand, 5µg purified ggapoA-V were added to the membrane strip. Bound apoA-V was detected using α-ggapoA-V antiserum. HRP-conjugated goat α-rabbit antibody was used as secondary antibody, and visualization of the blot was performed using ECL. **Right panel: Coomassie stain following Co-IP.** Proteins bound to the protein A-Sepharose beads were eluted with reducing Laemmli buffer and subjected to gradient-gel SDS-PAGE. In lane A, the specific α-ggapoA-V antiserum was used, whereas lane B shows the Co-immunoprecipitation with preimmune serum.

The performed Co-IP's (Figure 3.4, right panel) did not identify any binding partners of ggapoA-V. The strong band observed in both lanes (< 50 kDa) represents the rabbit IgG heavy chain (IgG dissociates in the presence of reducing agents into its heavy and light chains), which bound to the protein A-Sepharose with its conserved domain (Fc – domain). In lane A (which shows the Co-IP with the α-ggapoA-V antibody), but not in lane B (Co-IP with preimmune serum), a faint band underneath the IgG heavy chain can be seen. This band represents most probably the precipitated ggapoA-V. Nevertheless, no other proteins could be precipitated.
Rather than using a more sensitive method for the detection of co-IPed proteins (such as silver staining the gel), another method, reverse ligand blotting, was used for the identification of the

ggapoA-V binding proteins. Here, recombinant ggapoA-V was blotted onto the nitrocellulose membrane. After staining of the proteins with PonceauS, the specific region of the membrane, representing the ggapoA-V protein fraction (molecular mass of 40kDa), was cut out from the membrane. These membrane pieces were incubated with diluted yolk sac membrane extract to let the binding reaction proceed. After washing, proteins which bound to the membrane-immobilized ggpoA-V could be eluted with citric acid (see “Materials and Methods” and legend to Fig. 3.5). The eluted fraction was subjected to SDS-PAGE and subsequently silver staining was performed for the detection of the apoA-V binding proteins (Figure 3.5).

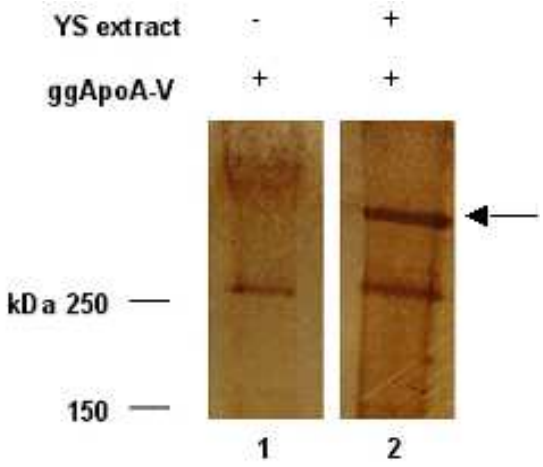

Figure 3.5. Silver stain gel following reverse ligand blot. 150µg recombinantly expressed ggapoA-V crude protein extract was subjected to SDS-PAGE under non-reducing conditions and then transferred to a nitrocellulose membrane by semi-dry blotting. The membrane piece containing the ggapoA-V protein band was cut out and incubated with diluted yolk sac membrane extract. After end-over-end rotation over night, the membrane pieces were washed. Binding partners of ggapoA-V were then eluted from the nitrocellulose membrane pieces with ~ 300µl 0.1M citric acid, pH 2.5. The elution was subjected to 4.5-12% gradient gel SDS-PAGE and proteins were detected using the silver stain method by Blum, a special method optimized for subsequent Mass Spectroscopy.

By reverse ligand blotting, it was possible to elute a protein, whose molecular mass resembled that of the larger interaction partner of ggapoA-V observed by forward ligand blotting (Figure 3.4, left panel). Other silver stains also revealed the second, smaller ggapoA-V binding partner (~110 kDa, figure3.3, lane 1), although in the reverse-ligand blotting experiments the amount of this protein was much lower.

For the identification of the binding partner of ggapoA-V (Figure 3.5, lane 2), the protein band was cut from the gel under sterile conditions and sent to the VBC Mass Spectroscopy facility.

The analysis of the protein via Mass Spectroscopy was successful and identified a huge protein, comprising 3728 amino acids, predicted to be the chicken homologue of mammalian cubilin. In figure 3.6, the identified peptides which span the whole sequence are highlighted in red.
As cubilin was the only chicken protein thus identified, the result is quite significant. Additionally, detailed reports in the literature revealing mammalian cubilin as a lipoprotein receptor underline the importance of this result even more. Cubilin, a peripheral membrane protein known to be involved in endocytic processes, also matched perfectly to the observed function of apoA-V in terms of binding to various cell surface receptors.

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   1 MVEEEEEEDE DEEEGSDEEE KGLLSFDVFG QPPLNQRTNP SDDSIPGDLG
  51 QQPIEQLKVR SSNVQCPDPT LCQAHIPQDH KLNQGMVAAA QATFSVDIFD
 101 DLFCIVALLL LAGLSCESNH YEKKRQKRNI YDEQPRLSSE QGNLVFHAGS
 151 SKNIEFRTGP LGKIKLNEED LAEMFSQMRE NKAEIQELKK ASGVSQNVSH
 201 QVSMLASKVT SLDMKLQSLE QTLQRKACSS NPCENSGTCV NSLDGFFCLC
 251 PSNWRGLRCS EDINECQIYA GTALGCQNGA TCVNTPGSYS CSCTPETYGP
 301 RCASKFDDCQ GGSQTLCEHG TCVDAERDTP NKPKYHCICD TGWMSPPGSP
 351 ACSADIDECS LPNPPCSQNP RVQCHNTLGS YSCDPCPTGW QGNGYSCQDI
 401 DECESDNGGC STAPMVQCIN TIGSFRCGVC PPGYEGDGQT CTQVDSCSIN
 451 NGGCHPSATC TSTPGLMPFC SCSPGYTGSG YGPNGCSPLT DICQLQNPCA
 501 NGQCLAVTSG YFCLCNAGWT GSNCTENIDE CISNPCQNGG SCTDGVNGYT
 551 CECTSAWTGP QCQTAQQACG GYLSGSRGTF SYPNNPSSQH YDTGVSCAWV
 601 IQTASNKILH ITFPFFHLEA STDCNSDFLQ IHDGASASMH MLGKYCGSNP
 651 PAELFSSHNS LYFWFHSNHA VTAGGFTVQW DSRDPECGGE LTATYGSISS
 701 PGYPGNYPVN RDCFWTISTN PGLLITFAFG TLSLEHHENC SYDYLEIRDG
 751 LLSQDSVLGK YCSTGSPPPL QTTGPYAWIH FHSDDEVTDK GFHIVYTTSA
 801 ADPTCGGNYT DSEGVITSPF WPNSFINSQQ CIYIIRQPED EKIHLNFTHL
 851 ELESHAGCSL NYIEVRDGDS EMSSLITRFC HSTVISPITS TSNSLWIKFK
 901 SDASVQRASF RAVYQVACGG SLSGEGTIHS PYFPRMSSQP KTCEWIISQS
 951 VSKVVILNFI DFDIRNTTTC DSDYVEVRDG NNTESPLLGK YCGTAVPSRV
1001 QSTRNNLYIK FRASSLTNLG FRAQYWPLDT VCGESLTGSE GTITSPGFPD
1051 VYPHGINCIW TINVQPGYLI RLTFTSFNLP FHSSCRMDYL EIYDNSTMQK
1101 LGRYCGRSIP PSLTSGGNVM MLYFVTDRSI SSEGFSANYI SLDASKVCSH
1151 NYNTETGVLT SPNYPNNYPV QTECIYTITV GINRQIVLRF TNFTLEGNLR
1201 CTEDYIEIRD GGYETSPYLG KYCGSGLPPV IISHGNKLWI KFVSDIFGTR
1251 KGFSAEWDGT SAGCGGTLTT SSGIFMSPNY PMPYYHSSEC YWLLRGSRGT
1301 PFEIQFEQFH LEYHPNCNFD YLAVYDGNSS NAKQLGKFCG NQIPQFINSS
1351 GDSVYIKLRT DSSVHGGGFL AKYKQVCHEV LTVNRSYGVL ESLNYPNNYP
1401 LGEHCRWTIQ TTKGNTLSYS FTAFDVEDGS NCDRDYLKLY DGPNVQSNLI
1451 GTFCGQSLPL AGNSTGTSLH VEFYSDGLQA RSGFQMLWHT NGCGGELSGP
1501 SGSFHSPGYP NRYPSNRECI WYIQTTPGSS IQLTIQEFDV EYHPNCNYDV
1551 LEVFGGPDFL SPRLAQLCIS RSAQNPLLLS TTGNSAVVRF KTDEAVTGKG
1601 FSASWQENLH GCGGIFQANS GEIHSPNYPE PYSNNTDCSW LIQVDYSHRV
1651 LLNFTDFDIE DHRLCNYDNV TVFDGPSNEA PLLRVLCGTQ HPSPITSSRN
1701 QMYVRLRSDR TFQHRGFSAR FSEACGSFIE SDSVGAAISS PLYPAKYPNN
1751 QNCSWIIQAQ EPFNHVTLSF TDFDIENNRQ NCTTDFVEIL DGNNYDAPLQ
1801 GRYCGTNMPH PITSFGNALV VNFISNNNIT TRGFHATYAA SSSSCGGTFH
1851 MDRGAFNSPG YPESYPLNTE CVWTILSSPG NRLQLSFTAF QVESSSGCTK
1901 DYLEIREGNA TGTLAGKFCG DSLPSNYTST VGHILWVKFV SDSSGTDVGF
1951 RATFSHLYGN DIVGNRGQIA SPQWPRSYPH NSNYQWRIST NASQVIHGRI
2001 LEMDIENHYR CYYDKLKVYD GPTIHSRPIA TYCGADPASF ASSGSTLTIQ
2051 FQSDSSVTGR GFLLEWYAME PSAPTRTIAR GACGGTVTSE ETPSFLYSPG
2101 WPLNYRNFAD CVWLIRAPGS TVEFNILALD IESHSSCYYD RLTIQDGDNG
2151 LSPLLATICG REPPGPVRST GETMFIRFVS DGSVTGAGFN ASYHKSCGGY
2201 LHADRGVITS PSYPQEYTPN LNCSWHVLVT SGYIITVHFE QPFQVKSEDA
2251 SCNSGDYVEL KNGLDASAPP LVSGRGNGRF CGSSPISTMY TTDNQLFVHF
2301 ISDSRNEGQG FKLKYEAKSL ACGGNIYISD FNPSGYTSSP NYPNNYPSHA
2351 DCVWTITAPN GHAVELQFED QFYIEPSPNC TSSYLELRNG ADSSAPVLAK
2401 LCGSLLPGSQ RSSGAVMYLR FRSDSSSTHV GFNAKYSIAP CGGTVAGRSG
2451 VIESVGYPDL HYPDNLLCEW FLQGPRGHYL TITLEDLDIQ NTSECASDFV
2501 EIREYNASGN LLGRYCGNTL PDAVDTSDSF AYVKFVADGS INARGFRLRF
2551 DSSTEECGGD FSAPVGMFTS PNYPNRYPHN RVCEWRITVE EGRRVILTIN
2601 DMRTEEHWRC SSDYVAVYNG LRQNSPRLVK LCGEVNPGTE VKSSGNTMKV
2651 VLVTDMTHAT RGFSVSYTSN EDSVCGGTLM GYTGGNFSSP GYDGVKNYTS
2701 KLNCEWTIEN PSHYNSSIYI SFEDFHLEHH QDCQYDYLEL RIGDADGELI
2751 ARLCGQAAPS VPLVIAAPQV WIHFVSDENT EDKGFLGRYI FEACGGIQSG
2801 ERGFISSPNY PEPYGNLNHC SWLLEAPEGE TITLNFTAFH VENHSLCKWD
2851 SVTILNGGSP GSPVIGRYCG NTSPGIIQSG SNKLLVIFNS DRSIQGGGFY
2901 ATWTAESLGC GGIIHSDSGM IKSPHWPQNF PMNTRCTWTI ITHESKHLEM
2951 IFHNNFQIPH SDGSCQSSFV KVWQGNHEEE EALLATGCGT SAPDPVIAPN
3001 NIVTTVFQSR DAPGRGFSAS FISRCGINFT SPAGRIVSPN YPSQYDNNLN
3051 CSYIIDRGPQ SLVILEFETF HLEAPALLSR ICLYDGVSIF RGTRVTPHPV
3101 ITLCGSEVPE PISVFGPMLL NFYTDSHIAG FGFQARYRTI ACGGTFSGSV
3151 GIISSPAHSV LDYHNNMNCS YHITVGSGKA VVLKFNTFQL ELSPSCYKDS
3201 VAVYDGSDTH APLLGKFCGS ELPPNMKSSS NQLFLVFNTD FSGSDRGWKA
3251 SFRETLGPQD GCGGYLTYSA YSFGSPVSNV SGRYERNLDC VWVITAPVNK
3301 LINLTFTSFV LEAHVAQTCQ YDYVKLYDGG NENANLVGTF CGSIVPAPFL
3351 STSNSLTLKF VTDNSVQREG FSATYTTVDR LCGGTYNATS TSLTATSPNF
3401 PSEYPPFTLC TWVIDAPPQQ QVKVVVETFH LHPSQDCSQN YLQLQDLPMH
3451 SQGSTHRFCG NETFPVPEFY SHDRTAIVTF KSDEYRINNG VRFTYQATGC
3501 SREYNQPFGY LKSPGWPGRH PNNMDCSIVL KAPLNHTISL FFHAFSLEDS
3551 IQCSHDFLEV RNGSNMQSPL LGRFCGNTVP SPIFPQSHVV FLRFKSDVSG
3601 AHDGYEITWT SSSSGCGGTL YGSTGTFASP RFPATYWNNT HCEWVITVPK
3651 GRIVTVNFDF ISIDDPGDCS SNYLILYNGP DTSYPQAGPY CGMDTNIAPF
3701 TATSHQVYVK FHAEYVTLPS GFRLSWTS
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Figure 3.6. Identification of the ggapoA-V binding protein via Mass Spectroscopy. The protein which was eluted from the reverse ligand blot (arrow in Fig. 3.7) was cut out from the silver stain gel and analyzed by the VBC Mass Spectroscopy facility. The peptides which could be identified are highlighted in red. These peptides were confirmed by database search to match perfectly to a chicken protein similar to mammalian cubilin.

Having identified cubilin as a potential ggapoA-V binding partner, it was now important to verify the presence of this protein in the chicken yolk sac. Because of the high degree of sequence conservation observed in the cubilin protein (Figure 3.7), we first tested whether an antibody directed against mouse or rat cubilin would also recognize chicken cubilin.

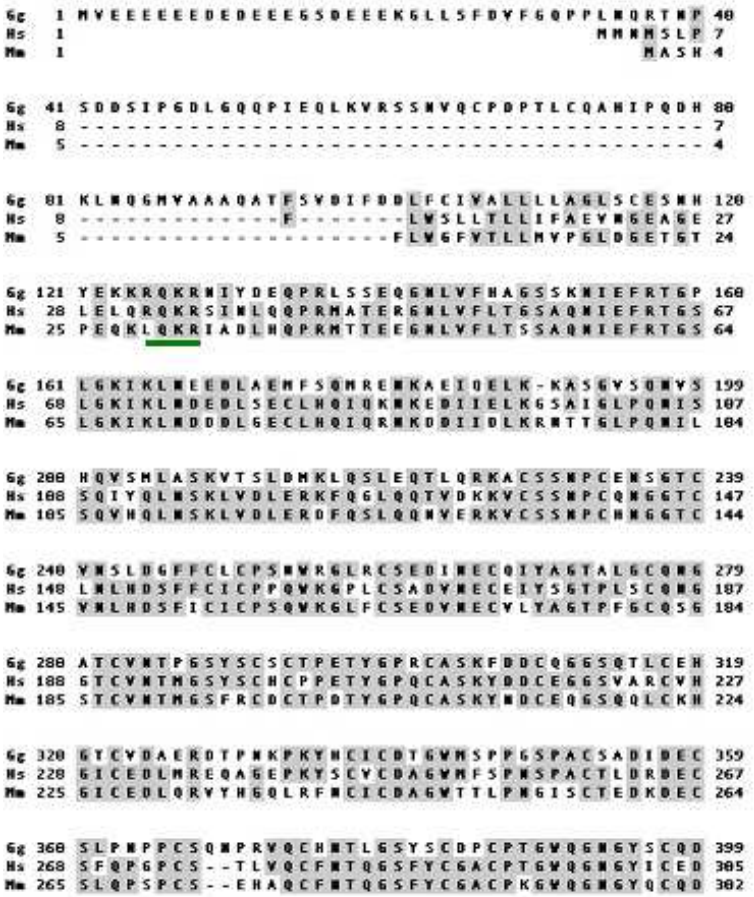

Figure 3.7. Sequence alignment of the amino terminus of chicken (Gg), human (Hs) and mouse (Mm) cubilin using ClustalW (MacVector). Identities are shown in grey, revealing a relatively high sequence conservation across species. Interestingly, the cubilin prepeptide which precedes the furin-cleavage site (underlined in green) in the chicken protein is by far the longest of the three.

Thus, we searched for an antibody that would recognize the chicken protein. Finally, we obtained an aliquot of antiserum from a research group in Texas, which had generated an antibody against mouse cubilin (Crider-Pirkle, Billingsley et al. 2002). Unfortunately, this antibody showed no cross reactivity with chicken cubilin, as shown in figure 3.8. This α-mouse cubilin antibody recognizes mouse and rat cubilin in kidney protein extracts (Figure 3.8, lanes 1, 3), but not chicken cubilin in the yolk sac (Figure 3.8, lane 5). Nevertheless, mouse and rat

cubilin (Figure 3.8, lanes 1,3) shows the same size as chicken cubilin, as determined by ligand blot analysis of chicken yolk sac extract (Figure 3.8, lane 4). Moreover, ggapoA-V shows binding to a protein present in the mouse kidney (Figure 3.8, lane 2). This protein migrates exactly at the same size as the second interaction partner detected in the chicken yolk sac (Figure 3.8, lane 4, see Fig. 3.4). In contrast, ggapoA-V shows no binding to mouse cubilin (Figure 3.8, lane 2).

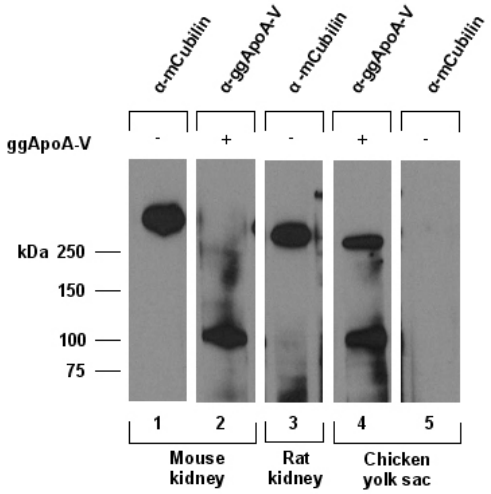

Figure 3.8. Western/Ligand blot analysis under non-reducing conditions for the detection of chicken cubilin in the yolk sac. The indicated protein extracts were subjected to gradient-gel SDS-PAGE and blotted to nitrocellulose membrane. Cubilin was detected using an α-mouse cubilin antibody (lanes 1, 3, 5). GgapoA-V (5µg pure, recombinant protein) was used as a ligand in lanes 2 and 3 and detected using an α-ggapoA-V antiserum. HRP-conjugated α-rabbit antibody was used for the detection of the primary antibodies.

Because it was not possible to detect chicken cubilin with the α-mouse cubilin antibody, I started with the generation of an α-chicken cubilin antibody, an important tool for further experiments. Because cubilin consists of repetitive structural domains also present in several other proteins, we decided to raise the antibody against the very N-terminal domain, a sequence stretch comprising 96 amino acids, which follows the furin cleavage site (Figure 3.7). This part of the protein is unique for cubilin, therefore presenting a good target for antibody formation (Fig. 3.9).

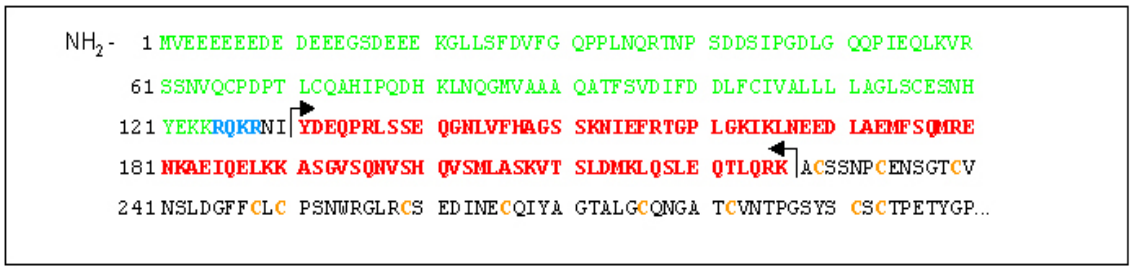

Figure 3.9. Cubilin peptide for antibody production. The protein region chosen for antibody generation is highlighted in red. This 96 amino acid stretch represents the only unique part of cubilin. The furin cleavage site is shown in blue, the prepeptide in green. The cysteine residues (yellow) mark the beginnig of the EGF domains which immediately follow the selected antigen region.

The DNA stretch corresponding to the cubilin antibody peptide, i.e., the predicted cDNA sequence for chicken cubilin in the PubMed Nucleotide database, is shown in figure 3.10. The sequence corresponding to the furin cleavage site (bold, black, underlined) and the primers used (blue and red, underlined) are shown.

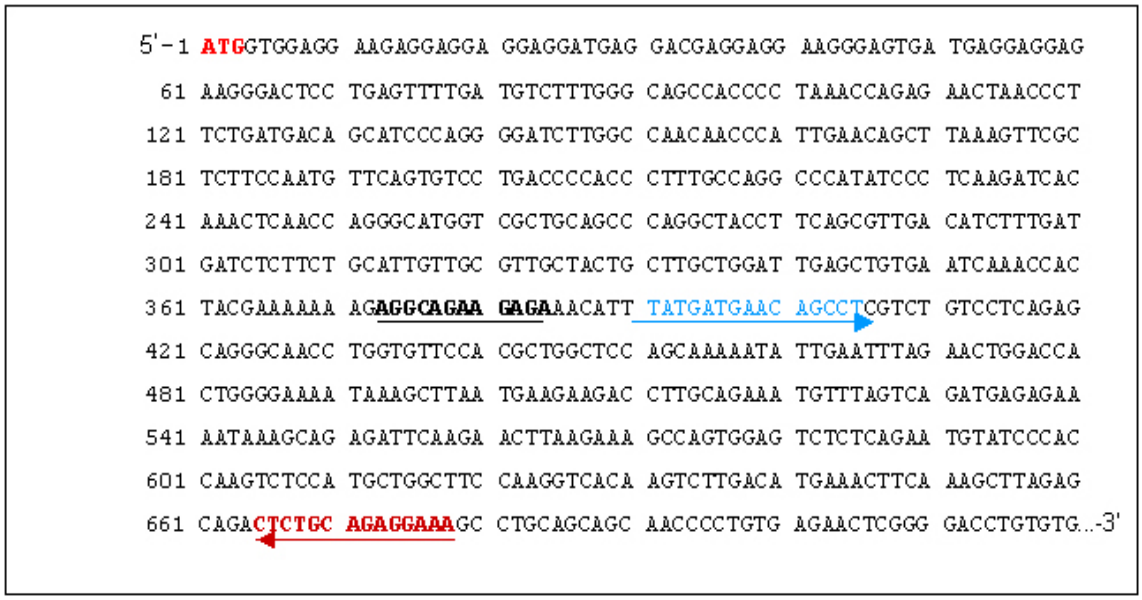

Figure 3.10. Start of the Chicken cubilin cDNA containing the fragment which encodes the antibody peptide. Light red = transcriptional start codon; underlined and bold bases = furin cleavage site; blue, underlined = forward primer with an additional NcoI restriction enzyme cleavage site (5'-AAC CAT GGT CTA TGA TGA ACA GCC T-3'); dark red, underlined = reverse primer with an additional EcoRI restriction enzyme cleavage site (5'-TTG AAT TCG GTT TCC TCT GCA GAG T-3'). Restriction enzyme cleavage sites are essential for the cloning of the fragment into the vector systems used.

For the amplification of the DNA fragment which encodes the antibody peptide, PCR was performed from yolk sac and kidney cDNAs following preparation of total RNAs from these tissues. Primers were used as described above, and the PCR reaction product was analyzed on a 2% agarose gel containing ethidium bromide (Figure 3.11). Bands were obtained in cDNAs of both tissues. It appears that cubilin may be more abundant in chicken kidney (Figure 3.11, lane 1) than in yolk sac (Figure 3.11, lane 2).

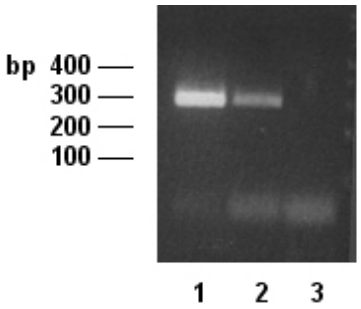

Figure 3.11. RT-PCR for amplification of the cubilin DNA fragment chosen for antibody generation. PCR products were analyzed on a 2% agarose gel containing ethidium bromide. Lane 1, the PCR product amplified from LH kidney cDNA; lane 2, yolk sac cDNA. PCR products were cut out from the gel and used for subsequent cloning reactions.

After cloning of the fragment into the pBluescript vector and confirmation of the sequence (obtained from the VBC sequencing facility), the DNA fragment encoding the antibody peptide was cloned into the expression vector pET-25b(+) using the restriction enzymes NcoI and EcoRI. The DNA fragment was again verified by DNA sequencing, and the vector containing the cubilin insert was transformed into the *E.coli* expression strain BL21. The recombinant chicken cubilin antibody fragment was expressed with a C-terminal 6x-His tag provided by the pET-25b(+) vector system. The induction of protein expression is shown in figure 3.12.

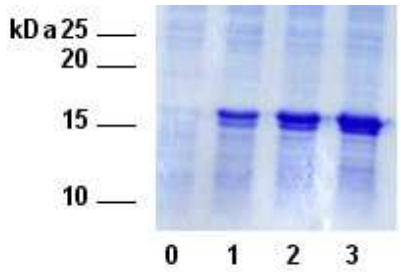

Figure 3.12. Induction of the expression of the His-tagged cubilin antibody peptide in *E.coli* strain BL21. The cubilin antibody peptide was expressed as a fusion protein containing a C-terminal His-tag. Bacteria were grown to an OD of 0.6 and protein expression was induced by addition of 1mM IPTG. 0 = before induction of protein expression; 1 = one hour after the induction of protein expression; 2 = after 2 hours of expression; 3 = after three hours of expression. At every time point, samples were removed and subsequently subjected to SDS-PAGE. Proteins were detected using a Coomassie Blue stain.

I was fortunate that the protein was expressed efficiently in high amounts. Therefore, the protein was purified using Ni-NTA Agarose, which binds proteins via their His-tag, and the purification results were analyzed by Western blotting using an α-His antibody (Figure 3.13).

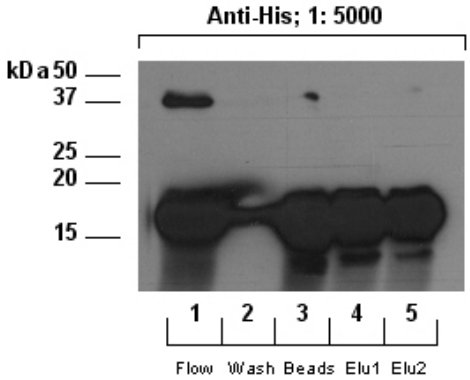

Figure 3.13. Western blot analysis for the detection of the recombinantly expressed and purified cubilin antibody peptide with α-His antibodies. After protein expression, the crude bacterial protein extract was incubated with Ni-NTA Agarose for the purification of the protein via its His-tag. Lane 1 shows the proteins which did not bind to the Ni-NTA beads; lane 2 shows the wash fraction (beads were extensively washed); lanes 4 and 5 show the eluted protein detected with α-His antibodies.

The soluble, purified protein was used for antibody generation. 300µg protein was injected into a New Zealand White rabbit and booster injections with the same amount of protein were administered 3 and 6 weeks after the primary immunization. After 8 weeks, the specificity of the

rabbit antiserum was tested by Western blot analysis on LH kidney and yolk sac protein extracts (Figure 3.14.). The antiserum, designated α-ggCubilin antiserum, clearly recognizes a protein of the expected size in kidney and yolk sac protein extracts, in agreement with the presence of cubilin in these tissues. No immunoreactive band was observed when probing the same blots with preimmune serum, confirming the specificity of the antiserum. Interestingly, the Western blot under reducing conditions in figure 3.14 shows, that the proteins in the kidney and yolk sac recognized by the α-ggCubilin antiserum seem to be slightly different in size.

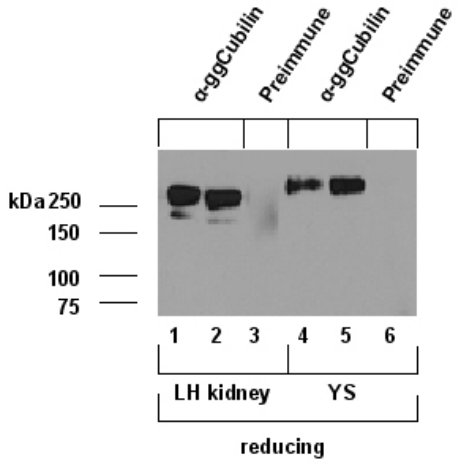

Figure 3.14. Western blot analysis of chicken cubilin in kidney and yolk sac under reducing conditions. Protein extracts were subjected to SDS-PAGE and blotted to a nitrocellulose membrane. For the detection of Cubilin in these extracts, lanes 1, 2, 4, and 5 were incubated with the α-ggCubilin antiserum (dilution 1:2000). Preimmune serum was used as a negative control (Lanes 3, 6). As secondary antibody, HRP-conjugated goat α-rabbit antibody was used and visualization was performed using ECL.

However, Western blots under non-reducing conditions show that the immunoreactive band recognized by the α-ggCubilin antiserum in the yolk sac appears diffuse (Figure 3.15, lanes 3, 4), whereas in the kidney the band was much narrower (Figure 3.15, lanes 1, 2). One explanation for this different migration pattern of the immunoreactive protein could be different extents of glycosylation of cubilin in kidney versus yolk sac. Furthermore, only in the yolk sac, the antibody recognizes a band at about 140 kDa under non-reducing conditions (Figure 3.15, lanes 3, 4).

Thus, to obtain supportive evidence that the α-ggCubilin antiserum recognizes chicken cubilin, the α-mCubilin antiserum was used as a positive control (Figure 3.15, lane 5, 6). Chicken and rat cubilin show almost the same size, indicating that the protein recognized by the α-ggCubilin antiserum indeed represents its target (compare also with Fig. 3.8).

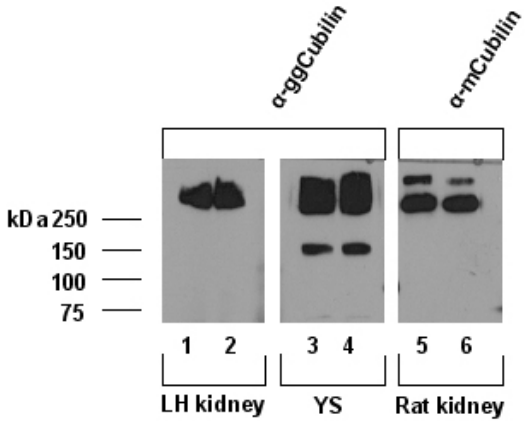

Figure 3.15. Western blot analysis of cubilin in chicken and rat tissues under non-reducing conditions. Protein extracts were subjected to SDS-PAGE and blotted to nitrocellulose membrane. For the detection of chicken cubilin, the nitrocellulose membrane strips were probed with the α-ggCubilin antiserum (lanes 1, 2, 3, 4). For the detection of rat cubilin, the α-mCubilin antiserum was used (lanes 5, 6). As secondary antibody, HRP-conjugated goat α-rabbit antibody was used, and visualization was performed using ECL.

To further confirm that ggapoA-V interacts with chicken cubilin, ligand blots were performed, and in adjacent lanes, cubilin was detected with the α-ggCubilin antiserum (Figure 3.16, lanes 1, 2, 3). The same size of the band detected with the specific antiserum (lanes 1, 3) and with the ligand ggapoA-V (lane 2, faint band) confirms that ggapoA-V binds to chicken cubilin. The ligand interaction was again solely observed under non-reducing conditions. Importantly, it needs to be pointed out that ligand blotting for the analysis of cubilin has not been previously reported.

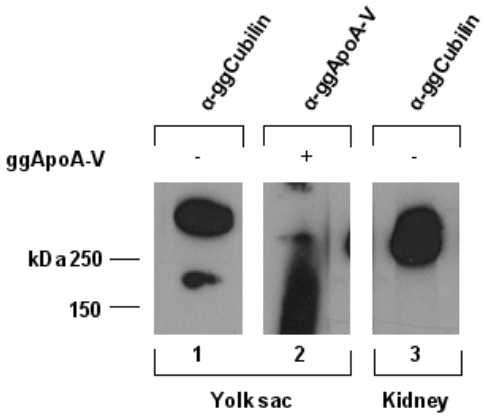

Figure 3.16. Western/Ligand blot for the confirmation of the ggapoA-V – cubilin interaction. Chicken yolk sac and kidney protein extracts were subjected to non-reducing SDS-PAGE and subsequently blotted to nitrocellulose membrane. Chicken cubilin was detected in both tissues using α-ggCubilin antiserum (lanes 1, 3). As a ligand, ggapoA-V was added to the membrane strip in lane 2, and after washing, bound ggapoA-V was detected using α-ggapoA-V antiserum. HRP-conjugated goat α-rabbit antibody was used as secondary antibody, and visualization was performed using ECL.

To identify new interaction partners of ggapoA-V in the yolk sac, and to further confirm the ggapoA-V – cubilin interaction, I performed affinity chromatography. To this end, I recombinantly expressed and purified 3mg of full-length ggapoA-V cloned into a pET25b(+) vector system, kindly provided by Andrea Dichlberger in the lab. The ggapoA-V was coupled to CNBr-activated Sepharose 4B beads (Figure 3.17, panel A), and affinity chromatography was performed using freshly prepared yolk sac membrane extract. The protein extract was allowed to circulate 2 hours through the affinity column containing the ggapoA-V-coupled beads. Following washing of the beads with approximately 350ml of wash-buffer, bound proteins were eluted from the column. The pooled eluted fractions were dialysed, lyophilized and dissolved in a small amount of ultrapure water. The sample was analyzed by silver stain for the detection of the eluted proteins (Figure 3.17, B). Interestingly, 2 proteins could be eluted from the affinity column, likely representing binding partners of ggapoA-V (lanes 5, 7). The protein in lane 7 has the size observed for chicken cubilin, in agreement with the above-presented evidence for a ggapoA-V – cubilin interaction. The protein in lane 5, with an apparent molecular mass of about 200 kDa may represent a hitherto unidentified ggapoA-V binding partner in the embryonic yolk sac. Whether this protein corresponds to the second immunoreactive band recognized by the α-ggCubilin antibody in the yolk sac seen under non-reducing conditions (Figure 3.16, lane 1) awaits analysis by mass spectroscopy.

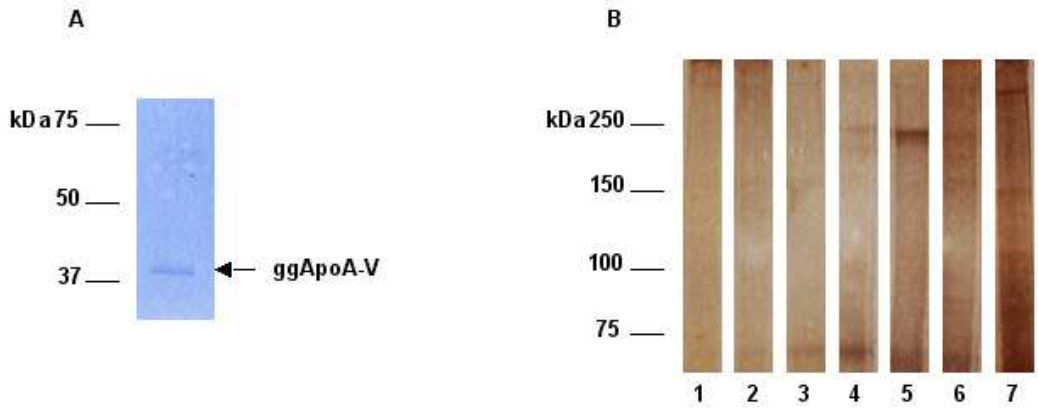

Figure 3.17. Affinity chromatography. 3mg of ggapoA-V were coupled to CNBr-activated Sepharose 4B beads, and the coupling efficiency was confirmed by elution of the bound proteins from the beads and Coomassie blue stain (Figure 3.16, A). The ggapoA-V coupled beads were incubated with freshly prepared yolk sac extract isolated from chicken embryos at day 10 of embryonic development. After extensively washing, the ggapoA-V binding proteins were eluted. Fractions of 1ml were collected, dialysed and concentrated by lyophilization. After dissolving in ~120µl ultrapure water, each fraction was subjected to silver staining following SDS-PAGE (B). Lane 1, last wash fraction before elution; lanes 2 and 3, consecutive 25mM EDTA elutions; lanes 4 and 5, consecutive 4M NaCl elutions; lanes 6 and 7, consecutive wash fractions after elution with 4M NaCl; All elutions were with 1ml of the corresponding buffer.

Furthermore, the presence of ggapoA-V in the yolk sac was observed using Western blot analysis (Figure 3.18, lane 1). The α-ggapoA-V antiserum clearly recognized an immunoreactive band of ~40 kDa matching with the well known size for ggapoA-V. The presence of ggapoA-V in the yolk sac is in good agreement with the fact that the protein was also found in the yolk compartment of chicken follicles (Andrea Dichlberger, unpublished results). Whether the detected protein is of maternal origin or synthesized by the cell layers of the yolk sac per se is not known. Nevertheless, the presence of the protein in the yolk sac supports the notion that ggapoA-V exerts a biological function during development of the chicken embryo.

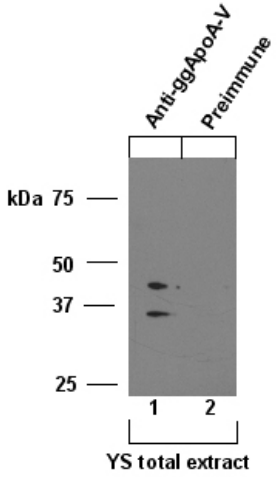

Figure 3.18. Western blot analysis of ggapoA-V in the chicken yolk sac. Yolk sac detergent extract was subjected to 10% reducing SDS-PAGE and blotted to nitrocellulose membrane. GgapoA-V was probed using α-ggapoA-V antiserum (lane 1). Preimmune serum was used as a negative control (lane 2). For detection of the immunoreactive proteins, HRP-conjugated goat α-rabbit antibody was used, and visualization was done using ECL.

Several results obtained in my work provide strong evidence that ggapoA-V interacts with chicken cubilin in the yolk sac, an important extraembryonic structure which supplies the developing embryo with nutrients. Because virtually nothing is known about the function of cubilin in the avian yolk sac, it will be interesting to conduct further experiments on this protein. In addition, further experiments will be necessary to learn more about the biological importance of the ggapA-V – Cubilin interaction.

4. DISCUSSION

Apolipoprotein A-V (apoA-V) has been established as an important and potent regulator of triglyceride (TG) levels. However, the exact detailed molecular mechanisms involving apoA-V are still unknown. Nevertheless, various properties, including the presence on certain classes of lipoproteins, lipid binding affinity, lipid droplet association, liver regeneration capacity, and the ability to bind cell surface- and sorting receptors have been described for this protein. In regards to this latter aspect, a search for new interaction partners of apoA-V is of prime interest.

Findings in model organisms like the chicken with its highly regulated metabolic pathways for TG-rich lipoproteins can be anticipated to be of general significance for our understanding the biology of apoA-V. Thus, the capacity of chicken apoA-V to bind to the major oocyte plasma membrane receptor LR8 revealed in our group (Dichlberger, Cogburn et al. 2007) provides a solid starting point for expanding our research on ggapoA-V in this direction.

4.1. Cubilin, a New Binding Partner of Chicken apoA-V

The major part of my diploma thesis was based on the above mentioned property of apoA-V to interact with cell surface receptors. Binding of hapoA-V to LR11, LRP1 and sortilin was reported by Nilsson et al using surface plasmon resonance in vitro binding studies for lipid-free as well as for DMPC-bound recombinant hapoA-V (Nilsson, Lookene et al. 2007; Nilsson, Christensen et al. 2008). They concluded that the protein is able to interact with the ligand binding domains present in LDLR related proteins and with Vps10p protein motifs in members of the Vps10p-domain receptor family. Furthermore, they demonstrated by internalization experiments and live imaging studies that hapoA-V is endocytosed by, and co-localizes with, these receptors in early endosomes (Nilsson, Christensen et al. 2008).

Moreover, ggapoA-V was shown to bind the major oocyte receptor LR8 in the chicken ((Dichlberger, Cogburn et al. 2007) and Figure 3.3). LR8, homologous to the mammalian VLDL receptor, is known to mediate chicken oocyte growth by binding and endocytosis of VLDL and VTG, the major components of yolk (Bujo, Hermann et al. 1994). Because ggapoA-V was also detected in the VLDL fraction of chicken plasma lipoproteins (Dichlberger, Cogburn et al. 2007), a proposed function of the protein (maybe in concert with apoB) is to enhance the uptake of VLDL particles into the yolk compartment of the oocyte.

With the evidence that chicken apoA-V shares the receptor binding capacity with its human counterpart, I started to search for new binding partners of ggapoA-V in various tissues. Indeed, using ligand blot analysis, I was able to detect two new binding partners of ggapoA-V in the chicken yolk sac (Figure 3.4, left panel).

The chicken yolk sac is an extraembryonic structure consisting of two loosely associated somatic cell layers (Figure 4.1). The outer layer is of mesodermal origin, whereas the inner layer, facing the yolk compartment, is composed of endodermal cells (Hermann, Mahon et al. 2000). These cells, called endodermal epithelial cells (EEC's) mediate the transport of yolk components to the embryonic circulation, thereby providing the growing embryo with nutrients. The molecular mechanisms underlying these transfer processes are largely unknown. Nevertheless, it was reported that the EEC's express a specific set of receptors which are exposed to the yolk compartment. In 2000, Hermann et al reported the presence of LR8 in the yolk sac, a highly active lipoprotein receptor with endocytic function (Hermann, Mahon et al. 2000). Furthermore, current studies have shown that the chicken yolk sac is one of the major expression sites of the LDLR-related protein LRP2 (Plieschnig 2008), which has a very broad ligand spectrum and also shows high endocytic activity.

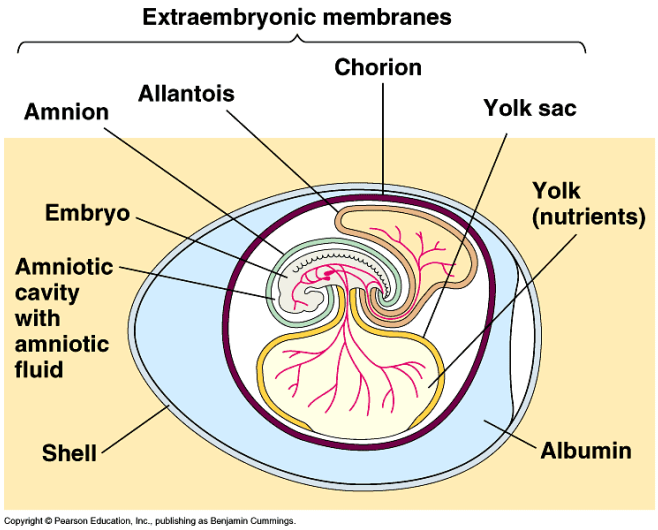

Figure 4.1. Schematic representation of a chicken embryo with its surrounding extraembryonic structures. Picture from http://io.uwinnipeg.ca/~simmons/16cm05/1116/chordate.htm

Because I could obtain strong evidence that none of the above mentioned receptors are interaction partners of ggapoA-V, the identification of the proteins identified by ligand blotting with apoA-V (Figs. 3.4 and 3.5) was of great interest.

The isolation of the >250 kDa ggapoA-V binding partner (Figure 3.5, lane 2) by reverse ligand blotting and its subsequent identification as chicken cubilin (Mass Spectroscopy data, Figure 3.6) provided evidence that ggapoA-V binds cubilin present in the total membrane fraction of chicken yolk sac.

Structure and ligands of cubilin: Cubilin, which to date has not been characterized in the chicken, is a 460 kDa large peripheral membrane glycoprotein in mammals known to be involved in endocytic processes (Christensen and Birn 2002). Formerly known as gp280, Cubilin was identified in 1997 as the intestinal intrinsic factor vitamin B_{12} receptor (Seetharam, Christensen et al. 1997). The gene for human cubilin is located on chromosome 10p (Kozyraki, Kristiansen et al. 1998), and the protein shows a high sequence conservation across species (canine cubilin shows 83% identity to human cubilin, (Christensen and Birn 2002)). The remarkable structure of cubilin is shown in Figure 4.2. Because cubilin lacks a transmembrane region as well as an intracellular domain, the protein is loosely attached to the plasma membrane, typical of known peripheral membrane proteins (Kristiansen, Kozyraki et al. 1999). The amino-terminal part of cubilin, a stretch of about 130 amino acids, has been shown to convey membrane association of the protein (Kristiansen, Kozyraki et al. 1999). Furthermore, helical plotting of this region showed the presence of a conserved amphipathic helix structure with a similarity to A-type amphipathic helices present in many apolipoproteins (Kristiansen, Kozyraki et al. 1999).

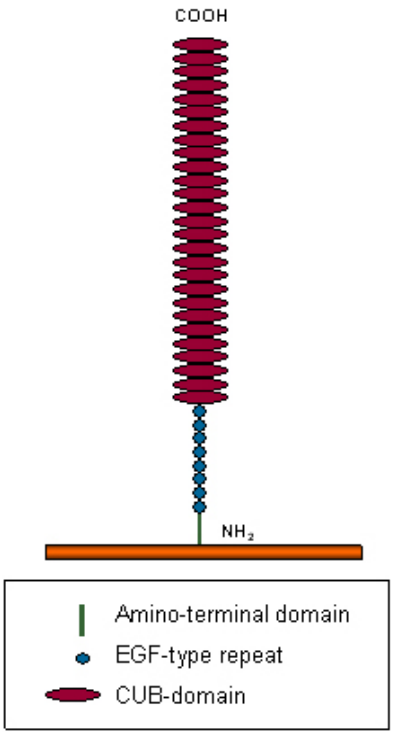

Figure 4.2. Schematic representation of the overall structural composition of cubilin. Cubilin displays neither a transmembrane nor a cytoplasmic domain. The protein's amino terminus, a stretch of about 130 amino acids, contains a putative class A amphipathic helix region and confers membrane association of the protein (Kozyraki 2001). This amino-terminal domain is followed by a domain of 8 EGF-repeats and 27 CUB-domains. This assembly of protein modules observed in cubilin is unique among the whole proteome.

At the extreme amino terminus of the protein, a recognition cleavage site for the transmembrane serine protease furin is present, indicating that post-translational processing of cubilin involves furin-mediated cleavage in the trans-Golgi network (Christensen and Birn 2002). The amino-terminal stretch is followed by 8 tandemly arranged EGF-type repeats. These cysteine-rich protein domains are also present in several membrane proteins including members of the LDLR family.

The largest part of cubilin is comprised of a cluster of 27 CUB (complement C1r/C1s, Uegf, and bone morphogenetic protein 1) domains. These CUB domains were initially observed in the C1r/C1s components of the complement system (Bork and Beckmann 1993) and subsequently in developmental control proteins like spermadhesins, tolloid protein, bone morphogenetic protein 1 (BMP1), and the tumor necrosis-factor stimulating protein 6 (TSG6) (Moestrup, Kozyraki et al. 1998). Each CUB domain is comprised of 110 amino acids including 4 conserved cysteine residues, except domain 13 of Cubilin, which lacks the first 2 cysteines (Moestrup, Kozyraki et al. 1998). The crystal structure of the CUB domain has been elucidated in spermadhesins (Romero, Romao et al. 1997). There it was shown that the CUB domain is composed of 2 five-stranded beta sheets, connected by surface exposed beta-turns (Romero, Romao et al. 1997). This predominant structural motif of cubilin has been proposed to constitute a potential ligand binding site for interaction with proteins, carbohydrates, and phospholipids (Moestrup, Kozyraki et al. 1998).

Compatible with this notion, cubilin acts as a multiligand binding protein for a broad spectrum of ligands including transferrin, albumin, haemoglobin, apolipoprotein A-I/HDL, transthyretin, intrinsic factor-vitamin B12, RAP, and for the membrane-anchored receptors LRP2 and amnionless (reviewed in Kozyraki and Gofflot 2007). Some of these ligands are also bound and endocytosed by LRP2, indicating a synergistic action of these two proteins.

Tissue distribution and intracellular trafficking of cubilin: The major site of cubilin expresssion is the surface of various polarized epithelial cells. Therefore, cubilin was shown to be synthesized by the epithelial cells of the renal proximal convoluted tubules (PCT), the visceral yolk sac (VYS) in rodents, small intestine, and the placenta (Sahali, Mulliez et al. 1988; Seetharam, Christensen et al. 1997). In addition, lung alveolar type II cells were reported to express low levels of cubilin (Kolleck, Wissel et al. 2002). Although it is well known that cubilin is exposed at the plasma membrane of polarized epithelial cells, only little is known about the subcellular trafficking and the mechanisms underlying the membrane anchoring of cubilin. However, recent studies propose a concerted action of cubilin with the 45- to 50 kDa transmembrane protein amnionless for membrane targeting and membrane anchoring of cubilin. In more detail, amnionless (AMN) was shown to bind to the EGF domain of cubilin (Coudroy, Gburek et al. 2005), thereby mediating the release and export of the complex from the endoplasmic reticulum (ER) (Coudroy, Gburek et al. 2005). Furthermore, both proteins seem to be absolutely required

for the expression of the complex at the plasma membrane (Coudroy, Gburek et al. 2005). Due to its high degree of glycosylation, cubilin is thought to be essential for the apical sorting of the receptor complex (Coudroy, Gburek et al. 2005).

The essential role of amnionless as a subunit of this receptor complex was also demonstrated in a study where cubilin failed to be targeted to the plasma membrane due to a mutation in the amnionless gene (He, Madsen et al. 2005). Dogs with this mutation (AMN c.1113-1145del) suffered from Imerslund-Gräsbeck syndrome, characterized by intestinal malabsorption of the intrinsic factor-cobalamin (vitamin B_{12}) (He, Madsen et al. 2005). Another binding protein of cubilin at the plasma membrane is LRP2, which most probably mediates the co-endocytosis of cubilin (Moestrup, Kozyraki et al. 1998). This was shown in vitro by a reduced uptake of the cubilin ligand HDL following the administration of α-LRP2 antibodies or LRP2 antisense oligonucleotides (Hammad, Barth et al. 2000). Figure 4.3 shows the proposed cubilin–amnionless–LRP2 trimeric complex.

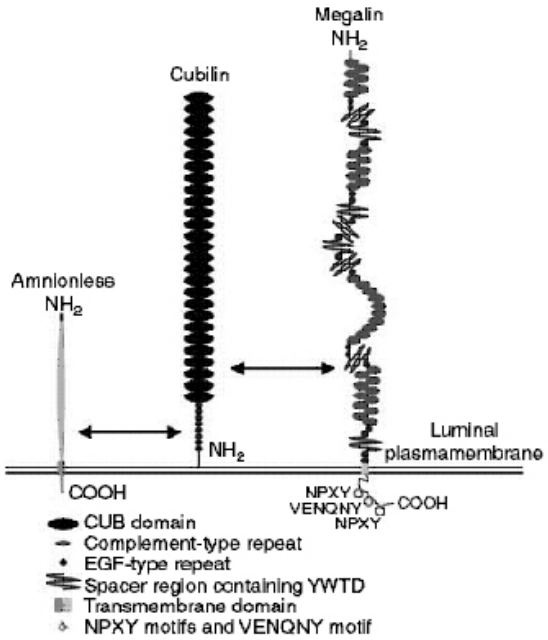

Figure 4.3. Cubilin, amnionless, and the large endocytic receptor LRP2 form a trimeric complex at the surface of various polarized epithelial cells. Cubilin and amnionless assist each other towards successful targeting to the plasma membrane. Additionally, LRP2 binds cubilin, thereby facilitating the endocytosis of cubilin ligands (Birn, Fyfe et al. 2000).

Physiological role of cubilin: The first physiological function described for cubilin was the ileal absorbtion of intrinsic factor cobalamin (vitamin B_{12}) (Seetharam, Christensen et al. 1997). Patients with mutations in the cubilin gene (and also with mutations in the amninonless gene)

suffer from the Imerslund-Gräsbeck disease (also known as autosomal recessive megaloblastic anemia, MGA), characterized by malabsorption of cobalamin (vitamin B_{12}).

In the kidney, high expression levels of cubilin were shown to contribute to the reabsorption of low molecular weight proteins such as albumin and apoA-I. This was again observed in dogs which lack functional cubilin. These dogs excrete high amounts of known cubilin ligands with their urine (Kozyraki, Fyfe et al. 1999; Birn, Fyfe et al. 2000), which is also the case for humans suffering from the Imerslund-Gräsbeck disease.

Finally, cubilin is known to be abundantly expressed in the visceral layer of the rodent yolk sac (Sahali, Mulliez et al. 1988). An indirect evidence for the physiological importance of cubilin at the feto-maternal interface was provided by the finding that the administration of monoclonal α-cubilin antibodies induced fetal resorption or malformations (Sahali, Mulliez et al. 1988). One reason for this dramatic phenotype could be the reduced or impaired uptake of cholesterol due to the functional inactivation of cubilin, shown to be responsible for the uptake of apoA-I/HDL in yolk sac epithelial cells (Kozyraki, Fyfe et al. 1999). Another function of cubilin at the feto-maternal interface was found by a research group studying the biology of uterine lectins. Quite surprisingly, they found that the lectin galectin-3 in the murine utero-placental complex, a protein found exclusively in the uteri of pregnant females and mainly located at the implantation site of the embryo, interacts with cubilin (Crider-Pirkle, Billingsley et al. 2002).

Interestingly, to date there have been no reports about cubilin in any avian species. However, the findings reported here, i.e., that ggapoA-V binds chicken cubilin in the chicken yolk sac, provides even more evidence for the functional importance of cubilin at feto-maternal interfaces. Chicken yolk sac, which functions analogous to the mammalian placenta, absorbs components from the yolk compartment to provide the chicken embryo with essential nutrients. Compared to other species, it is likely that this uptake is mediated via endocytic receptors, which localize to the surface of the EEC layer of the yolk sac. The finding that LRP2 is one of these receptors (Plieschnig 2008) makes the presence of cubilin in the chicken yolk sac even more interesting. Therefore, it is most likely that in the chicken yolk sac, cubilin and LRP2 also act as a highly efficient endocytic complex. In addition, chicken amnionless transcripts were detected by Northern blot analysis in the yolk sac (Christian 2006). The expression of *ggAMN* and the presence of cubilin and LRP2 proteins in the yolk sac suggest that an active trimeric complex is produced in this tissue. Nevertheless, because we have only very limited information about the amnionless protein in chicken yolk sac, further investigations are required to characterize the cubilin-amnionless-LRP2 complex in the chicken.

Furthermore, the ggapoA-V–cubilin interaction in the yolk sac is supported by at least two interesting observations. First, Western blot analysis showed that ggapoA-V is present in the yolk compartment of growing follicles (Andrea Dichlberger, unpublished reults). Secondly, ggapoA-V could also be detected in yolk sac extracts (Figure 3.18). These findings are

compatible with the uptake of ggapoA-V into cells of the yolk sac via cubilin as part of the trimeric cubilin-amnionless-LRP2 complex, but this possibility needs to be tested formally.

Figure 4.4. shows a proposed model for the migration and/or transport of ggapoA-V from its site of synthesis in the maternal liver to its endocytosis by cubilin into cells of the yolk sac. After its synthesis, ggapoA-V leaves the liver either bound to VLDL and HDL particles or in lipid-free form. At the plasma membrane of the growing oocyte, ggapoA-V binds to and is internalized by LR8 into the yolk compartment of the germ cell. After fertilization of the oocyte, embryonic structures begin to form. During embryogenesis, yolk becomes enclosed by two cell layers giving rise to the extraembryonic structure of the yolk sac. These cells, most probably the EECs, express cubilin at the surface facing the yolk compartment. Consequently, ggapoA-V becomes bound and endocytosed, resulting in its localization in the cells of the yolk sac. Whether ggapoA-V is targeted to the embryonic circulation or fulfills a hitherto unknown function in the cell layer of the yolk sac is not known. However, the EEC layer of the chicken yolk sac is highly enriched in phagolysosomes containing yolk granules (Donaldson, Bogenman et al. 1990). These yolk droplets contain lipids and apolipoproteins, which were previously taken up by the EECs from the yolk compartment (Kanai, Soji et al. 1997). The fact that apoA-V was shown to associate with lipid droplets (Shu, Ryan et al. 2008) suggest the presence of ggapoA-V within the yolk granules of the yolk sac EECs. There, the protein could be used for the synthesis of new lipoprotein particles, which are subsequently transferred to the embryonic circulation to supply the embryo with nutrients (Kanai, Soji et al. 1997). Interestingly, early studies suggested that apoA-V may be a limiting factor for the production of triglyceride-rich lipoproteins (Dichlberger, Cogburn et al. 2007); thus, sufficient amounts of apoA-V may be provided via the multi-step process described here.

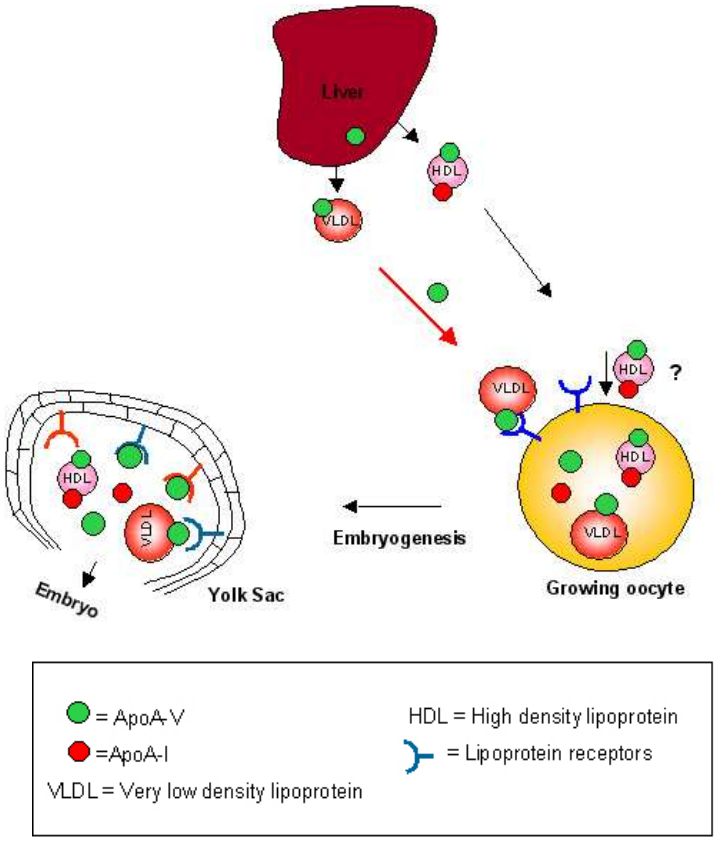

Figure 4.4. Schematic representation of the proposed metabolic pathway of ggapoA-V. GgapoA-V (green) enters the maternal circulation after its synthesis in the liver. At the plasma membrane of the oocyte, the protein is endocytosed by LR8 and accumulates in the yolk compartment of the oocyte. After fertilization and formation of the yolk sac, ggapoA-V is internalized into the EEC layer of the yolk sac by an uptake mechanism involving the peripheral membrane protein cubilin.

As determined by Western blot analysis, yolk sac cubilin showed a slightly slower migration in SDS gels (Figure 3.14) than kidney cubilin. One of the reasons for this difference could be a tissue specific, alternative posttranslational modification of the protein. Two mechanisms by which cubilin is posttranslationally modified are cleavage of the cubilin leader peptide by the TGN proteinase furin and glycosylation of the protein, as cubilin was shown to contain 42 potential N-glycosylation sites (Moestrup, Kozyraki et al. 1998). Differences in the glycosylation pattern between yolk sac- and kidney cubilin could also contribute to different binding affinities of ggapoA-V to cubilin. This may explain why ligand blot analysis detected the binding of ggapoA-V to cubilin present in yolk sac extracts only.

In conclusion, the results gained during my diploma research provided strong evidence for the identification of cubilin as a new binding partner of ggapoA-V, and allows the proposal of a model for the action of cubilin in the chicken yolk sac. The newly generated data are a solid basis for further studies into the functions of apolipoproteins and their receptors in the chicken, which is a powerful model due to the highly regulated pathways orchestrating massive lipid flow in the laying hen.

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6. ABBREVIATIONS

A

α	alpha, anti
α-2M	alpha-2 macroglobulin
A	adenin
Apo	apolipoprotein
APS	ammonium persulfate
Amp	ampicillin
AMN	amnionless

B

BMP1	bone morphogenetic protein 1
bp	base pair

C

C	cytosine
CE	cholesterolester
CM	chylomicrons
cDNA	complementary DNA
$CaCl_2$	calcium chloride
C-terminus	carboxy-terminus
CD	circular dichroism
CUB	complement C1r/C1s, Uegf, and bone morphogenetic protein 1
CNBr	cyanogen bromide

D

DNA	deoxyribonucleic acid
DTT	dithiothreitol
DMPC	dimyristoil phosphatidylcholin

E

EDTA	ethylenediaminetetraacetic acid
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
EEC	epidermal endothelial cells
EGF	epidermal growth factor

G

G	guanine

gg	gallus gallus
GC	granulosa cells
GPIHBP1	glycosylphosphatidylinositol-anchored high-density lipoprotein – binding protein 1
gp	glycoprotein

H

HDL	high-density lipoprotein
HRP	horseraddish peroxidase
HNF4A	hepatocyte nuclear factor-4 alpha
HSPG	heparan sulfate proteoglycane
hs	homo sapiens
His	histidine
hr	hour

I

IPTG	isopropyl β-D thiogalactoside
IL 1-B	interleukine 1-beta
IP	immunoprecipitation
IgG	immunoglobulin G
IF	intrinsic factor

K

kDa	kilo dalton
kb	kilo bases
kbp	kilo basepairs

L

LDL	low-density lipoprotein
LRP	low-density lipoprotein receptor related protein
LB	luria broth
LCAT	lecithin:cholesterol-acyltransferase
LDLR	low-density lipoprotein receptor
LR8	low-density lipoprotein receptor related protein with 8 ligand binding domains
LPL	lipoprotein lipase
LA	type A ligand binding domains
LH	laying hen
LXRA	liver X receptor alpha

M

µ	micro
mM	milli molar
mA	milli ampere
MWCO	molecular weight cut-off
mRNA	messenger ribonucleic acid
MTP	microsomal transfer protein
Mm	mus musculus
M6P	mannose-6-phosphate
MGA	megaloblastic anemia

N

nm	nano meter
n.d.	not defined
NTP	nucleoside triphosphate
N-terminus	amino terminus
Ni-NTA	nickel nitriloacetic acid

O

o.n.	overnight
OD	optical density

P

PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PAA	polyacrylamide
PPARA	peroxisome proliferator-activated receptor alpha
PCT	proximal convoluted tubule
PAGE	polyacrylamide gel electrophoresis

R

RT	room temperature
rpm	rounds per minute
RNA	ribonucleic acid
R/O	restricted ovulator
RAP	receptor associated protein
RT-PCR	reverse transcriptase polymerase chain reaction

S

SDS	sodium dodecyl sulfate
SNP	small nucleotide polymorphism

SPR	surface plasmon resonance

T

TG	triglyceride
T	thymine
TEMED	N,N,N',N'-Tetramethylethylenediamine
TBS	tris-buffered saline
TNF-α	tumor necrosis factor-α
TGN	trans-golgi network
TSG6	tumor necrosis-factor stimulating protein 6

U

UV	ultra violette
USF1	upstream-stimulating factor 1

V

VLDL	very-low density lipoprotein
Vps10p	vacuolar protein sorting 10 protein
VTG	vitellogenin
VBC	Vienna Biocenter
VYS	visceral yolk sac

Y

YS	yolk sac

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