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Studies on CAR and CLMP, two proteins of epithelial tight junctions

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To the ones I love

"There is no use trying," said Alice; "one can't believe impossible things."
"I dare say you haven't had much practice," said the Queen. "When I was your age, I always did it for half an hour a day. Why, sometimes I've believed as many as six impossible things before breakfast."

Lewis Carroll

Populärvetenskaplig sammanfattning

Den mänskliga kroppen består uppskattningsvis av 100 000 miljarder celler som delas upp i 200 olika celltyper. Varje cell fungerar som en egen levande enhet som kan försörja sig med energi, ta hand om sitt eget avfall och föröka sig. Trots att alla dessa celler fungerar på ett likartat sätt så har de specialiserat sig för att kunna tillgodose kroppens olika funktioner. De specialiserade cellerna delas in i fem vävnadstyper; epitellagret, muskelvävnad, bindväv, nervvävnad och blodceller. Dessa vävnadstyper bildar i sin tur kroppens alla organ och mjukdelar.

Epitellagret täcker utsidan av kroppen, samt dess ihåligheter så som mag- och tarmkanalen, lungor och njurar. Dessa celler fungerar i att skydda kroppen från farliga ämnen som den kommer i kontakt med via luft, mat och vätska, att ta upp vatten och näringsämnen från matsmältningen samt att utsöndra olika typer av substanser som är viktiga för kroppens olika funktioner som t.ex. hormoner och enzymer. I njuren och levern är epitellagret viktig för rening av blodet och att i njuren återuppta vatten och mineraler från urin-filtratet.

Ett kännetecken för epitellagret är att cellerna sitter tätt ihop i ett eller flera lager och på så sätt skapar de en barriär mellan utsidan av cellerna och underliggande vävnad. Två intilliggande celler hålls ihop av cellfogar som sitter fast i cellmembranet som omsluter cellen. Fogarna är också viktiga för epitelcellens uppbyggnad och utseende samt för utbyte av olika substanser cellerna emellan. Cellfogarna är uppbyggda av specifika proteiner som ger fogarna dess karaktäristiska utseende och funktion. Den här avhandlingen fokuserar på en typ av epitelcells fogar, de täta fogarna, samt ett av dess proteiner, den så kallade coxsackie och adenovirus receptorn (CAR).

Funktionen av CAR i levande celler är att reglera fogarnas täthet samt att hålla ihop cellerna. I levande organismer är inte funktionen av CAR helt kartlagd. Det är dock känt att proteinet bland annat är viktigt för hjärtats utveckling och CAR har en viktig uppgift vid inflammation, eftersom proteinet hjälper till att rekrytera kroppens försvarsceller till inflammation härden. Målet med detta arbete är att klargöra vilken funktion CAR har i levande organismer, så som möss.

I artikel I beskrivs identifieringen av proteinet ligand-av-Numb proteinet-X (LNX) som binder till CAR. Här visas att CAR är viktig för att rekrytera LNX till kontaktytan mellan två intilliggande celler. Artikel II visar att i vuxna möss så är CAR uttryckt i epitelceller från flertal organ. I dessa celler lokaliserades CAR enbart till den delen av cellmembranet som innehåller de täta fogarna, och denna studie visar också att mängden CAR korrelerar med tätheten av dessa fogar. I artikel III analyserades vilken biologisk funktion CAR har i epitelcellerna. För denna studie användes zebrafiskar som har en liknande uppsättning av celltyper och uppbyggnad av organ som möss. När CAR proteinets uttryck experimentellt förhindrades i zebrafisken kunde njuren inte fungera och utvecklas normalt, vilket tyder på att CAR har en viktig funktion i detta organ. Artikel IV beskriver identifieringen av det CAR-liknande membran proteinet (CLMP). Även detta protein hittades i epitelcellernas täta fogar. Studien visar att CLMP är viktig för att hålla ihop celler samt att reglera fogarnas täthet.

Dessa upptäckter är viktiga för att kunna förstå vilken roll CAR och CLMP har i uppbyggandet och funktionen av epitelcellers täta fogar samt vilken funktion CAR proteinet har i levande organismer.

Abstract

Tight junctions are important structures for the function of epithelial cells. They are composed of multi-protein complexes that connect the plasma membranes of two adjacent epithelial cells to each other and function as paracellular barriers, which regulate flux of ions, solutes, proteins and cells across the epithelium. Classical transmembrane components of tight junctions include the tetra-membrane-spanning proteins claudins and occludin, which are involved in the formation of these junction complexes.

In this thesis, a newly identified group of tight junction proteins is described. These proteins belong to the cortical thymocyte marker in *Xenopus* (CTX)-subfamily, which belongs to the larger immunoglobulin superfamily of cell adhesion molecules. All CTX-like proteins are type-1 single spanning transmembrane proteins that are composed of two extracellular immunoglobulin loops and an intracellular tail containing a PDZ-domain binding motif, which is used for interactions with PDZ-domain containing proteins that serve as scaffolds, anchoring the transmembrane components of tight junctions to the cytoskeleton. CTX-like proteins mediate cell-cell adhesion and have the capacity to increase transepithelial electrical resistance (TER) across epithelial cells, which suggests a role in barrier function. One member of the CTX-family has been of particular interest: the coxsackie and adenovirus receptor (CAR). CAR was originally identified as a virus receptor and has, as such, been in focus in various adenovirus-based gene transfer and gene therapy studies. Its *in vitro* properties as being a cell adhesion molecule, expressed at epithelial tight junctions, have also been analyzed. The physiological role of CAR *in vivo*, however, is less clear.

The major aims of these studies were to determine the function of CAR and a newly identified member of the CTX-family, CAR-like membrane protein (CLMP), in adult tissues and during embryonic development. The ligand-of-numb protein-X (LNX) was identified as a novel interacting partner of CAR and co-localized with CAR at cell-cell contacts in epithelial cells. Moreover, CAR was essential for the recruitment of LNX to this sub-cellular localization. In the adult mouse, CAR was predominantly expressed in epithelial cells, where it localized to tight junctions. The intracellular tail of CAR was essential for correct tight junction localization *in vivo*. A positive correlation between CAR expression and tight junction maturity was found in epithelial cells lining different segments of the tubules in the kidneys. This suggested a role for CAR in regulating permeability and tissue homeostasis, which was further supported by results from studies of the effect of CAR knockdown during zebrafish development. Fish embryos lacking CAR expression in all cells and tissues developed abnormalities that were associated with kidney failure, such as pericardial and body edema and formation of renal cysts. These studies showed that CAR is essential for proper kidney development and function and thus uncovered a novel function for CAR, which had not been detected in mouse embryos lacking CAR expression.

CLMP, which was identified through computer-based database searches, was similarly to CAR, expressed in epithelial cells, where it localized to tight junctions. Functional studies showed that CLMP could mediate cell-cell adhesion and increase TER across epithelial cells in culture.

In conclusion, the findings presented in this thesis expand our knowledge of the physiological function of CAR and give new insights into the complexity of the CTX-subfamily group of proteins.

List of original publications

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I **The coxsackievirus and adenovirus receptor (CAR) forms a complex with the PDZ domain-containing protein ligand-of-numb protein-X (LNX)**
Sollerbrant K, Raschperger E*, Mirza M*, Engstrom U, Philipson L, Ljungdahl PO, Pettersson RF
J Biol Chem. Feb 28;278(9):7439-44, 2003

II **The coxsackie- and adenovirus receptor (CAR) is an *in vivo* marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis**
Raschperger E, Thyberg J, Pettersson S, Philipson L, Fuxe J*, Pettersson RF*.
Exp Cell Res. May 15;312(9):1566-80, 2006

III **The role of the coxsackie and adenovirus receptor (CAR) in zebrafish kidney development**
Raschperger E, Tryggvason K, Majumdar A*, Pettersson RF*
Manuscript, 2006

IV **CLMP, a novel member of the CTX family and a new component of epithelial tight junctions**
Raschperger E, Engstrom U, Pettersson RF, Fuxe J
J Biol Chem. Jan 2;279(1):796-804, 2004

*equal contribution

Abbreviations

aa	amino acid
ACAM	adipocyte adhesion molecule
BBB	blood-brain-barrier
BEAS-2B	human normal lung epithelial cells
BT-IgSF	brain and testis immunoglobulin superfamily
C2 domain	constant 2 domain
Caco-2	human colon carcinoma cells
CAR/CXADR/CVADR	coxsackie and adenovirus receptor
CHO	chinese hamster ovary cells
CLMP	CAR-like membrane protein
CTH/CTM/CTX	cortical thymocyte marker in *Human/Mouse/Xenopus*
CVB	group B coxsackievirus
DAF	decay-accelerating factor
dpf	days post fertilization
EMT	epithelial to mesenchymal transition
ESAM	endothelial cell-selective adhesion molecule
GBM	glomerular basement membrane
GPI	glycosylphosphatidylinositol
GST	glutathione-S-transferase
hpf	hours post fertilization
IFN-γ	interferon-γ
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
JAM	junctional adhesion molecule
JAML	junctional adhesion molecule-like
J-segment	juxtapose-segment
kDa	kilo Dalton
LNX	ligand-of-numb protein-X
MAGI-1b	membrane-associated guanylate kinase 1b
MAGUK	membrane associated guanylate kinase
MDCK	Madin-Darby canine kidney cells
MUPP1	multi-PDZ domain protein-1
PDZ-domain	PSD95/DLG/ZO-1-domain
PICK1	protein interacting with protein C kinase
PSD-95	postsynaptic density 95
TER	transepithelial electrical resistance
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
V domain	variable domain
ZO	zonula occludens

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Preface

This thesis will most certainly be read by the opponent and the members of the graduation committee, and hopefully by all my closest colleagues. However, I would like to introduce a broader public to the fascinating world of epithelial cells and their cellular junctions. Therefore, the two first sections of this thesis, An overview of the epithelium and Cellular junctions, are written for the educated layman and is free from to many complicated scientific words and phrases.

Elisabeth Raschperger
Stockholm, September 22, 2006

1 Introduction

Mammalians are multicellular organisms, which are composed of five basic tissue types all specialized to perform different biological tasks. These tissue types are the epithelium, connective tissue, muscle, nervous tissue and blood. The epithelium of different organs differs in morphology depending on cellular functions, and serves as a protective barrier, in secreting various components and in absorbing nutrients and water. Nevertheless, epithelial cells have some features in common. They grow in a polarized fashion, which is defined as cells having two distinct morphologically and physiologically different surfaces of the plasma membranes. The apical surface is facing the luminal side of the organs, while the basolateral surface of the epithelium is facing the underlying tissues. Another common feature is that they contain several distinct cellular junctions at the plasma membrane. One of these junctions is the tight junctions. They are important for preserving the morphology of the epithelium and for regulating and maintaining the paracellular permeability. In addition, tight junctions act as a barrier between the apical and basolateral surface. Tight junctions are composed of several transmembrane and cytosolic proteins that are important for their functions. The transmembrane proteins of the cortical thymocyte marker in *Xenopus* (CTX) subfamily are one group of tight junctional proteins. This family contains several proteins, such as the coxsackie and adenovirus receptor (CAR), which mediate cell adhesion and regulate permeability. The role of the CTX-subfamily, and several other important tight junctional components, will be reviewed in the following sections.

1.1 Epithelial cells and their cellular junctions

The epithelium has the fundamental roles in the living organism to separate different cellular compartments and to regulate the exchange of oxygen, nutrients and water between them. These functions are highly dependent on the structure of the epithelial cells and the presence of different cellular junctions at the plasma membrane. Although, the endothelium of the blood and lymphatic vessels share several structural and functional similarities with the epithelium (Bazzoni, 2006), the following sections will focus on the epithelial cells.

1.1.1 An overview of the epithelium

The human body is constantly exposed to harmful environmental substances such as pathogens, pollution, and UV-radiation. The epithelial cell layer that

lines all the external and internal surfaces of the body is protecting it from these agents. In addition, the epithelium has several other physiological functions. The epithelium of the skin protects the body from mechanical injury and from excessive loss of water, and it is also important for excretion of sweat for regulating body temperature. Epithelial cells that line the external surface of the internal organs mediate passive transport of fluid, gases, nutrients and metabolites. The thin epithelial lining of the respiratory system allows oxygen from the inhaled air to diffuse to the endothelial capillaries. Another feature of the respiratory epithelium is that the ciliated epithelium removes inhaled dust and pollution from the lungs. Epithelial cells of various glands and the intestine produce and secrete hormones, enzymes, and protective mucous, and the intestinal epithelium is also important for fluid and nutrient absorption from the digested food. Finally, the epithelium of the kidney functions in filtering waste products from the blood and reabsorbing needed ions and water from the filtrate back to the blood stream.

The epithelial tubules are made up by epithelial cells that are packed closely together with almost no intercellular spaces, allowing the cells to form a tight barrier to the external environment. The epithelium is surrounded by connective tissue and smooth muscle cells that support the epithelial tubule. In most cases, the epithelial tubules are surrounded by a specialized epithelium, the mesoepithelium, which protects the epithelial tubule from friction with surrounding organs. The epithelium itself is nonvascular, and are therefore supplied with oxygen, nutrients and metabolites through diffusion from the blood vessels in the underlying connective tissue (Figure 1). Depending on the structure and function of the epithelium, it can be divided into three groups, the simple epithelium, the pseudostratified epithelium and the stratified epithelium. The simple epithelium lines the external surface of the digestive organs, lungs and heart (called mesoepithelium). The internal surface of

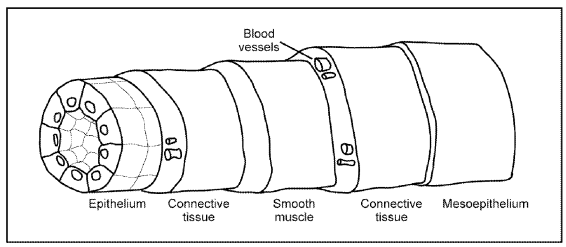

Figure 1. Structure of an epithelial tubule. The inner layer of an epithelial tubule is composed of tightly packed epithelial cells, which is surrounded by connective tissue and smooth muscle cells. The external epithelial layer, the mesoepithelium, protects the tubule from friction from surrounding organs. Since the epithelium is nonvascular, it is supplied with oxygen, nutrients and metabolites by diffusion from the blood vessels, which are located in the surrounding connective tissue. This schematic representation is adapted and modified from Eroschenko *et al.*, 2000

excretory ducts, tubules in kidneys, stomach, gall bladder and small and large intestine are also lined with simple epithelium. Pseudostratified epithelium lines the respiratory passages and epididymis, while stratified epithelium covers the urinary system, skin, excretory ducts and moist cavities, such as the mouth, esophagus, vagina, and the anal canal (Eroschenko, 2000).

A special feature of epithelial cells are that they grow in a polarized fashion. This means that the cellular plasma membrane is divided into three surfaces (Figure 2A). The apical surface is facing the lumen of the epithelial tubule, the lateral is connecting one epithelial cell to another and the basal surface anchors the epithelium to the underlying basement membrane or connective tissue. This cellular structure is common for the majority of epithelial cells in the body. However, there are two important exceptions. The renal podocytes and hepatocytes of the liver are two specialized epithelial cells, which both have an important role in barrier function. Although these cells are of epithelial origin and grow in a polarized fashion, they display a unique cellular structure. Podocytes faces an extremely large apical lumen. The small basolateral surface of podocyte foot processes are anchored to the glomerular basement membrane (GBM) that is in close contact with the fenestrated endothelium (Figure 2B) (Pavenstadt et al., 2003). Podocytes have the vital function to filtrate the blood. The blood plasma exits the blood stream via fenestrated endothelium, passes the GBM and is then filtrated by the slit diaphragms, which only allow waste products, water, and ions to pass. Water and ions are then reabsorbed along the renal nephron while the urine is transported out from the kidneys (Tryggvason and Wartiovaara, 2005). In contrast to the podocytes, the apical surface of hepatocytes faces the small lumen of the bile canaliculi (Figure 2C). The large basolateral surface is in close contact with neighbouring hepatocytes or with the fenestrated endothelium (also called sinusoids). Hepatocytes and the fenestrated endothelium are separated by a thin basement membrane, which allows the blood to come in direct contact with the basolateral surface of the plasma membrane, a feature not seen in any other epithelial tissue in the body. This allows the hepatocytes to absorb drugs and poisonous substances from the blood, an important function of the liver (Eroschenko, 2000).

1.1.2 Cellular junctions

The plasma membrane of epithelial cells have three classes of cellular junctions that are important for its structure and function. These are the occluding, anchoring and communicating junctions (Alberts et al., 2002).

The occluding junctions are referred to as ***tight junctions***. They localize at the apex part of the lateral side of the plasma membrane where they form a

belt-like structure that encompasses the cell (Figure 2A). Tight junctions have several important functions. They closely connect the plasma membrane of two neighbouring epithelial cells and are therefore important for holding these cells together. The tight junctions prevent the movement of lipids between the apical and basolateral plasma membrane of the cell. This feature is important for the maintenance of polarization and in preserving the specialized functions of each surface, such as uptake of fluid and proteins from the apical surface and release of a variety of agents from the basolateral surface. This tightly associated junction, forming virtually an impermeable barrier to fluid, proteins and ions, is protecting the underlying tissue from direct contact with the external environment. All materials are preferentially transported by active transport through the epithelial cells rather than between them. Tight junctions are composed of the transmembrane proteins occludin, claudins and CTX-subfamily proteins that are linked to the actin cytoskeleton via cytoplamic proteins such as, ZO-1, cingulin and MAGI-1 (Alberts et al., 2002; Gonzalez-Mariscal et al., 2003). The tight junctions will be further discussed in section 1.1.3.

The anchoring junctions are composed of adherens junctions, desmosomes and hemidesmosomes (Figure 2A). These junctions are important for holding epithelial cells together and for anchoring cells to the underlying basement membrane or connective tissue (Alberts et al., 2002). The ***adherens junctions*** localize beneath the tight junctions, and they surround the cell in a similar belt-like structure (Figure 2A). Adherens junctions are composed of four major proteins, the transmembrane protein cadherin and the intracellular proteins α- and β-catenin and p120. This protein complex connects the actin cytoskeleton of two adjacent epithelial cells, thus holding them together. The adherens junctions also have several other important functions. They are central players in several signalling pathways, and their regulation is important during development and in cancer progression. Adherens junctions are also important for the formation and maintenance of the tight junctions. The next group of anchoring junctions are the ***desmosomes***, which are spot-like junctions that connect epithelial cells via the transmembrane protein desmosomal cadherin. This protein is found in a complex with the cytosolic proteins desmoplakins and plakoglobins and the intermediate cytoskeleton filament. Desmosomes are randomly arranged on the lateral side of the plasma membranes and help to resist shearing forces (Figure 2A). Finally, the last type of anchoring junctions, the ***hemidesmosomes***, are structurally similar to desmosomes, except that these junctions mediate cell to extracellular matrix attachment and are therefore only found at the basal portion of the plasma membrane (Figure 2A). The adhesive properties are mediated by transmembrane integrins, which connect via

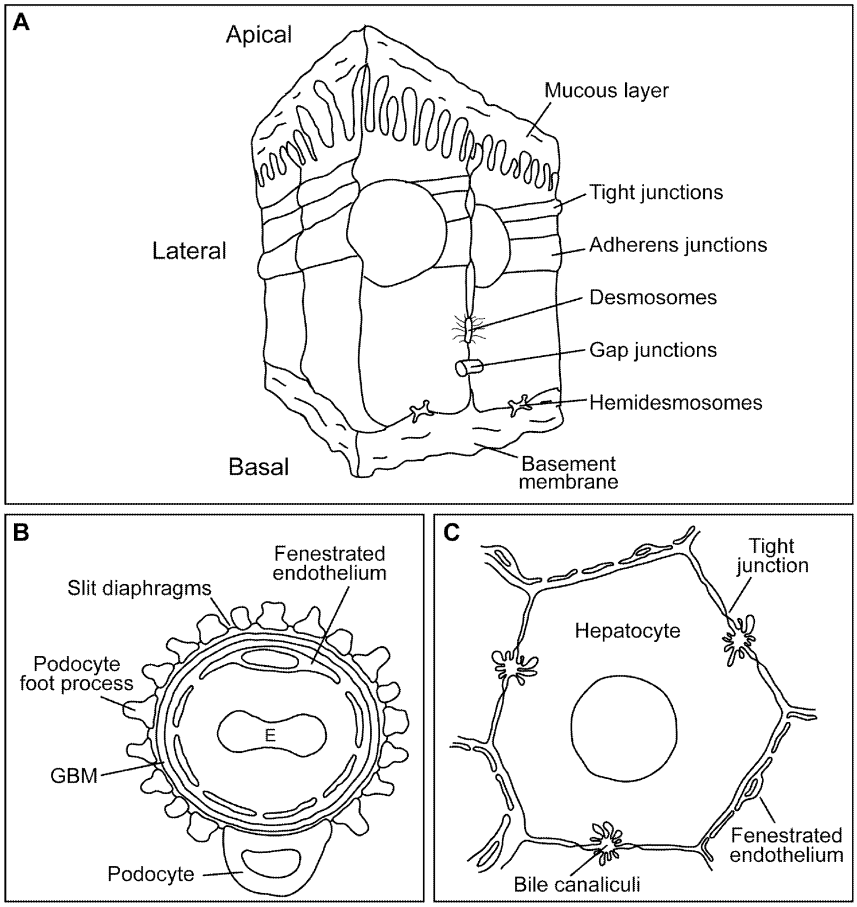

Figure 2. Cellular junctions of regular and specialized epithelial cells. (A) The majority of epithelium, here represented by ciliated epithelial cells, grow in a polarized fashion. This is characterized by a plasma membrane with an apical surface facing the lumen, a lateral surface connecting to neighbouring cells, and a basal surface anchoring cells to the basement membrane. A protective mucous layer often covers the apical surface of the epithelium. The plasma membrane of regular epithelium harbours three classes of cellular junctions. The tight junctions are located as a belt-like structure at the most apical portion of the lateral surface. Adherens junctions, desmosomes and hemidesmosomes anchor the cell to neighbouring cells and to the basement membrane. While adherens junctions are surrounding the plasma membrane, the desmosomes and hemidesmosomes are randomly arranged on the basolateral surface. Gap junctions are only found at the lateral surface. **(B and C)** Renal podocytes and liver hepatocytes are two specialized epithelial cells with important barrier functions. **(B)** The foot processes of podocytes have small basal surfaces that are anchored to the glomerular basement membrane (GBM). Podocytes harbour specialized epithelial junctions, the slit diaphragms, which prevent large molecules to exit the blood stream via the fenestrated endothelium (erythrocyte (E)). **(C)** The luminal surfaces of two adjacent hepatocytes are forming the smallest bile ducts, the bile canaliculi. Tight junctions prevent backflow of bile from bile canaliculi to the underlying fenestrated endothelium and the blood stream. These schematic representations are adapted and modified from Alberts *et al.*, 2002, Pavenstadt *et al.*, 2003 and Ross *et al.*, 1989.

desmoplakins and plakoglobins to the intermediate cytoskeleton filament (Alberts et al., 2002).

The last group of epithelial junctions are the communicating ***gap junctions***, which are only found at the lateral plasma membrane (Figure 2A). These junctions are composed of different connexin proteins, which depending on their composition form channels with different levels of permeability. The function of the gap junctions is to allow free passage of ions and water-soluble molecules between two adjacent epithelial cells (Alberts et al., 2002).

As mentioned earlier, there are two specialized epithelial cells that have an important barrier function. The renal podocytes lack epithelial cellular junctions such as tight and adherens junctions, instead these cells have ***slit diaphragms*** (Figure 2B). The slit diaphragm is a modified epithelial junction that have emerged from the adherens and tight junctions, since proteins such as cadherin, ZO-1 and CAR are expressed at the slit diaphragms (Nagai et al., 2003; Pavenstadt et al., 2003). In the liver, hepatocytes are connected to each other as regular epithelium. The importance of the barrier function of the tight junctions is obvious in these cells, as these junctions prevent the bile, which is secreted into the bile canaliculi, to backflow into the blood stream (Figure 2C) (Anderson, 1993).

1.1.3 Tight junctions

The tight junctions were visualized by electron microscopy for the first time in 1963 (Farquhar and Palade, 1963). On ultrathin electron micrograph sections, this cellular structure was observed as a series of fusion points between the plasma membranes of two adjacent epithelial cells. It was also found that the tight junctions appear as a network of continuous and connecting filaments where the intercellular space is completely missing, as seen on freeze-fracture replica electron micrographs of this junction (Gonzalez-Mariscal et al., 2003). Since its discovery, the structure and function of the tight junctions have gradually become clarified.

1.1.3.1 Structure

There are more than 50 proteins expressed at epithelial tight junctions, demostrating the complexity of this cellular junction. These proteins can be divided into two major groups, the transmembrane proteins and the cytoplasmic proteins. Only proteins of relevance to this thesis will be discussed here.

Occludin was the first tight junctional protein to be identified (Furuse et al., 1993). The protein is exclusively expressed in epithelial and endothelial tight

junctions where it integrates into the plasma membrane as a tetraspanning protein, composed of two extracellular loops and both an N- and C-termini on the cytoplasmic side. The intracellular portion of occludin has been found to interact with several PDZ-containing proteins, such as ZO-1, -2, -3 (Figure 3C). These interactions link occludin to the actin filament and mediate its cell-cell adhesive properties (Aijaz et al., 2006; Feldman et al., 2005). There are several indications that occludin is modulating cellular permeability. *In vitro* studies show that overexpression of occludin in Madin-Darby canine kidney

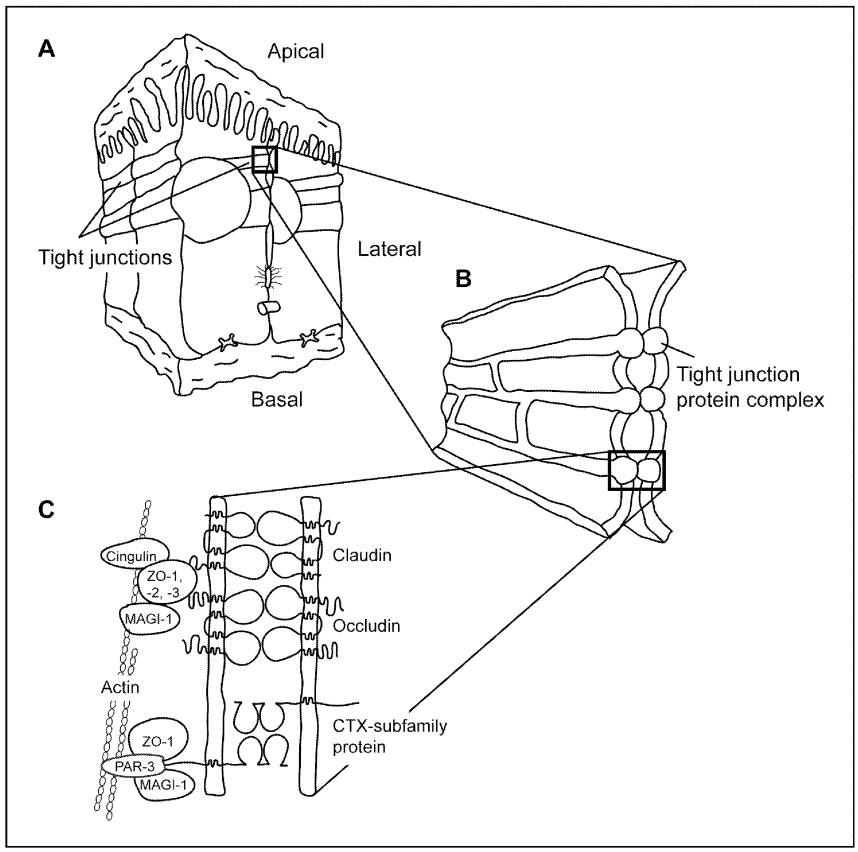

Figure 3. Sub-cellular localization and structure of epithelial tight junctions. (A) The tight junctions are located as a belt-like structure at the most apical part of the lateral membrane. **(B)** The permeability of the epithelium is generally dependent on the number of tight junctional strands, here illustrated as three parallel strands, and on the composition of the tight junctional protein complex. **(C)** A simplified illustration of the tight junction protein complex. Tight junctions are composed of the transmembrane proteins claudin, occludin and CTX-subfamily members. These proteins are linked to the actin cytoskeleton via interaction with the cytosolic proteins ZO-1, -2 and -3, cingulin, MAGI-1, and PAR-3. These schematic representations are adapted and modified from Mitic and Anderson 1998.

(MDCK) cells increases the transepithelial electrical resistance (TER) and number of tight junctional strands (Furuse et al., 1996; McCarthy et al., 1996). *In vivo* expression levels of occludin correlate well with tight junction permeability, as seen along the renal nephron. High expression levels of occludin are observed in distal impermeable parts of the renal nephron in contrast to proximal leaky tubules, which display low or absent levels of the protein. Higher levels of occludin have also been observed in the "tighter" tight junctions of endothelial cells forming the blood-brain-barrier (BBB) when compared to the less "tighter" ones of arteries and veins (Feldman et al., 2005). Post-translational modifications of occludin, such as phosphorylation and dephosphorylation, play an important role in the regulation of paracellular permeability. In addition, regulation of occludin expression was found to be mediated by different cytokine signalling pathways (Feldman et al., 2005). Originally, occludin was thought to be an essential protein for tight junction formation and function. However, occludin deficient mice did not show any obvious developmental defects or alterations of the tight junctions. Nevertheless, these mice displayed reproductive defects and several other non-lethal, complex phenotypes including defects in epithelial differentiation in various tissues (Saitou et al., 2000; Schulzke et al., 2005). In conclusion, occludin is a component of the tight junctions that regulates its permeability, although it appears not to be essential for tight junction formation and maintenance.

Claudins are a large group of cell adhesion proteins that are important components of epithelial and endothelial tight junctions. These proteins are mediating the formation of tight junctions, since overexpression of a single claudin type is sufficient to induce tight junction assembly in cell cultures that lack these junctions (Furuse et al., 1998). To date there are more than 20 claudins identified, which all have a similar protein structure as occludin (Figure 3C). The majority of claudins harbour a potential PDZ-binding motif in their intracellular domains and several interacting proteins, such as ZO-1, -2, -3, MUPP-1 and PATJ, have been identified (Aijaz et al., 2006). The expression pattern of the claudins are quite variable among different endothelial and epithelial tissues. While claudin-1 is ubiquitously expressed, other claudins are restricted to only a specific cell type or certain stages of development. In addition, there are cell types that express several claudins, while others only express one. The different combinations of claudins result in epithelial tight junctions with variable permeability. It has been determined that the extracellular loops of the claudins are mediating the ion and size selective permeability of the paracellular pathway. The expression of the claudins is regulated by several factors. Phosphorylation of claudins is associated with both an increase and decrease in the permeability of the tight junctions, while in other cases no obvious effect of phosphorylation was

observed. Numerous signalling pathways have been found to regulate claudin expression and consequently also the paracellular barrier. Several claudins have been targeted in gene deletion studies in order to elucidate the physiological function. The claudin deficient mice display phenotypes such as skin or Swann cell barrier defects, size-selective BBB defects, and loss of endocochlear potential (Van Itallie and Anderson, 2006). The majority of these phenotypes are non-lethal, which is quite surprising since claudins are important in the assembly of the tight junctions and in regulating the permeability. However, the mild phenotypes might be due to redundancy among different claudins.

The last group of transmembrane proteins, which are components of epithelial and endothelial tight junctions, are the members of the ***CTX-subfamily*** of the larger immunoglobulin superfamily (IgSF). Several of these single spanning transmembrane proteins interact with cytosolic PDZ-domain containing proteins at the site of tight junctions (Figure 3C). In addition, some of the CTX-members have been found to mediate cell-cell adhesion and to alter TER across epithelial and endothelial cells. The CTX-subfamily members are further discussed in sections 1.2 and 1.3.

The second group of tight junctional proteins are the cytosolic proteins. The majority of these proteins contain PDZ (PSD95/DLG/ZO-1) domains, which are 80-90 amino acid units that bind to specific PDZ-domain binding motifs at the very end of the C-termini of transmembrane proteins (shown for the CTX-subfamily in Table I) (Malergue et al., 1998; Schultz et al., 1998; Songyang et al., 1997). However, there are several indications that PDZ-domains also interact with internal binding sites of their ligands and that they can bind to other proteins that contains PDZ-domains (Bezprozvanny and Maximov, 2001; Hung and Sheng, 2002). PDZ-domain containing proteins are important for clustering and anchoring transmembrane proteins at the plasma membrane and to link these proteins to the actin cytoskeleton. They also act as scaffolding proteins that interact with signalling and cytosolic proteins at specific regions of the plasma membrane (Gonzalez-Mariscal et al., 2003; Kim et al., 1995).

The zonula occluden proteins, ***ZO-1***, ***-2*** and ***-3***, are members of the membrane associated guanylate kinase homologue (MAGUK) family. MAGUKs have, in addition to PDZ-domains, also structurally conserved src homology 3 (SH3) and guanyl kinase (GK) domains (Gonzalez-Mariscal et al., 2000a; Gonzalez-Mariscal et al., 2003). The ZO proteins localize to the submembranous domain of epithelial and endothelial tight junctions. However, during junction assembly or in the absence of tight junctions these proteins localize to the adherens junctions. The ZO proteins interact with several tight junctional proteins, such as occludin, claudin, cingulin, JAMs and CAR, and with each other in a heterophilic manner (Figure 3C). In addition, the ZO proteins also interact with proteins of the adherens junctions, such as catenins,

and with the actin cytoskeleton (Aijaz et al., 2006; Cohen et al., 2001b; Gonzalez-Mariscal et al., 2003). Regulation of ZO-1 and -2 by phosphorylation results in tight junctions with low or absent TER, and redistribution of the ZO proteins to the cell nucleus were observed in cells cultures with low confluence. As ZO-1 and -2 contain both nuclear localization and export signals these proteins are able to shuttle between the plasma membrane and the nucleus, and are thought to be involved in regulating cell differentiation and cell growth (Gonzalez-Mariscal et al., 2003; Matter and Balda, 2003). ZO-1 and -2 are considered to act as tumor suppressors, and down-regulation of these proteins have been found to correlate with progression of some cancers (Chlenski et al., 1999; Hoover et al., 1998). In a recent published paper, depletion of ZO-1 and -2 in cultured epithelial cells resulted in the total lack of tight junctions. However, these cells were still able to polarize. It was also shown that the ZO proteins are able to independently determine whether and were claudins are polymerized (Umeda et al., 2006).

The MAGUK-inverted protein membrane-associated guanylate kinase 1b, ***MAGI-1***, is structurally similar to the PDZ-domain containing proteins, although the SH3 domain is replaced by two WW domains. MAGI-1 interacts with ZO-1, CAR and JAM4 at epithelial tight junctions (Figure 3C), as well as with the adherens junctional proteins E-cadherin and β-catenin during junction formation (Dobrosotskaya et al., 1997; Dobrosotskaya and James, 2000; Excoffon et al., 2004; Hirabayashi et al., 2003; Ide et al., 1999). It is believed that MAGI-1 acts as a tumor suppressor, since the tumorigenic potential of several human viral oncoproteins depends on their ability to sequester and target MAGI-1 for degradation (Glaunsinger et al., 2000; Lee et al., 2000).

Cingulin is an epithelial and endothelial tight junctional protein that lacks PDZ-domains (Citi et al., 1991; Citi et al., 1988; Citi et al., 1989; Stevenson et al., 1989). Instead, cingulin is composed of an globular head and tail domain separated by a central α-helical rod region (Cordenonsi et al., 1999a). Cingulin interacts with several tight junctional proteins, such as ZO-1, -2 and -3, occludin and JAM-A (Figure 3C), as well as with the cytoskeletal proteins F-actin and myosin (Bazzoni et al., 2000; Cordenonsi et al., 1999a; Cordenonsi et al., 1999b; D'Atri and Citi, 2001; D'Atri et al., 2002). Although cingulin is post-translationally phosphorylated, this modification does not seem to be altered by the protein C agonist PMA, protein kinase inhibitor H-7 or by the calcium chelator EGTA (Citi and Denisenko, 1995). A function of cingulin in Rho GTPase activity in epithelial proliferation and differentation has been observed as binding of cingulin to the guanine nucleotide exchange factor for RhoA (GEF-H1) inhibited RhoA activation and signalling (Aijaz et al., 2005). Recently it was shown that cingulin-depleted MDCK cells have an increased level of claudin-2 and ZO-1. These cells also displayed an increased RhoA activity, activation of G1/S phase transition and an increased cell density.

However, these cingulin-depleted MDCK cells showed no alteration in the overall molecular composition or barrier function of the tight junctions (Guillemot and Citi, 2006). Interestingly, targeted deletion of cingulin in embryoid bodies derived from embryonic stem cells developed normal tight junctions, although altered gene expression of several tight junctional proteins, such as occludin and claudin-2, -6 and -7, was observed (Guillemot et al., 2004).

1.1.3.2 Function

Tight junctions have three crucial cellular functions, generally referred to as the gate, the fence and the trafficking and signalling platform. These functions are dynamic processes that involve different proteins and cellular events. In the context of this thesis it would be impossible to address all these processes in detail. Therefore a brief summary will be presented in the following section.

Tight junctions are important for creating a barrier, or a gate, between the apical and basolateral surface of the epithelium (Figure 3A). The transport of solutes and fluids between these two compartments are mediated either by the transcellular or the paracellular pathway. The transcellular pathway uses energy-dependent transporters and channels that are located at the plasma membrane (Powell, 1981). The paracellular pathway is controlled by transmembrane tight junctional proteins, which regulate passive diffusion of small, noncharged solutes and ions. As mentioned earlier, occludin and especially claudins play a major role in the regulation of paracellular diffusion. Depending on the protein composition and the number of tight junctional strands (Figure 3B and C), the epithelial cells display different levels of tightness (Feldman et al., 2005; Powell, 1981; Van Itallie and Anderson, 2006). This is especially evident along the renal epithelial tubule. The renal tubular segments, important for reabsorbing water and ions back to the blood stream, are leaky and have less “tighter” tight junctions. In contrast, the tubular segments that are transporting the urine out of the kidney are extremely tight, thus preventing the urine to come in contact with the underlying tissue (Balkovetz, 2006; Claude and Goodenough, 1973; Gonzalez-Mariscal et al., 2000b).

Another crucial function of the tight junctions is to generate and maintain the polarity of epithelial cells. This feature, called the fence, prevents intramembrane mixing of lipids from the apical and basolateral plasma membranes (Dragsten et al., 1981; van Meer and Simons, 1986). This preserves the special features of the apical and basolateral surfaces of epithelial cells, such as specific lipid composition and the presence of ion carriers, water channels, and receptors (Vize et al., 2003). Occludin is important for this function, since overexpression of a dominant negative mutant resulted in

disruption of the fence, indicating that occludin is either involved in the formation of the fence or in its regulation. It was hypothesized that the fence also prevents proteins to freely diffuse between the apical and basolateral surface of epithelial cells. However, this was found not to be the case, as disruption of the fence without entirely disrupting the tight junctional complex, resulted in diffusion of lipids, but not proteins (Balda et al., 1996; Jou et al., 1998).

The epithelial tight junctions also serve as a trafficking and signalling platform that regulate proliferation, differentiation, and polarization. Signals transmitted from the tight junctions to the cell interior regulate gene expression, cell proliferation, and differentiation, while signals directed from the cell interior to the tight junctions mediate tight junction assembly, disassembly, and paracellular permeability (Matter and Balda, 2003). Polarization has been closely linked to junctional assembly in epithelial cells and this process has been extensively studied in epithelial cell lines and in different *in vivo* model organisms such as *C. elegans*, *D. melanogaster* and mice (Aijaz et al., 2006). Interestingly, the mechanisms of junction formation are well conserved between different species. The first event of polarization and junctional assembly involves the formation of primordial cell-cell contacts, so called “kissing points”, that is mediated by the adherens junctional proteins nectins. The micro-clusters of nectins then anchor to the actin cytoskeleton before they recruit E-cadherin, which stabilizes this primordial junction. The lateral plasma membrane then expands, and JAM and ZO-1 are recruited, followed by the recruitment of claudins and occludin. Finally, the adherens and tight junctional proteins separate into two distinct junctional complexes as the cells mature into a polarized epithelium. The assembly of tight junctions is obviously dependent on the formation and maintenance of the adherens junctions, thus showing the importance of both these cellular junctions for epithelial polarization and function (Miyoshi and Takai, 2005). There are several key regulators that are involved in tight junction assembly. Activation of protein kinase A, heterotrimeric G proteins, Rho GTPases and protein kinase C signalling have been shown to regulate both assembly and disassembly of tight junctions. The actin cytoskeleton is also important in the regulation of tight junctions as it interacts with several tight junctional proteins. In addition, components of the two conserved signalling complexes, CRB3/Pals1/PATJ and PAR3/PAR6/aPKC, have been found to be important in regulating epithelial junction assembly and polarization (Aijaz et al., 2006; Matter and Balda, 2003).

1.1.4 Disease

The epithelium that lines all external and internal surfaces of a living organism is designed to protect the living organism from the surrounding environment. However, the constant exposure to harmful substances are linked to several pathological conditions that are associated with disruption or targeting of epithelial cellular junctions. Interestingly, several tight junctional proteins were found to be involved in these processes.

Microorganisms, such as bacteria and enteropathogenic viruses, are adapted to either target epithelial cells directly or to disrupt the tight junctions as they invade the gastrointestinal tract and cause diarrhea, a disease affecting a large population of the third world. Inflammatory diarrhea leads to cell death while noninflammatory diarrhea causes secretion of a large amount of electrolytes and water in excess of the reabsorptive capacity of the epithelium. Epithelial tight junctions have also been found to be targeted by different allergens. Dust mite allergens harbour several proteolytic enzymes that cause degradation of the tight junctions. An alteration of tight junctions is also seen in chronic allergic rhinitis, as nasal epithelium shows an increased permeability (Mullin et al., 2005).

During the inflammation response, tight junctional proteins are mediating leukocyte transmigration over endothelial and epithelial cells to the site of infection. This occurs without visible disruption of the tight junctional integrity, or by down-regulation or redistribution of tight junctional proteins (Huber et al., 1998; Muller, 2003; Mullin et al., 2005). Several members of the CTX-subfamily have been found to be important for leukocyte transmigration. The JAMs and ESAM mediate leukocyte transmigration over endothelial and epithelial cells, while CAR mediated leukocyte transmigration is restricted to epithelial cells (Mandell and Parkos, 2005; Wegmann et al., 2006; Zen et al., 2005). It has also been found that neutrophile transepithelial migration is mediated by occludin (Huber et al., 2000). Interestingly, occludin and claudin-3 down-regulation, and consequently alteration of epithelial and endothelial barrier function, are seen in chronic inflammatory diseases such as collagen colitis, psoriasis, systemic peripheral inflammation and encephalitis. Reduced levels of occludin have also been associated with increased permeability of endothelial cells in the blood-retina-barrier of diabetic rat models (Mullin et al., 2005).

Cancer is the second cause of death in the western world. This disease can progress from almost any organ in the body, although the majority of all tumors are of epithelial origin. A characteristic feature of epithelial cancer progression is the epithelial to mesenchymal transition (EMT), in which the junctional complexes of the epithelial cells are disrupted and the cells loose their polarity. As a consequence of EMT, the stationary epithelial cells are

transformed into migratory fibroblast-like cells as the cancer cell becomes more invasive or metastatic. Disorganization or loss of tight junctions is seen in the majority of tumors, and is therefore considered to be a hallmark for tumor progression. Indeed, down-regulation or absence of the tight junctional proteins occludin, claudins, ZO-1 and -2 are often observed in tumors when compared to normal tissue (Aijaz et al., 2006; Mullin et al., 2005). Down regulation of CAR expression is also frequently seen in tumors. This will be further reviewed in section 1.2.4.

Finally, there are several kidney diseases linked to alteration of epithelial differentiation, depolarization or disrupted barrier function. EMT-derived fibroblasts have been found to cause renal fibrosis, and there are several indications that alteration of the apical junction complex and dysfunction of polarity as well as cell growth are the major cause of autosomal dominant polycystic kidney disease. Additionally, acute renal failure is often associated with epithelial barrier and polarity dysregulation (Lee et al., 2006).

1.2 The coxsackie and adenovirus receptor (CAR)

The first report on the isolation of a protein involved in binding and infection of group B coxsackievirus (CVB) in cervical cancer cells (HeLa) was published two decades ago (Mapoles et al., 1985). This protein was later found to be the receptor for both the CVB and adenovirus, and was consequently named coxsackie and adenovirus receptor (CAR, CVADR or CXADR) (Bergelson et al., 1997a; Carson et al., 1997; Tomko et al., 1997). CAR is a type-I transmembrane protein that belongs to the CTX-subfamily, which belongs to the large IgSF of adhesion molecules. The family name and the gene and protein hallmarks of the CTX-subfamily was defined by the first member to be identified, namely the cortical thymocyte in *Xenopus* (CTX) protein (Chretien et al., 1998; Du Pasquier et al., 1999). The proposed hallmarks are *i:* the complete conservation of the exon-intron structure of the prototype CTX, *i.e.* presence of seven exons in the gene where each Ig-domain is encoded by two-half domain exons, *ii:* secondary protein structure containing a variable (V) and a constant 2 (C2) domain linked by a juxtapose (J) -segment, one transmembrane region and an intracellular tail, and *iii:* an extra disulfide bridge between the two cysteine residues that encompasses the C2 domain. To date there are over 10 members of the CTX-subfamily that share similar protein structure (Figure 5). Other characteristic features of the CTX-subfamily members are that they are likely to be post-translationally modified by glycosylation and phosphorylation, and that several of the members harbour type I or type II PDZ-domain binding motif at the C-termini that mediates interaction with cytoplasmic tight junctional proteins (Table I). In the following sections a comprehensive review of the published data on CAR is presented, followed by a short summary in section 1.3 of what is known about the other CTX-subfamily members.

1.2.1 Gene and protein structure

The genomic and protein structure of CAR has been characterized in great detail. The human *CAR* (*hCAR*) gene localizes to chromosome 21q11.2 and four pseudogenes are found on chromosomes 15, 18 and 21 (Bowles et al., 1999). The gene encoding mouse *CAR* (*mCAR*) is located on chromosome 16 (Chen et al., 2003). The intron-exon boundaries have been determined for both *hCAR* and *mCAR*, and the eight *CAR* encoding exons have been identified and sequenced (Figure 4A) (Andersson et al., 2000; Bowles et al., 1999; Chen et al., 2003). The core promoter region of the *hCAR* gene is predicted to be located at residues –470 to –719 upstream of the ATG site (Pong et al., 2003), and the transcription initiation sites of *hCAR* and *mCAR* have been localized to

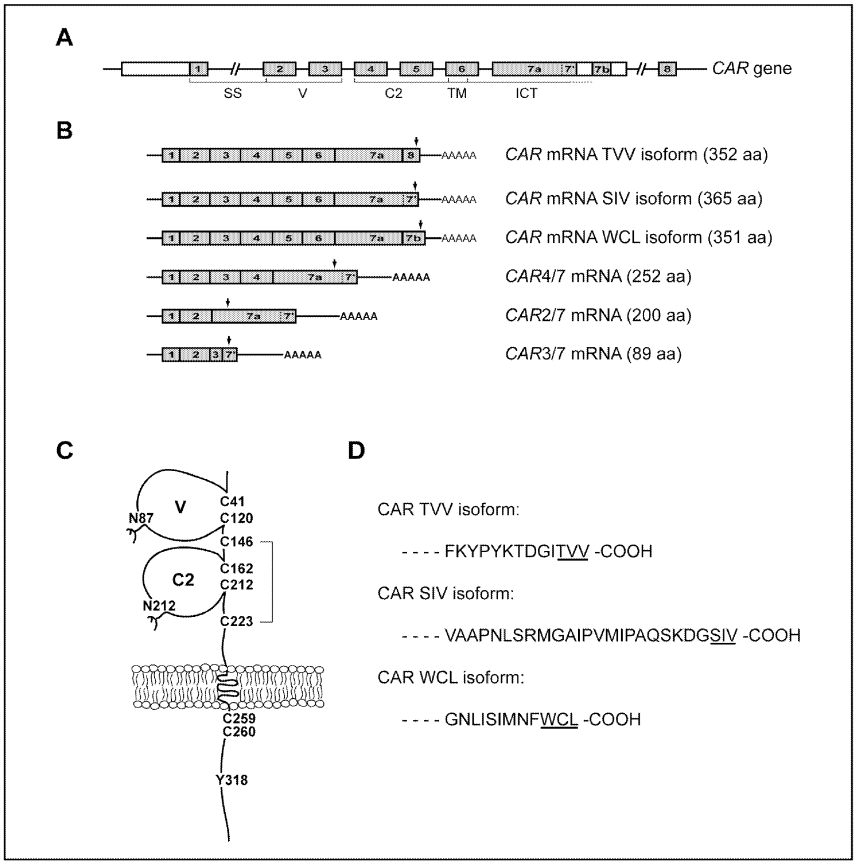

Figure 4. Genomic, mRNA and protein structure of mouse CAR. (A) The *CAR* gene harbours 8 exons that encode the different *CAR* mRNA species. Gray boxes indicate coding regions while white boxes represent the untranslated regions. Exons encoding the signal sequence (SS), variable (V) and the constant 2 (C2) domains, transmembrane region (TM) and the intracellular tail (ICT) are indicated. **(B)** The different *CAR* mRNA species with the encoding exons are shown in grey. Arrows indicate translation termination. The predicted length of translation products are shown in brackets. **(C)** A schematic representation of the secondary structure of the membrane bound CAR TVV, SIV and WCL isoforms. The four cysteine residues forming the V and C2 domains, and the two extra cysteine residues encompassing the C2 domain are indicated. The V and C2 domains harbour two putative N-linked glycosylation sites. The intracellular tail contains two S-acylation motifs at cysteine residues and a tyrosine sorting signal. **(D)** The difference in amino acid sequences at the extreme C-terminus from which the sequence of the membrane bound CAR TVV, SIV and WCL isoforms diverge. The PDZ-domain binding motifs are underlined. **(A-C)** These schematic representations are not drawn to scale.

approximately 150 base pairs upstream of ATG (Chen et al., 2003; Pong et al., 2003). The N-terminal signal sequence of *CAR* is encoded by exon 1 and partially by exon 2, while the remaining of exon 2, and exon 3 encode the first Ig-loop (V domain). The second Ig-loop (C2 domain) is encoded by exon 4

and 5 and the transmembrane region is encoded by exon 6. Due to alternative splicing of exon 7 and 8, three *CAR* mRNA species are transcribed, differing only in the very end of their 3' sequences (Figure 4A and B) (Andersson et al., 2000; Chen et al., 2003; Thoelen et al., 2001b). There are also three different exon-skipping *hCAR* mRNA transcripts, all lacking exon 6 that encodes the transmembrane region, potentially resulting in truncated and soluble forms of CAR (Figure 4A and B) (Bernal et al., 2002; Dorner et al., 2004; Thoelen et al., 2001b). The CAR isoform that ends with the residues TVV is referred to as either CAR TVV, CAR-1, CAR 2 or H1/M1, while the CAR isoform that ends with the residues SIV is referred to as either CAR SIV, CAR-2, CAR 1 or H2/M2 (Andersson et al., 2000; Chen et al., 2003; Fechner et al., 1999; Mirza et al., 2005; Shaw et al., 2004; Thoelen et al., 2001b). In addition the third CAR isoform that ends with the residues WCL is named H3/M3 (Andersson et al., 2000). This is quit confusing and should therefore be simplified by all CAR investigators. To avoid any confusion here, the membrane-bound CAR isoforms that are discussed in this thesis will be referred to as CAR TVV, SIV, or WCL isoforms, referring to the three last C-terminal amino acid residues of the intracellular tail.

The membrane-bound mouse CAR isoforms share identical amino acid (aa) composition and protein structure until the very end of their cytoplasmic tails (Figure 4C and D). The mature protein, after the removal of the 19 residues long N-terminal signal sequence, contains an ectodomain (216 aa), one transmembrane region (23 aa) and an intracellular tail (TVV isoform; 92 aa, SIV isoform; 107 aa, WCL isoform 91 aa). The ectodomain of CAR harbours conserved cysteine residues that are involved in the formation of the V domain (80 aa) and the C2 domain (51 aa). Two additional cysteine residues at position 146 and 223 probably form an extra disulfide bound, which encompasses the C2 domain (Figure 4C). In addition, the V and C2 domains are separated by a J-segment. The predicted size of CAR is 38 kilo Dalton (kDa), although the mature protein has a size of approximately 46 kDa, probably due to post-translational modification, such as N-linked glycosylation and phosphorylation (Beausoleil et al., 2004; Honda et al., 2000). In addition, the intracellular tail of CAR contains two motifs for S-acylation, which has been found to be post-translationally modified with the fatty acid palmitate (Figure 4C). This modification was found to be important for correct sub-cellular localization of CAR to the basolateral surface (van't Hof and Crystal, 2002). The CAR TVV, SIV, and WCL isoforms also harbour potential PDZ-binding motif in the very C-terminus of the intracellular tail (Figure 4D and Table I).

Finally, CAR homologues have been identified in several mammalian species such as rat, canine, porcine, and bovine (Bergelson et al., 1998; Fechner et al., 1999; Thoelen et al., 2001a; Tomko et al., 2000). These CAR homologues show an overall identity of about 90% with the human and mouse

CAR. CAR homologues in non-mammalian vertebrates such as the zebrafish and frogs (Petrella et al., 2002) have also been identified. Here an overall identity of 44% between the human and zebrafish CAR was seen. Interestingly, the intracellular domain of CAR is highly conserved between many different species indicating an important biological function for this domain.

1.2.2 Tissue distribution

Characterizing the expression pattern of a protein in a living organism is an important step towards the understanding of its biologic function. The expression pattern of CAR has primarily been determined by analyzing the mRNA expression by RT-PCR, Northern blotting, and *in situ* hybridization. Attempts to analyze CAR expression on the protein level have been hampered due to the lack of specific and sensitive antibodies against the CAR protein, therefore the protein expression data on CAR is limited.

CAR mRNA expression in adult human tissues was detected in epithelial tissues such as kidney, pancreas, brain, heart, lung, liver, small intestine, colon, testis, and prostate, whereas *CAR* transcripts were undetectable in the placenta, peripherial blood lymphocyte, ovary, thymus, and spleen (Bergelson et al., 1998; Fechner et al., 1999; Tomko et al., 1997). CAR protein expression was immunohistochemically detected at the cell surface of primary human airway epithelial cells in culture, as well as in epithelial tissues of the human respiratory system (Pickles et al., 2000). CAR has also been detected in human muscle tissues. Both CAR SIV and TVV isoforms are expressed in cardiac muscle, whereas only the CAR SIV isoform was observed in skeletal muscle. Instead, the CAR TVV isoform was found to be expressed in the vasculature of skeletal muscles (Shaw et al., 2004). Recently, CAR protein was detected in mature human sperms (Mirza et al., 2006).

CAR expression in rodents has been more extensively studied. During mouse embryogenesis, CAR protein is expressed in several regions of the brain, including the central nervous system, dorsal root ganglia, peripheral nerves, primary neurones hippocampus, ependyma and in the developing spinal cord. During embryogenesis, CAR expression was also observed in several epithelial tissues, such as esophagus, liver, lung, pancreas, skin, intestine and in the sensory organs. CAR expression was also seen in the myocard and pericard of the developing heart (Dorner et al., 2005; Honda et al., 2000; Hotta et al., 2003; Mallam et al., 2004; Mirza et al., 2005; Tomko et al., 2000; Xu and Crowell, 1996). In the adult mouse and rat, *CAR* mRNA expression was detected in several epithelial tissues, such as lung, testis, liver, heart, brain, spleen, kidney and pancreas. Interestingly, transcripts encoding the third membrane-bound CAR WCL isoform were found to be restricted to

the heart. However, there are no reports on the expression pattern of this heart specific isoform at the protein level (Andersson et al., 2000; Bergelson et al., 1998; Fechner et al., 1999; Honda et al., 2000; Tomko et al., 1997). The expression of CAR protein in the adult mouse corresponds well with the mRNA expression data. In the adult mouse brain, CAR expression was found in the ependymal cells lining the ventricles. CAR was also detected in several epithelial tissues in the adult mouse, such as in the respiratory system, liver, intestine, sensory organs and kidney (Excoffon et al., 2006; Mallam et al., 2004; Tomko et al., 2000). Interestingly, the expression of CAR in the mouse kidney was restricted to the podocytes of glomeruli and in the distal and collecting epithelial ducts, whereas CAR was absent from the rest of the renal epithelial nephron (Nagai et al., 2003). In addition to the broad epithelial expression, CAR protein has also been detected in several non-epithelial tissues, such as in sperms and in cardiac and skeletal muscle (Kashimura et al., 2004; Mirza et al., 2006; Shaw et al., 2004). These data indicate that CAR has a rather restricted expression pattern in epithelial cells both during development and in adult humans and rodents, although the protein was also detected in other cell types.

1.2.3 Sub-cellular localization

Early it became evident that CAR localizes to a defined area of the epithelial plasma membrane. Several attempts to use adenovirus-mediated gene transfer to target CAR from the apical side of airway epithelial cells from patient with cystic fibrosis failed, indicating a restricted localization of the receptor on these polarized epithelial cells (Grubb et al., 1994; Knowles et al., 1995). It was later found that CAR expression is restricted to the lateral portion of the plasma membrane in human respiratory epithelial cells *in vitro* and *in vivo*, where it appeared to localize at the site of the tight junctions (Pickles et al., 2000; Walters et al., 1999). This was confirmed by exogenously expressing full length or truncated CAR in well-polarized MDCK cells. Full-length CAR localized to the basolateral plasma membrane. In contrast, both apical and basolateral expression was seen in MDCK cells over-expressing truncated CAR lacking the entire intracellular tail or where the extracellular domain was attached to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor (Pickles et al., 2000). These data suggested that the intracellular tail of CAR targets the protein to the basolateral side of the plasma membrane. Detailed analysis of the intracellular tail located the important region for basolateral sorting to residues between 315 and 345. This region of the protein harbours a potential tyrosine-based sorting signal (318YNQV321). And indeed, exchange of the tyrosine residue into alanine (Y318A) resulted in

altered sub-cellular targeting of CAR to the apical surface of the plasma membrane of polarized MDCK cells (Figure 4C) (Cohen et al., 2001a). These *in vitro* data does not comment on the effect of overexpressed truncated protein on localization and function of the endogenously expressed CAR. However, transgenic overexpression of truncated CAR lacking the intracellular tail under the control of the human ubiquitin-C promoter, generated mice that abundantly expressed truncated CAR in all tissues. Interestingly, no dominant-negative effect of the truncated protein was observed as the CAR-transgenic mice developed normally with no visible defects (Tallone et al., 2001). In a recent study, the subcellular localization of truncated CAR, either lacking the V or the C2 domain, was analyzed and it was found that the extracellular domain is also important for proper localization of CAR to the lateral side of the plasma membrane (Excoffon et al., 2005). Finally, it was determined that CAR and the tight junction protein ZO-1 co-localize specifically to the most apex part of the lateral plasma membrane of epithelial cells *in vitro* (Cohen et al., 2001b). The sub-cellular localization of CAR *in vivo* was later confirmed on mouse kidney sections, as CAR clearly localized to tight junctions of the distal epithelial tubular cells (Nagai et al., 2003). In addition, in non-epithelial tissue such as adult human and mouse cardiac and skeletal muscle, CAR was detected at the intercalated disks between cardiomyocytes and at neuromuscular junctions, respectively (Shaw et al., 2004).

1.2.4 Regulation of expression

There are several reports showing that CAR is regulated both during physiological and pathological conditions. CAR expression is altered during the development, during inflammatory responses, and in cancer, indicating that CAR may have several different biological functions.

1.2.4.1 Regulation during embryonic development

CAR is highly regulated during embryogenesis. The protein is expressed in several areas of the developing mouse brain. *CAR* mRNA levels increase until the perinatal period then rapidly decrease to almost undetectable levels in the adult brain and protein is only detected in the ependymal cell layer lining the ventricles (Honda et al., 2000; Hotta et al., 2003; Tomko et al., 2000). CAR is in addition regulated during mouse cochlear development. The broad perinatal CAR expression found at cellular junctions of most cochlear cell types became more restricted to pillar cells in the adult organism (Excoffon et al., 2006). CAR was also found to be regulated during mouse skeletal muscle maturation, as high developmental levels of CAR declined to barely detectable levels in the adult mouse (Nalbantoglu et al., 1999). The same tendency of CAR regulation

occurs during rat development (Fechner et al., 2003; Ito et al., 2000). These data suggests that CAR may have an role during embryogenesis and that the physiological function of CAR may differ during the development and in the adult organism.

1.2.4.2 Regulation in muscle disease

Altered CAR expression has been found in several pathological cardiac and skeletal muscle conditions. While CAR is undetectable in normal human hearts, induction of CAR expression was observed at intercalated discs and sarcolemma in human hearts with dilated cardiomyopathy (Noutsias et al., 2001). Elevated CAR expression was also detected in adult rat hearts with experimental autoimmune myocarditis or after myocardial infarction (Fechner et al., 2003; Ito et al., 2000). There are several studies suggesting that CAR is important during the onset of cardiac muscle cell regeneration *in vitro*. Overexpression of CAR in neonatal rat cardiomyocytes resulted in accumulation of CAR at early cell-cell contacts (Noutsias et al., 2001). In cultured cardiomyocytes from newborn rats, CAR levels first declined, indicating a suppression of CAR, then increased at the time of cell proliferation, clustering and the onset of beating. In addition, this induction of CAR increased when cultured cardiomyocytes were treated with the inflammatory mediator concanavalin A (Ito et al., 2000). In a mouse model, regulation of CAR was observed during skeletal muscle regeneration in dystrophic *mdx* mice. CAR levels increased in regenerating muscle cells, declined in fully regenerated muscle and was not observed in normal skeletal muscle (Nalbantoglu et al., 1999). Since CAR localizes to the intercaleted discs in the cardiac muscle and at neuromuscular junctions in the skeletal muscle in healthy rodents (Shaw et al., 2004), these data suggests a role for CAR in cell-cell contact formation and in the regeneration of muscle cells and its junctions.

1.2.4.3 Regulation in cancer

Expression levels of cell adhesion molecules, such as cadherins, integrins and IgSF proteins, are known to be regulated in several cancers. The progression of cancer cells from low malignancy to metastatic forms are accompanied with several cellular changes. During this transformation, tumor cells lose their ability to form close attachment to surrounding cells and to underlying basement membrane. Consequently, these cells become more motile, metastasize, and invade new tissue. Expression of cell adhesion proteins is highly regulated throughout this process. Cell adhesion molecules are also involved in a variety of cellular processes associated with the development and progression of cancer, such as signal transduction, cell growth, differentiation,

site specific gene expression and immunological functions (Okegawa et al., 2004).

CAR expression appears to inversely correlate with tumor progression. Decreased CAR levels were observed in invasive human bladder and prostate cancer when compared to less invasive cancer (Okegawa et al., 2000; Okegawa et al., 2001; Sachs et al., 2004; Sachs et al., 2002). The same tendency was observed in head and neck squamous cell carcinoma when compared to normal tissue (Jee et al., 2002). Reduction of CAR was also observed in human astrocytomas as the tumor cells progressed from a low to a highly malignant form (Grade II to IV) (Fuxe et al., 2003). Additionally, inhibition of CAR expression in CAR positive bladder cancer cells promoted *in vitro* growth, suggesting that CAR may function as a tumor suppressor. Accordingly, transient expression of CAR in human prostate and bladder cancer cell lines induced growth-inhibitory activity (Okegawa et al., 2000; Okegawa et al., 2001). CAR overexpression in human ovarian cancer cells mediated cell aggregation and reduced cell migration (Bruning and Runnebaum, 2004). Furthermore, it was shown that both the extracellular and the cytoplasmic domain of CAR is mediating its tumor suppression activity (Kim et al., 2003; Okegawa et al., 2001). Although most studies indicate a negative correlation of CAR expression and tumor cell progression, there are some indications to the contrary (Hemmi et al., 1998). In addition, CAR expression has also been found to be induced in xenografts as compared to parental primary glioblastomas (Fuxe et al., 2003).

There are several regulatory mechanisms that alter the expression of CAR in cancer cells *in vitro*. CAR promoter studies in human bladder cancer cell lines showed that low expression levels was caused by epigenetic regulation of the *CAR* promoter by histone acetylation but not DNA methylation (Pong et al., 2003). Treatment of different human cancer cell lines with histone deacetylase inhibitors such as FR901228, sodium butyrate and trichostatin-A, activated the *CAR* gene and increased transcription (Goldsmith et al., 2003; Hemminki et al., 2003; Kitazono et al., 2001; Kitazono et al., 2002; Pong et al., 2003; Sachs et al., 2004). CAR expression was also affected by altered cell density. Increased cell density could both induce and suppress CAR expression in both normal and cancer cells (Carson et al., 1999; Fechner et al., 2003). This suggests a cell-cell contact dependent signalling as one possible regulatory mechanism of CAR gene transcription. Inflammatory agents such as cytokines have been found to regulate CAR expression *in vitro*. Treatment of human cancer cell lines with the inflammatory cytokine tumor necrosis factor-α (TNF-α) could both induce and suppress CAR expression dependent on tumor type, while decreased CAR levels were seen in transforming growth factor-β (TGF-β) treated cells (Bruning and Runnebaum, 2003). In addition, in normal human endothelial and epithelial cell cultures, TNF-α and interferon-γ (IFN-γ)

decreased CAR levels and reduced adenovirus transduction efficiency, and when cells were treated simultaneously with TNF-α and IFN-γ, a synergistic effect was obtained (Vincent et al., 2004). Treatment of several human cancer cell lines with the anti-inflammatory reagent dexamethasone, a synthetic glucocorticoid, decreased CAR expression and adenovirus transduction (Bruning and Runnebaum, 2003). Interestingly, CAR expression is suppressed in mouse and human cancer cell lines that have undergone EMT in response to TGF-β. Treatment of Ras-transformed mammary epithelial cells that have undergone EMT *in vivo* with LY2109761, a specific pharmacological inhibitor of TGF-β receptor types I and II kinases, increased *CAR* transcript and protein expression (Lacher et al., 2006). The Raf/MEK/ERK signalling pathway is frequently induced in cancer cells and are known to down-regulate cell surface proteins. Indeed, disruption of the Raf/MEK/ERK pathway by inhibition of MEK in mouse and human cancer cell lines up-regulated the expression of CAR while induction of Raf-1 decreased CAR levels (Anders et al., 2003).

1.2.5 Biological functions

Since CAR localizes to epithelial cell-cell contacts and the fact that many Ig-containing proteins are mediating cell-cell adhesion, suggested that CAR function as a cell adhesion molecule. Indeed, transfection of CAR into cell cultures with low adhesive properties, when grown in suspension, resulted in the formation of cell aggregates via homophilic CAR interactions. This homophilic interaction is mediated both by the V and the C2 domain, which was abolished in the presence of antibodies directed against the ectodomain (Cohen et al., 2001b; Excoffon et al., 2005; Honda et al., 2000; Okegawa et al., 2001). CAR also mediates heterophilic binding to other CTX-subfamily proteins, as CAR and JAM-C could be co-immunoprecipitated from mouse testis homogenates. Since both proteins are expressed during spermatogenesis and JAM-C has been reported to be important during this developmental process, CAR might have a potential role in the differentiation of mouse germ cells (Gliki et al., 2004; Mirza et al., 2006). Interestingly, CAR was recently found to mediate transepithelial migration of leukocytes via direct interaction with the junctional adhesion molecule-like (JAML) protein (Zen et al., 2005). In addition, the V domain of CAR was found to interact with the C2 domain of JAML, and the binding of leukocytes to CAR was inhibited by antibodies directed against the extracellular domain of CAR or JAML.

CAR has been suggested to mediate epithelial barrier permeability since the protein localizes at epithelial tight junctions and possesses cell-adhesion properties. This was found to be the case as overexpression of CAR in polarized epithelial cells *in vitro*, increased the TER. This alteration in

permeability could be inhibited by treatment of the cells with soluble CAR or CAR specific antibodies (Cohen et al., 2001b; Walters et al., 2002). It was also reported that the endogenous amounts of CAR in primary human airway epithelia cells correspond with the degree of TER. Interestingly, overexpression of a truncated CAR lacking the C-terminal PDZ-domain binding motif failed to increase the TER to the same degree as full-length CAR in well-differentiated human airway epithelial cells. In addition, the PDZ-domain binding motif was also found to play a role in cell growth, as mouse fibroblast L-cells transfected with truncated CAR grew slower than cells over expressing the full length protein (Excoffon et al., 2004).

The sub-cellular localization of CAR and the presence of a PDZ-domain binding motif in the intracellular tail of both CAR isoforms TVV and SIV (Table I), indicates that CAR might interact with PDZ-domain containing scaffolding proteins that are expressed at the site of tight junctions. Indeed, the CAR SIV isoform was found to interact with, and to recruit, ZO-1, MAGI-1b, protein interacting with protein C kinase (PICK1), and postsynaptic density 95 (PSD-95) to newly formed cellular junctions *in vitro* (Cohen et al., 2001b; Excoffon et al., 2004). Although not all these interactions may be mediated by direct binding of CAR with these proteins, it shows that CAR is present in larger protein complexes at epithelial tight junctions and that CAR may be important for the recruitment of PDZ-domain containing proteins to this site of the plasma membrane. However, the CAR SIV isoform interacted directly with the multi-PDZ domain protein-1 (MUPP-1). CAR and MUPP-1 were observed to co-localize at tight junctions in epithelial cells *in vitro* and it was found that CAR is important for the recruitment of MUPP-1 to early cell-cell contacts (Coyne et al., 2004). A direct interaction of the CAR TVV and SIV isoforms to the PDZ-domain containing protein ligand-of-numb protein-X2 (LNX2) was also recently demonstrated. However, the physiological function of this interaction is yet unknown (Mirza et al., 2005).

Recently, several reports have demonstrated the importance of CAR during mouse cardiac development as CAR deficient mice die between embryonic day E11.5 to E13.5 due to cardiac failure (Asher et al., 2005; Chen et al., 2006; Dorner et al., 2005). Interestingly, some phenotypic differences between these CAR null mice were observed. Asher *et al.* found that the CAR null mice displayed thinner myocardial wall with foci of degenerating cardiomyocytes. Absence of normal elongated epicardial and endocardial monolayers and disruption of the cardiac wall was also observed (Asher et al., 2005). The CAR deficient mice, generated by Dorner *et al.*, showed smaller ventricular lumen with enlarged cushions, resulting in only one atrioventricular canal instead of two. These CAR null mice displayed a decreased number of myofibrils and lack of close attachments between them (Dorner et al., 2005). Chen *et al.* found in their CAR null mice an abnormal thickening of the left ventricular wall due

to an increased number of cardiomyocytes. These CAR deficient mouse embryos displayed moderate thickening of the atrial myocardium and abnormalities in the junction between the sinus venosus and atrium. Another finding was that the junctions between cardiomyocytes were abnormal. It was also found that CAR is crucial during a critical developmental window, between E10 and E11, during cardiogenesis (Chen et al., 2006). No reports of other gross abnormalities in the fetus and placenta were observed prior to death, except for enlargement of the pericardium due to edema. Surprisingly, dilatations of the cardiac veins and aortas at E11.5 were seen, although CAR was found to be absent in endothelial cells. Both these phenotypes are probably due to secondary effects of cardiac dysfunction (Chen et al., 2006; Dorner et al., 2005). It is noteworthy to mention that knockout mice have been generated for several tight junction and CTX-subfamily proteins, such as occludin, several claudins, ESAM, JAM-A, -B and -C. So far CAR has been the only component of tight junctions that have a complete lethal embryonic phenotype, compared to the others that show none or limited number of dead embryos (Cera et al., 2004; Cooke et al., 2006; Feldman et al., 2005; Gliki et al., 2004; Sakaguchi et al., 2006; Van Itallie and Anderson, 2006).

1.2.6 CAR as a virus receptor

Except for the biological functions of CAR mentioned earlier, the protein is also utilized as a virus receptor for CVB and adenoviruses. In the following sections a brief review is presented of the CVB and adenoviruses, the properties of CAR-virus interaction as well as the possibility of using CAR in adenoviral gene transfer and therapy.

1.2.6.1 CVB and adenovirus binding and infection

The CVB, belonging to the *picornaviridae* family, initially infects epithelial cells of the respiratory system and gastrointestinal tract. The virus then replicates, spreads and causes secondary infections in the heart, pancreas, skeletal muscle and the central nervous system (Knipe and Howley, 2001). CAR serves as the receptor for all six CVB serotypes (CVB1-6), although CVB serotypes 1, 3 and 5 also use the decay-accelerating factor (DAF) as a co-receptor (Bergelson et al., 1997b; Bergelson et al., 1995; He et al., 2001; Martino et al., 2000; Shafren et al., 1995). The mechanism used by CVB to access CAR at the epithelial tight junctions has been unknown. However, recent published data show that the attachment of CVB to DAF on the apical surface of the epithelium, activates Rac-dependent actin rearrangements, resulting in virus movement to the tight junctions. At this site, the CVB comes

in contact with CAR that mediates virus transduction into the epithelial cells (Coyne and Bergelson, 2006).

The human adenovirus family, that belongs to the *Adenoviridae* family, consists of 51 serotypes which fall into six subgroups (A-F). They can infect and replicate in a wide range of organs, such as the respiratory tract, eye, urinary bladder, gastrointestinal tract and liver. Adenovirus subgroups A, C, D, E and F binding to cells is mediated by CAR at least *in vitro*, while the internalization appears to be mediated by cell surface integrin molecules ($\alpha_v\beta_1$, $\alpha_v\beta_3$ or $\alpha_v\beta_5$) (Roelvink et al., 1998; Wickham et al., 1993). *In vitro* studies of adenovirus infection of primary human airway epithelial cells revealed that after adenovirus infection and replication, progeny virions and adenovirus fiber proteins are released only to the basolateral surface of the epithelium. Virions and fiber then travels to the site of tight junctions were CAR is localized. Adenovirus fiber knob then binds CAR and mediates disruption of junctional integrity, allowing the virions to escape to the apical side of the epithelial cells (Walters et al., 2002).

In vitro studies of adenovirus transduction efficiency of cells expressing full length or truncated CAR, lacking either the V or the C2 domain, demonstrated that the V domain mediates adenovirus binding (Freimuth et al., 1999; Kim et al., 2003). The complex between the V domain and the adenovirus fiber knob was crystallized and characterized. It was found that the CAR V domain folds into a β-sandwich, that is characteristic for Ig V domains, and that three CAR V monomers can bind a fiber knob trimer. In this complex the V domain binds at the interface between two adjacent adenovirus knob monomers. It was also determined that the V domain forms homodimers in the crystal using the same surface which interacts with the adenovirus fiber knob (Bewley et al., 1999; van Raaij et al., 2000). Analyzes of the interaction properties by residue substitutions in the CAR V domain, showed that residues Val70 and Ile72 are crucial for adenovirus binding. However, none of these residue substitutions affected CVB binding and infection. This was found to be due to that fact that adenoviruses and CVB interact with different parts of the CAR extracellular domain. While adenovirus binding was restricted to the V domain of CAR, CVB was found to bind to the distal end of the V domain and to the C2 domain (He et al., 2001; Tomko et al., 2000). Several studies have shown that the transduction efficiency of adenovirus and CVB is not dependent on the transmembrane region or the intracellular tail of CAR, since efficient virus infection was observed in cells overexpressing either GPI-anchored extracellular domain of CAR or truncated CAR lacking only the intracellular tail (Leon et al., 1998; Nalbantoglu et al., 1999; Okegawa et al., 2001; Wang and Bergelson, 1999). It was also found that both recombinant soluble CAR as well as endogenously secreted CAR could abrogate adenovirus and CVB infection of cells in culture. In addition, recombinant soluble CAR could also

block CVB mediated pancreatitis and myocarditis in mice (Bernal et al., 2002; Dorner et al., 2004; Freimuth et al., 1999; Goodfellow et al., 2005; Yanagawa et al., 2004).

1.2.6.2 CAR in adenovirus gene transfer and therapy

Adenovirus-based vectors were believed to be useful tools in model systems for curing of monogenetic diseases, such as cystic fibrosis, and for cancer therapy. Recombinant adenoviruses can be produced in high titers and they show low pathogenicity in humans at physiological amounts. These viruses bind with high affinity to CAR and can transduce cells *in vivo* with transgenes of more than 30 kb. Finally, they have a very efficient nuclear entry mechanism and they do not integrate its viral genome into the host cell genome (Volpers and Kochanek, 2004). Unfortunately, several experimental challenges of using recombinant adenoviruses in gene transfer or disease treatment have emerged during recent years.

The route of adenovirus administration to the diseased area has been found to be one limiting factor in using recombinant adenovirus for gene transfer studies. Clinical and preclinical studies of adenovirus-mediated gene transfer to lung epithelium of patients with cystic fibrosis showed insufficient adenovirus infection of the respiratory epithelium (Grubb et al., 1994; Knowles et al., 1995). Later *in vitro* studies showed that this was due to the restricted expression of CAR at the basolateral side of the plasma membrane. Administration of the adenovirus from the basolateral side of polarized epithelial cells in culture resulted in efficient adenovirus transduction (Pickles et al., 1998; Walters et al., 1999). The basolateral localization of CAR is not the only limiting factor for adenoviral gene transfer from the apical side of polarized epithelial cells. *In vitro* and *in vivo* studies of epithelial cells expressing GPI-anchored extracellular domain of CAR on the apical surface of the cells, did not increase adenovirus transduction. This was found to be due to the protective mucous layer, or glycocalyx, which cover the luminal surface of the respiratory epithelium (Figure 2A). When the surface mucous was treated with reagents that remove glycocalyx-components, induction of adenovirus infection was observed (Pickles et al., 2000; Stonebraker et al., 2004). These data show the relevance of administrating the adenoviruses from the basolateral surface of epithelial cells to achieve virus transduction. Upon systemic administration of adenovirus, a majority of virus is either depleted in a CAR-independent way by Kupffer cells or infect liver hepatocytes. As the hepatocytes are in close contact with the fenestrated epithelium, the virus passes easily from the blood to the basolateral side of the hepatocytes (Figure 2C) (Alemany et al., 2000; Shayakhmetov et al., 2004). Virus then migrates along the lateral surface and reaches the tight junction. Since CAR has been

found to localize at these junctions, implies a role of CAR in binding and uptake of adenovirus, hense mediating virus infection of hepatocytes (Paper II). Another limiting factor for using recombinant adenoviruses for cancer therapy is the frequent down-regulation of CAR in tumor cells. These cells are consequently not susceptible to adenovirus infection (Hemmi et al., 1998; Li et al., 1999; Okegawa et al., 2000). To overcome the low expression of CAR in different human tumor cells, treatment with potential cancer chemotherapeutic agents such as FR901228, sodium butyrate and trichostatin-A was found to increase CAR expression and induce adenovirus transduction efficiency *in vitro* (Hemminki et al., 2003; Kitazono et al., 2001). However, these reagents are often associated with negative side effects, which are harmful for the patients. Finally, the restricted expression of CAR in epithelial cells *in vivo* hampers adenovirus gene transfer of CAR-negative cell types such as leukocytes and endothelial cells. To overcome this problem, several CAR transgenic mouse models have been developed that have allowed for recombinant adenoviral transfer into cells that usually are not susceptible to adenoviral infection (Schmidt et al., 2000; Tallone et al., 2001; Wan et al., 2000).

Together these data suggest that the sub-cellular localization and expression levels of CAR must be taken into account when recombinant adenoviruses are to be used in gene transfer or therapy studies. These limiting factors have prompted the development of retargeted adenovirus vectors to achieve CAR-independent gene delivery. This could be one way to overcome the problems of low accessibility and expression of CAR while maintaining the properties of efficient gene delivery into host cells (Krasnykh et al., 2000; Mizuguchi and Hayakawa, 2004).

1.3 The other members of the CTX-subfamily

The remaining members of the CTX-subfamily are all type I transmembrane proteins that have a similar protein structure as have been described for CAR (Figure 5). Many of the CTX-proteins are cell-adhesion molecules that are mainly expressed at tight junctions of epithelial and endothelial cells. In the following sections, a brief review of each member will be presented.

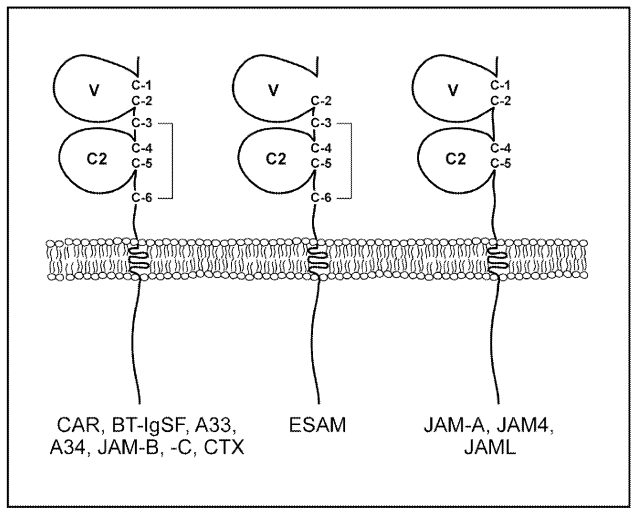

Figure 5. Protein structure of the CTX-subfamily. All proteins of the CTX-subfamily contain two extracellular immunoglobulin loops, the V and C2 domains, one transmembrane region and an intracellular tail. The variability in conservation of the six cysteine residues (C-1 to C-6) among the CTX-subfamily members are shown. This schematic representation is not drawn to scale.

1.3.1 CTX

The cortical thymocyte in *Xenopus* (CTX) is the founding member of the CTX-subfamily (Figure 5). It was isolated as a protein expressed by a large fraction of cortical thymocytes in *Xenopus* (Chretien et al., 1996) (Robert et al., 1997b). Little is known about the physiological function of CTX. *In vitro* cross-linking of CTX on lymphoid tumor cell lines with monoclonal anti-CTX antibodies inhibited cell growth and caused abnormal tumor cell mitosis and multinucleation (Robert et al., 1997a; Robert et al., 1997b). The human (CTH) and mouse (CTM) homologues were found not to be specifically expressed in lymphocytes as was seen for CTX. In addition, CTH and CTM transcripts were detected in stomach, prostate, and colon, while expression of CTH transcripts

was also observed in the trachea, thyroid glands, bladder, and lung (Chretien et al., 1998).

1.3.2 JAMs

The junctional adhesion molecules-A, -B, -C, 4 (JAM-A, -B, -C, JAM4), and the junctional adhesion molecule-like protein (JAML) have been extensively studied. The expression and biological function of JAMs are briefly discussed below.

The JAMs share a similar protein structure with other members of the CTX-subfamily (Figure 5) (Mandell and Parkos, 2005). JAMs function as cell adhesion proteins since they mediate both homophilic and heterophilic interactions via their ectodomains. JAM-A and -B interacts in a homophilic fashion, while heterophilic interaction has been observed between JAM-B and -C. In addition, several extracellular ligands that interact with JAMs have been identified. JAM-A interacts with LFA-1, $\alpha_v\beta_3$ integrin, and the attachment protein $\sigma 1$. JAM-B interacts with $\alpha 4\beta 1$ integrin and JAM-C binds CD11b/CD18 (Mandell and Parkos, 2005). JAML was recently found to interact with CAR (Zen et al., 2005). The intracellular tails of JAM-A, -B, -C and JAM4 contain type I or type II PDZ-domain binding motifs (Table I), which interact with several PDZ-domain containing cytosolic proteins. JAM-A interacts with cingulin, MAGUK protein CASK/LIN-2, ASIP/PAR-3, and MUPP-1. JAM-B and -C bind ZO-1 and PAR-3, while JAM4 interact with MAGI-1 and LNX (Kansaku et al., 2006; Mandell and Parkos, 2005).

The cell specific expression and sub-cellular localization of the JAMs have been extensively investigated. JAMs are predominantly expressed in epithelial and endothelial cells as seen in the digestive system, respiratory system, heart, brain, and placenta. In addition, some members are also expressed in haematopoietic cells. Interestingly, JAM-A is the only CTX-subfamily member known to date that is expressed both on epithelial and endothelial cells (Mandell and Parkos, 2005; Moog-Lutz et al., 2003; Zen et al., 2005). At the sub-cellular level, JAMs associate with cellular junctions both when endogenously expressed in a variety of cells *in vitro* and *in vivo*, and when overexpressed in JAM-negative cells in culture. More specifically, the sub-cellular localization of JAMs in epithelial cells has been detected at, or adjacent to tight junctions (Mandell and Parkos, 2005). The expression of JAM-A and -C at cellular junctions, was found to be regulated by inflammatory cytokines and by phosporylation of the intracellular tail, indicating that the localization of JAMs can be affected by specific signals from the extracellular or intracellular environment (Ebnet et al., 2003; Ozaki et al., 2000; Ozaki et al., 1999; Shaw et al., 2001).

Table I. The amino acid composition of the extreme C-terminus of the CTX-subfamily members and their classification into PDZ-domain binding motif I or II*.

	Residue position:				
	-4	-3	-2	-1	0**
A. PDZ-domain binding motif type I	**X**	**X**	**S/T**	**X**	**Φ**
CAR TVV isoform	G	I	T	V	V
CAR SIV isoform	D	G	S	I	V
BT-IgSF	A	G	S	L	V
ESAM	A	G	S	L	V
JAM4	N	V	T	L	V
B. PDZ-domain binding motif type II	**X**	**X**	**Φ**	**X**	**Φ**
CAR WCL isoform	T	F	W	C	L
JAM-A	S	S	F	L	F
JAM-B	K	S	F	I	I
JAM-C	S	S	F	V	I
C. CTX-subfamily members lacking a PDZ-domain binding motif:					
A33	D	Q	P	F	Q
A34	G	V	V	K	A
JAML	T	Q	Q	A	F
CTH	L	P	M	V	V

* Songyang *et al.*, 1997 and Schultz *et al.*, 1998
** Residue position 0 refers to the last C-terminal residue
X denotes any amino acid
Φ denotes a hydrophobic amino acid
Note: Group A and B refers to mouse secuences, while group C refers to human secuences

The JAMs have been found to be involved in several physiological and pathological processes that involve cellular adhesion. JAM-A is important in tight junction assembly, since the protein recruits several tight junction proteins such as ZO-1, CASK, occludin, PAR3, and AF6 to early cellular contacts (Bazzoni et al., 2000; Ebnet et al., 2000; Ebnet et al., 2001; Itoh et al., 2001; Liang et al., 2000; Liu et al., 2000; Martin-Padura et al., 1998; Martinez-Estrada et al., 2001). Several JAMs may play a role in inflammatory processes. JAM-A, -B, -C, and JAML are mediating leukocyte transmigration across the endothelium and epithelium by forming homophilic and heteroplilic interactions with proteins expressed on these cells (Cunningham et al., 2000; Cunningham et al., 2002; Johnson-Leger et al., 2002; Liang et al., 2002; Martin-Padura et al., 1998; Ostermann et al., 2002; Santoso et al., 2002; Zen et al., 2004; Zen et al., 2005). JAM-A also mediates platelet activation, aggregation, and spreading (Kornecki et al., 1990; Ozaki et al., 2000; Sobocka et al., 2000). A role for JAM-A in angiogenesis was suggested in knockdown experiments in which siRNA inhibition of JAM-A decreased endothelial cell

motility and adhesion. In addition, overexpression of JAM-A increased endothelial cell proliferation (Naik et al., 2003a; Naik et al., 2003b). To investigate the biological role of JAMs *in vivo* several JAM deficient mice have been generated. JAM-A and JAM-B null mice were born in the correct Mendelian ratios and did not display any significant alteration of organ development or morphology, compared to wild type mice (Cera et al., 2004; Sakaguchi et al., 2006). However, in a recent report JAM-A null mice were shown to be born with a frequency 40% lower than expected. Although the live born JAM-A null mice developed normally, they displayed a reduced litter size and a lower male to female ratio than expected (Cooke et al., 2006). JAM-C has also been targeted for gene deletion in mice. This study revealed that 60% of the JAM-C deficient mice died during postnatal development. In addition, JAM-C was found to be required for spermatid differentiation as the viable JAM-C null males were found to be infertile (Gliki et al., 2004). Finally, JAM-A is a virus receptor, utilized by reovirus and feline calicivirus (Barton et al., 2001; Forrest et al., 2003; Makino et al., 2006; Prota et al., 2003).

1.3.3 ESAM

The endothelial cell-selective adhesion molecule (ESAM) is preferentially expressed in vascular endothelium (Hirata et al., 2001; Nasdala et al., 2002). Remarkably, ESAM harbours only five conserved cysteine residues in the extracellular domain, the first cysteine residue that forms the V domain in other CTX-subfamily proteins is missing (Figure 5). Nevertheless, computer analysis based on highly conserved residues in the ESAM V domain suggests that this immunoglobulin domain is present (Nasdala et al., 2002). ESAM is expressed in tissues with high content of endothelial cells, such as in digestive and respiratory systems, heart and brain, during both development and in the adult mouse (Hirata et al., 2001; Nasdala et al., 2002). Investigation of the *in vivo* sub-cellular localization of ESAM revealed that the protein co-localized with ZO-1, occludin and claudin-5 in endothelial tight junctions (Nasdala et al., 2002). Several studies deal with the physiological functions of ESAM. The protein was found to be a cell adhesion molecule, since it mediates Ca^{2+}-independent homophilic cell-cell interactions (Harita et al., 2006; Nasdala et al., 2002). ESAM interacts via its C-terminal PDZ-domain binding type I motif with MAGI-1 (Table I), and recruits MAGI-1 to newly formed endothelial cell-cell contacts *in vitro* (Wegmann et al., 2004). The localization of ESAM to tight junctions was not altered by the removal of the last five C-terminal residues harbouring the PDZ-domain binding motif, suggesting that its interaction with MAGI-1 is not essential for proper sub-cellular localization. Instead, these data suggest that ESAM is important in anchoring MAGI-1 at

endothelial tight junctions (Nasdala et al., 2002). To elucidate the biological function, ESAM deficient mice were generated. Surprisingly, these null-mice developed normally without any obvious morphological defects in the vasculature. However, further analyzes showed that ESAM is important for *in vivo* tumor vascularization. The protein was also found to be involved in several inflammatory processes, such as in signalling events that trigger the opening of endothelial cell contacts and in mediating neutrophil recruitment to inflammatory tissue (Ishida et al., 2003; Wegmann et al., 2006).

1.3.4 A33 and A34

The A33 antigen was initially discovered by the highly specific murine monoclonal antibody A33, hence the name of the protein. A33 is a cell surface antigen which is specifically expressed in normal human gastrointestinal epithelial cells as well as in the majority of primary or metastatic colon and rectal cancer cells (Garin-Chesa et al., 1996; Heath et al., 1997; Johnstone et al., 2002; Sakamoto et al., 2000; Welt et al., 1994; Welt et al., 1990; Welt et al., 1996). The human A33 antigen has been used for cancer diagnosis due to its tissue-restricted expression pattern, and the protein was targeted with radiolabeled monoclonal A33 antibodies in several phase I/II radioimmunotherapy studies with great success on patients with metastatic colorectal cancer (King et al., 1995; Scott et al., 2005; Welt et al., 1994; Welt et al., 2003a; Welt et al., 2003b; Welt et al., 1996). The mouse A33 homologue is exclusively expressed in epithelial cells of the intestinal tract. In contrast to many other CTX-subfamily members, the mouse A33 antigen was found to localize to the basolateral side of the epithelial plasma membrane (Johnstone et al., 2000).

The human A34 antigen was recently identified in a screen for novel tissue-restricted proteins. The A34 and A33 antigens are approximately 30% identical and show an overall similar protein structure (Figure 5). Human A34 transcripts and protein expression were detected in normal stomach, pancreas, and testis tissues, as well as in various human tumors (Scanlan et al., 2006). Its expression pattern, which is similar to the A33 antigen, and the presence of a specific A34 monoclonal antibody, suggest that the A34 antigen could be used as a target in different types of cancer therapies.

1.3.5 BT-IgSF

The brain- and testis-specific IgSF (BT-IgSF or IGSF11) was identified as a protein that is structurally related to the CTX-subfamily (Figure 5). BT-IgSF is

a cell adhesion molecule that mediates cell-cell adhesion in a Ca^{2+}/Mg^{2+}-independent manner (Harada et al., 2005; Katoh, 2003; Suzu et al., 2002). The expression of BT-IgSF has been mainly characterized at the mRNA level. High levels of human and murine BT-IgSF transcripts have been detected in testis and brain, and at lower levels in the kidney, adrenal gland and ovary (Suzu et al., 2002; Watanabe et al., 2005). By comparing the expression levels of BT-IgSF in different human and murine tumors, both induction and suppression of BT-IgSF were observed (Katoh, 2003; Watanabe et al., 2005). Interestingly, inhibition of BT-IgSF by siRNA decreased the growth of human gastric cancer cells *in vitro* (Watanabe et al., 2005).

2 Aims of the study

There is an extensive amount of data available on the role of CAR being utilized as the receptor for adenovirus-based gene transfer vectors. The molecular characteristics and adhesive properties of CAR have been thoroughly examined *in vitro*, but less is known about its biological role in the living organism. The major aim of this study was to broaden the understanding of the physiological function of CAR *in vivo* as a cell adhesion molecule, which localizes to epithelial tight junctions. In addition, we set out to identify new members of the CTX-subfamily in the attempt to gain new insights into this group of cell adhesion proteins.

The specific aims were to:

- elucidate the functional properties of CAR by identifying novel proteins that interact directly and specifically to its intracellular tail (Paper I)
- determine the expression pattern and sub-cellular localization of CAR in the adult mouse (Paper II)
- investigate the physiological role of CAR in kidney development and glomerular function (Paper III)
- study the expression pattern and the functional properties of CLMP, a new member of the CTX-subfamily (Paper IV)

3 Results and discussion

The results of the publications are briefly summarized and discussed in the following sections. For a more detailed description of each study and the experimental procedures used, the reader is referred to the original papers that can be found in the manuscript section of this thesis.

3.1 The coxsackievirus and adenovirus receptor (CAR) forms a complex with the PDZ domain-containing protein ligand-of-numb protein-X (LNX) (Paper I)

Transmembrane components of tight junctions are commonly anchored on the intracellular side to PDZ-domain containing proteins that serve as scaffolds, which connect plasma membrane proteins to the cytoskeleton (Harris and Lim, 2001; Roh and Margolis, 2003). CAR is a component of the same complexes as several PDZ-domain containing scaffolding proteins, such as ZO-1, MAGI-1b, PSD-95 and PICK-1 (Cohen et al., 2001b; Excoffon et al., 2004). Although direct interactions of these proteins with CAR have not been shown, CAR seems to be required for their recruitment to newly formed cell-cell contacts. To learn more about the role of CAR at epithelial tight junctions, we performed a screen to identify novel interacting partners to the intracellular tail of CAR.

CAR harbours potential PDZ-domain binding motif in the extreme C-terminus of the intracellular tail (Table I). In a yeast two-hybrid screen using a 17-day mouse embryo cDNA library, we identified ligand-of-numb protein-X (LNX) as a novel PDZ-domain containing protein that interacts directly with the intracellular tail of the mouse CAR TVV isoform (referred to as mCAR-1 in Paper I). The interaction between these two proteins was confirmed by glutathione-S-transferase fused CAR (GST-CAR) pulldown of LNX from transfected cell lysates and of *in vitro* transcribed and translated LNX. LNX is a multi-PDZ-domain containing protein that interacts via its first PDZ-domain with the cell fate determinant Numb and co-expression of these two proteins was observed in the developing and adult mouse brain (Dho et al., 1998; Rice et al., 2001). Numb is implicated in asymmetric cell division and regulation of Notch activity in the developing nervous system in *Drosophila* (Dho et al., 1998; Knoblich, 2001). There are two known LNX isoforms, LNXp70 and LNXp80, only differing in their N-terminus, in which the LNXp80 has a RING finger domain. This domain of LNXp80 functions as a RING type E3 ubiquitin ligase that targets Numb for degradation (Nie et al., 2002). In addition, the human homologue of LNX was identified and found to be down regulated in human gliomas (Chen et al., 2005a; Chen et al., 2005b). Recently, the CTX-

subfamily member JAM4, was shown to interact directly with LNX. JAM4 forms a tripartite complex *in vitro* with LNX and Numb, in which Numb mediates endocytosis of JAM4 and LNX from the plasma membrane. It was therefore suggested that this complex is important in the remodelling of cellular junctions. LNX was also shown to be involved in TGFβ-induced EMT of mammary epithelial cells as a redistribution of JAM4 from cell-cell contacts to intracellular clusters was seen (Kansaku et al., 2006).

Next we set out to identify the interacting sites in LNX and CAR. By GST-CAR pulldowns of several truncated LNX constructs lacking one or several PDZ-domains, the second PDZ-domain of LNX was found to mediate the interaction with CAR. This is the same domain of LNX that binds JAM4 (Kansaku et al., 2006). It is not known whether binding of CAR and JAM4 to LNX is mutually exclusive. Since both CAR and JAM4 localize to epithelial tight junctions, their binding to LNX might be cell type specific or depend on different cellular conditions. Two different deletion constructs were made to determine the amino acid residues of the intracellular tail of the CAR TVV isoform that bind LNX. We observed that the interaction between LNX and CAR was completely abolished in the truncated CAR lacking the last three amino acid residues (GST-CARΔTVV). This result was expected since PDZ-domains previously have been shown to interact mainly with the extreme C-terminus of target proteins that end with a conserved PDZ-binding motif. However, we found that this interaction was partly re-established in a truncated CAR lacking the last 13 amino acid residues (GST-CARΔCterm). This suggest that LNX is able to interact with a second site in the intracellular tail of CAR, a feature observed for several other PDZ-domain containing proteins that bind internal amino acid sequences within their ligands (Hung and Sheng, 2002). Why GST-CARΔTVV does not bind LNX even though the second binding site is present is still unclear. We speculate that this maybe due to secondary conformational changes of the intracellular tail of the truncated CAR that masks the internal LNX binding site.

CAR was previously reported to recruit PDZ-domain containing proteins to newly-formed cell-cell contacts (Cohen et al., 2001b; Excoffon et al., 2004). We therefore speculated that CAR might function in targeting LNX to the plasma membrane. Indeed, we found that overexpression of CAR in cell cultures recruited LNX to cell-cell contacts, although some LNX protein was also detected in the cytoplasm.

To summarize, we found that the CAR TVV isoform interacted with the second PDZ-domain of LNX. We identified the last three amino acids of the intracellular tail of CAR to be crucial for binding to LNX, although LNX also utilizes a secondary internal binding site. CAR was crucial for the recruitment of LNX to cell-cell contacts, and since LNX binds and regulates the expression of the cell-fate determinant Numb, these data may suggest a role for CAR in

targeting these two proteins to specific parts of the plasma membrane. Co-expression of CAR, LNX and Numb in tissues, such as in the developing brain, further supports the idea that these proteins might form a functional complex. Finally, since Numb is regulating Notch activity in the developing nervous system, it is tempting speculate that CAR has a potential role in this important developmental process.

3.2 The coxsackie- and adenovirus receptor (CAR) is an *in vivo* marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis (Paper II)

CAR has been extensively studied for its cell-adhesion properties and expression pattern *in vitro* (see sections 1.2.2 and 1.2.5). However, less is known about its physiological function and expression pattern *in vivo*. Elucidating the expression pattern and the sub-cellular localization of CAR in a living organism is an important step towards a greater understanding of its biological function. We therefore performed a comprehensive study of the CAR expression pattern in the adult mouse. As there were no commercial antibodies available against CAR to perform this study, we produced several specific antibodies that immunoreacted either with the common part of the CAR TVV and SIV isoforms or that were isoform-specific.

Our studies showed that CAR was predominantly expressed at epithelial cell-cell contacts of the digestive, respiratory, and urinary systems in the adult mouse. We also detected CAR in a few non-epithelial tissues, such as in the intercalated discs between the cardiomyocytes and at neuromuscular junctions of skeletal muscle, as was previously reported (Shaw et al., 2004). Earlier reports showed CAR expression in endothelial cell cultures (Carson et al., 1999; Vincent et al., 2004). However, although we performed a systematic and thorough analysis of endothelial cells in both blood and lymphatic vessels we were unable to detect the protein. At this point, we cannot rule the possibility that CAR is expressed at very low levels in this cell type, or in other cell types that were CAR-negative in our studies.

In kidneys, CAR expression was restricted to glomeruli and to certain segments of the epithelial renal tubule. A characteristic feature of the renal tubule is that it is composed out of several distinct segments of variable permeability, reflecting its reabsorptive functions (Figure 6A). Permeable segments, such as the proximal tubule, express low levels of the tight junctional proteins occludin and ZO-1. In contrast, high levels of these proteins are expressed in impermeable segments, such as the distal tubules and collecting ducts (Gonzalez-Mariscal et al., 2000b). CAR expression was undetectable in tubular segments with low expression of ZO-1 and occludin. In

contrast, CAR expression was observed in tubular segments that expressed high levels of ZO-1 and occludin. These data demonstrated a restricted expression of CAR to impermeable segments of the renal epithelial tubule. Indeed, kidney sections stained for CAR and the lectin PNA, a marker for impermeable distal tubules and collecting ducts, revealed an overlapping expression pattern. However, CAR did not co-localize with PHA-E, a lectin marker for more permeable proximal tubules, confirming the restricted expression pattern of CAR to the impermeable renal tubular segments. We observed a similar restricted expression pattern of CAR in choroid plexus and ventricular ependymal lining in the brain. Both these structures originate from epithelial cells, however, a clear difference is seen in the permeability properties of their tight junctions. The epithelium of the choroid plexus displays mature and non-leaky junctions where high levels of CAR were detected. In contrast, low levels of CAR were observed in the highly permeable epithelium of the ventricular ependymal lining. Together these data suggest that CAR may have a role in regulating epithelial permeability and in tissue homeostasis, a feature that has, until now, not been reported for any other member of the CTX-subfamily.

To investigate the sub-cellular expression of CAR in relation to other tight junctional proteins, we performed high-resolution confocal microscopy analysis on epithelial tissues that were co-immunostained for CAR and the tight junction marker occludin. We found that CAR is indeed a component of epithelial tight junctions *in vivo*, which supports earlier findings that CAR is expressed at epithelial tight junctions (Cohen et al., 2001b; Nagai et al., 2003). The intracellular tail of CAR harbours sorting signals that are essential for correct targeting of the protein to the basolateral side of polarized epithelial cells in culture (Cohen et al., 2001a; Pickles et al., 2000). To investigate the role of the intracellular tail of CAR for correct targeting of the protein to tight junctions *in vivo*, we took advantage of a transgenic mouse model in which a tailless CAR is overexpressed in all cell types (Tallone et al., 2001). We found that tailless CAR was diffusely localized in the epithelial plasma membrane in contrast to the restricted tight junctional localization of endogenous CAR, further demonstrating the importance of the intracellular tail for correct targeting of CAR. Moreover, these results showed that overexpression of tailless CAR does result in a dominant-negative effect on the proper sub-cellular localization of the endogenously expressed CAR to epithelial cell-cell contacts. This could explain why the CAR transgenic mice developed normally with no visible defects (Tallone et al., 2001).

Although the two alternative CAR TVV and SIV isoforms were identified several years ago (Andersson et al., 2000; Chen et al., 2003; Thoelen et al., 2001b), very little is known about their *in vivo* expression pattern and biological function. Both isoforms are expressed in sperms and in cardiac and

skeletal muscle, and they interact with several PDZ-domain containing proteins (Paper I, (Cohen et al., 2001a; Excoffon et al., 2004; Mirza et al., 2006; Mirza et al., 2005; Shaw et al., 2004)). To gain new insights about the biological role of the two CAR TVV and SIV isoforms, we investigated their expression pattern in several adult mouse tissues. In most organs analyzed, such as pancreas, colon and choroid plexus, no difference in expression pattern was observed. In the kidney and liver, however, a restricted expression of CAR isoforms was seen. CAR TVV isoform levels were lower in the renal collecting ducts when compared to the CAR SIV isoform. Surprisingly, a complete lack of the TVV isoform was observed in hepatocytes, as only the CAR SIV isoform was detected. The biological implications of the different expression pattern of CAR isoforms in liver and kidney are not known. Morover, our data indicates that CAR expression in specific organs is regulated, not only at the transcriptional level but also by alternative mRNA splicing or by isoform-specific degradation. We speculate that one explanation for the different expression patterns for the CAR isoforms could be that they differ in the regulation of the permeability properties of the epithelium.

In conclusion, we showed that CAR is a component of epithelial tight junctions *in vivo* and that CAR expression levels correlated with permeability of the epithelium. We also found that although the CAR TVV and SIV isoforms are co-expressed in most epithelial tissues, there are some organs that show a restricted isoform expression. Altogether, these data suggest that CAR may have a function in regulating epithelial permeability and tissue homeostasis. In addition, the epithelial tight junctional localization of CAR could partially explain the difficulties of using adenovirus based gene transfer experiments *in vivo* (see section 1.2.6).

3.3 The role of the coxsackie and adenovirus receptor (CAR) in zebrafish kidney development (Paper III)

In paper II we concluded that CAR could be involved in regulating epithelial permeability and tissue homeostasis, based on the specific expression pattern of CAR in the glomerular podocytes and in more impermeable segments of the renal tubule (Paper II) (Nagai et al., 2003). We chose to investigate the potential role of CAR in kidney function and development using the zebrafish as a model organism.

There are several anatomic advantages for using zebrafish to study biological functions of higher vertebrates such as mice. The anatomic simplicity of the zebrafish pronephros is striking in comparison to the complex mammalian kidney. The mammalian nephron contains approximately one million glomerular filtering units, each of them connecting to an epithelial renal tubule. The ultrafiltrate passes from the glomerulus to the renal epithelial

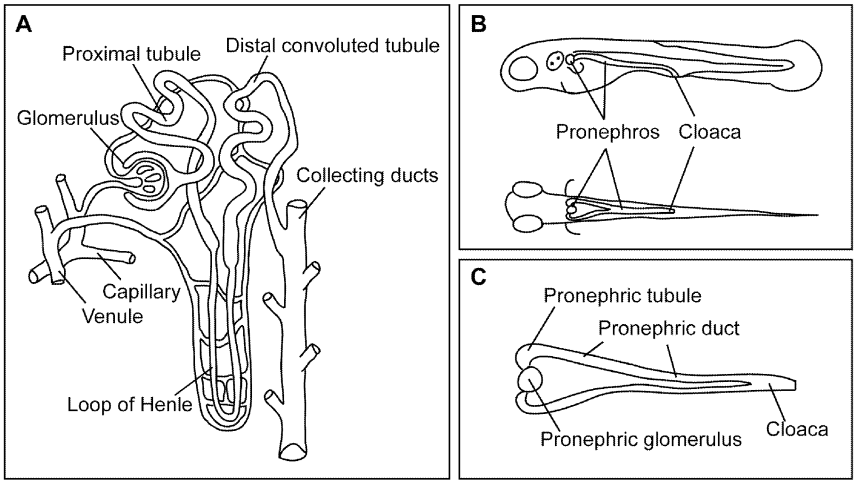

Figure 6. Anatomical structure of the mammalian nephron and the zebrafish pronephros. (A) Schematic representation of the mammalian nephron. Each nephron consists of a filtering unit, the glomerulus, which is connected to the renal epithelial tubule. The epithelial tubule consists of several distinct segments that differ in morphology, permeability and function. The permeable proximal tubule is important for the reabsorption of glucose, amino acids, ions and water from the glomerular ultrafiltrate. The descending segment of the loop of Henle shows high permeability to water and ions when compared to the less permeable ascending tubular segment. The distal convoluted tubule is impermeable to water and the epithelial cells actively reabsorb ions. Finally, the epithelium of the impermeable collecting duct is important for controlling the blood pressure by regulating reabsorption of the remaining water. These cells also regulate the acid-base balance by secreting bicarbonate. The nephron is surrounded by capillaries that collect the reabsorbed glomerular filtrate, which is then drained by venules. **(B and C)** Schematic representation of the zebrafish pronephros. **(B)** The location of the pronephros and the cloaca in a zebrafish larvae, seen from the lateral and dorsal view. **(C)** The zebrafish pronephros is composed of two renal epithelial tubules, which share a common midline pronephric glomerulus. The glomerular ultrafiltrate passes from the pronephric glomerulus into the pronephric tubule, which transition into the pronephric duct. Water and ions are reabsorbed along the renal epithelium before the urine exits the larvae via the cloaca. These schematic representations are adapted and modified from Drummond, 2000 and Vize *et al.*, 2003.

tubule, which is composed of several distinct and morphological different segments. Here water and ions are reabsorbed before the urine is transported out of the kidney (Figure 6A) (Vize et al., 2003). In contrast, the zebrafish pronephros is composed of only two renal tubules that share a common, midline glomerulus. Glomerular ultrafiltrate passes from the glomerulus to the pronephric tubule and duct segments which pass the urine out of the body via the cloaca (Figure 6B and C) (Drummond, 2000; Drummond, 2005). The simplicity of the zebrafish pronephros is therefore an advantage in studying developmental alteration of the kidneys. Another advantage is that the zebrafish have highly conserved kidney-specific genes as well as remarkable similarities in pronephric cell types compared to higher vertebrates. In addition, the zebrafish as a vertebrate model system has also several

experimental advantages over higher vertebrates. The zebrafish embryos are born *ex utero* and can be bread in large numbers. They develop rapidly from newly fertilized eggs to free swimming larvae in approximately 48 hours post fertilization (hpf), at which time most organ systems are physiologically functioning. The rapid organogenesis together with the ease to experimentally manipulate the embryos make the zebrafish an ideal model system for mutagenesis screens or gene specific morpholino knockdown studies (Corey and Abrams, 2001; Drummond, 2000; Drummond, 2005; Heasman, 2002).

Although zebrafish CAR (zCAR) has been identified, its expression pattern and physiological function in the zebrafish is not known (Petrella et al., 2002). In agreement with our CAR expression data in the mouse (Paper II), immunohistochemical analysis of zCAR expression revealed that the protein was mainly expressed at epithelial cell-cell contacts in tissues such as the gut, skin and liver during development and also in the adult zebrafish. zCAR expression was also observed in heart and brain. However the protein was undetectable in structures that resembled blood vessels. To determine the sub-cellular localization of zCAR, we co-immunostained tissues from zebrafish larvae for zCAR and the tight junctional proteins ZO-1 and cingulin. By high-resolution confocal microscopy we showed that zCAR co-localizes with these proteins at epithelial tight junctions of the skin and pronephric tubules/ducts.

Since the main focus of this study was to investigate the role of zCAR in kidney development and function, we examined the expression pattern of zCAR in the pronephros in more detail. zCAR was co-expressed with ZO-1 in a sub-population of cells in the glomerulus. We also observed that zCAR co-localizes with the tight junctional proteins ZO-1 and cingulin in epithelial cells of pronephric tubules/ducts. In agreement with the restricted expression pattern of CAR in impermeable renal tubular segments in the mouse (Paper II), we observed that zCAR was partially co-expressed with the Na^+/K^+ATPase alpha1 subunit, a protein that is expressed along the entire pronephric tubules and ducts. This suggests that the restricted zCAR expression in the pronephros may correspond to the tightness of the epithelial tubules.

In order to determine the biological role of CAR in kidney development and function, we developed a zCAR specific morpholino knockdown strategy. Morpholinos are antisense oligonucleotides that are stable, nuclease-resistant and display low toxicity. They are highly target-specific due to their small size (25-mer). Morpholinos can be designed either to bind to splice donor/acceptor sites and inhibit RNA splicing, or to block translation through binding directly to mRNA (Corey and Abrams, 2001; Heasman, 2002). We designed two translation blocking morpholinos specific for the *zCAR* gene. While control morphants developed normally at all time points, the *zCAR* morphants developed several phenotypes, such as pericardial and body edema, and renal cysts. These developmental defects became progressively more severe until 96

hpf, at which time the embryos displayed significant slower heartbeat and several embryos died.

Finally, we also histologically examined the consequences of zCAR knockdown during zebrafish kidney development by comparing sections of the *zCAR* morphants with un-injected and control larvae. The pronephric glomeruli of the controls displayed densely packed podocytes covering all parts of the glomerulus basement membrane (GBM) and a small Bowman's space filled with glomerular filtrate. In the *zCAR* morphants, however, we frequently observed naked GBM suggesting a loss of GBM/podocyte contact and detachment of podocytes from the GBM. The *zCAR* morphants also displayed enlarged Bowman's space with accumulation of glomerular filtrate, a phenotype, which is probably related to dysfunctional glomerular filtration. Interestingly, naked GBM is not present in morphants that lack podocyte specific proteins, such as Nephrin and Podocin, two proteins that are crucial for the development of podocyte slit diaphragms (Kramer-Zucker et al., 2005). Taken together these data, suggest a novel role of zCAR during zebrafish kidney development and function.

Except for the pronephric glomerular phenotype, we could not detect any other gross developmental defects in the *zCAR* morphants. Although zCAR is abundantly expressed in the epithelium of the gut and the pronephric tubules/ducts, no alteration either in polarization or organization of epithelial cells was seen. Neither could we observe any gross morphological alteration of the cardiac development in the *zCAR* morphants. This is in contrast to recently published reports of the phenotypic findings in CAR deficient mice. Knockout of the *CAR* gene generated mice with defects in the differentiation of the cardiomyocytes. These null-mice died due to cardiac failure between E11.5-E13.5 of embryonic development (Asher et al., 2005; Chen et al., 2006; Dorner et al., 2005). We cannot rule out that there are alterations in the cardiogenesis of the *zCAR* morphants, resulting in the pericardial edema and the altered heartbeats. However, we speculated that these cardiac phenotypes might be caused by edema due to kidney failure.

In summary, we show that zCAR is expressed predominantly in epithelial cells in the developing and adult zebrafish. The sub-cellular localization of zCAR was detected at the tight junctions where the protein co-localized with ZO-1 and cingulin. Interestingly, we could also show for the first time that zCAR is crucial for proper kidney development and function in the zebrafish.

3.4 CLMP, a novel member of the CTX family and a new component of epithelial tight junctions (Paper IV)

New members of the CTX-subfamily have been identified recently. Several of these proteins are cell-cell adhesion molecules that localizes to intercellular

junctions in epithelial and endothelial cells (see sections 1.2 and 1.3). In order to understand the complexity and physiological function of this group of proteins it is important to identify all members of this family.

To identify new members of the CTX-subfamily we searched human and mouse EST and genomic databases using the most conserved regions within the J segment and the C2 domain of the previously characterized CTX-members as search strains. We identified a sequence that was 31% identical to CAR, and was therefore named CAR-like membrane protein (CLMP). Based on a phylogenetic analysis of the evolution of the CTX-members, we proposed that CLMP together with CAR, ESAM, and BT-IgSF form a sub-group within the CTX-subfamily. The protein structure of CLMP harbours all hallmarks of being a member of the CTX-subfamily, *e. g.* an extracellular domain with conserved cysteine residues forming the V and C2 domains, one transmembrane region and an intracellular tail. We also identified two potential N-linked glycosylation sites in the ectodomain.

We next set out to analyze the expression pattern of CLMP in cell lines and tissues. *CLMP* transcripts were detected in several organs such as the lung, spleen, kidney and cardiac and skeletal muscle. In addition, human *CLMP* transcripts were also detected in the intestine and placenta. We also detected *CLMP* mRNA by RT-PCR in several human glioma cell lines. Interestingly, *CLMP* mRNA of a different size was detected in the mouse testis, suggesting the presence of an alternative splice variant of CLMP in this tissue. To be able to analyze the distribution of CLMP in cells and tissues, we generated two peptide antibodies directed against either the N-terminal (NP1 antibodies) or C-terminal (CP1 antibodies) domain of the human CLMP protein. These antibodies were characterized for their specificity against CLMP by immunoblot analysis of exogenously expressed human or mouse CLMP in cell cultures. Both NP1 and CP1 antibodies immunoreacted with human and mouse CLMP, both seen to migrate with a size of approximately 44 to 48 kDa under reduced conditions on SDS-PAGE. We further analyzed the specificity of the CP1 antibodies against endogenous CLMP in several human cell lines of epithelial and glioma origin by immunoblot analysis. We could detect CLMP in normal lung epithelial cells (BEAS-2B), in colon carcinoma cells (Caco-2), and in glioma cell cultures. Since several members of the CTX-subfamily localize at intracellular junctions, and more specifically to tight junctions (see sections 1.2.3 and 1.3), we were interested to determine the sub-cellular localization of CLMP in epithelial cells. CLMP was detected at cell-cell contacts of confluent epithelial cells in culture, as seen in CLMP transfected MDCK cells as well as endogenously in BEAS-2B and Caco-2 cells. Furthermore, endogenous CLMP was also detected at early cell-cell contacts in sub-confluent epithelial cells *in vitro*, suggesting that CLMP may have a function in early junction assembly. We also analyzed the sub-cellular

localization of CLMP in epithelial cells *in vitro* by high resolution confocal microscopy. CLMP was observed to co-localize with the tight junctional proteins ZO-1 and occludin, suggesting that CLMP is a novel component of epithelial tight junctions. Indeed, a more apical localization of CLMP compared to the adherens junctional protein E-cadherin, further supported its tight junctional localization. Immunohistochemical analysis of CLMP distribution in several adult mouse tissues, showed expression of CLMP in epithelial cells that line the trachea, respiratory bronchioli, subluminal glands and colon. Furthermore, CLMP was detected in skeletal muscle. These *in vivo* data corresponded well with the mouse *CLMP* mRNA expression data.

The structure of the extracellular domain of CLMP and the specific expression of the protein at cell-cell contacts indicated that CLMP might act as a cell-adhesion molecule. Indeed, CLMP overexpression in chinese hamster ovary cells (CHO) cells, a cell line that poorly forms aggregates when grown in suspension, resulted in the formation of large cell-cell aggregates. The adhesive properties of CLMP and its specific tight junctional localization suggested that CLMP could be involved in regulation of epithelial permeability. Experiments showing that CLMP transfected MDCK displayed a higher TER when compared to untransfected cells, further supported this theory.

Taken together these results show that CLMP is a new member of the CTX-subfamily, which is predominantly expressed in epithelial tissues. The expression of CLMP at early cell-cell contacts in sub-confluent cells and at tight junctions in fully polarized epithelial cells. This suggests a potential role of CLMP in the assembly of tight junctions. We also show that CLMP is a cell adhesion molecule, which regulates epithelial permeability. Further characterization of the expression and biological function of CLMP will be needed to further reveal its role in epithelial tight junctions in the context of the other CTX-subfamily members and tight junction components.

Noteworthy to mention is that the rat homologue of CLMP (OL-16) was identified by Makino an co-workers as a gene up-regulated and exclusively expressed in the visceral adipose tissue of the type 2 diabetes obese Otsuka Long-Evans Tokushima fatty rats when compared to their diabetes-resistant counterpart (Hida et al., 2000). OL-16 was renamed to adipocyte adhesion molecule (ACAM), and proposed to be a new member of the CTX-subfamily. Human and rodent *ACAM* mRNA was detected mainly in white adipose tissue, although transcripts were detected also in heart and brain. Furthermore, is was shown that *ACAM* mRNA expression is up-regulated during the progression of obesity and that the protein mediates homophilic cell-cell interaction (Eguchi et al., 2005).

4 Future perspectives

Some interesting questions have emerged from this thesis work referring to the biological role of CAR and CLMP in comparison to other members of the CTX-subfamily and to classical tight junction components, such as occludin and claudins.

Most CTX-like proteins localize to tight junctions in epithelial and endothelial cells, and have therefore been suggested to be important in regulating permeability. To understand the biological function of the CTX-subfamily as a group of proteins, it is crucial to identify all members of this family. We have identified CLMP as a novel protein of epithelial tight junctions, which mediates cell adhesion and regulates epithelial permeability. To get new insights into the biological role of CLMP, it will be necessary to further analyze the properties of this protein. It will be important to gain knowledge of the regulation of CLMP, its potential role in junction assembly and epithelial polarization, as well as identifying possible interacting proteins.

It will also be interesting to further investigate the sub-cellular localization of the CTX-members and other tight junction components at junction assembly and in mature tight junctions. By elucidating whether these proteins are organized in a specific order or if they mix randomly within, or in proximity to tight junctions, it may be possible to learn more about their physiological role in junction assembly and in regulating permeability of the epithelium and endothelium. In this context it will also be important to further examine the role of the different CAR isoforms in the abovementioned processes. It will be interesting to find out if alternative isoforms have different biological functions, if they interact with similar PDZ-domain containing proteins and if they have the capacity, in a redundancy way, to replace the function of one another.

We found that the CAR is crucial for kidney development and function in the zebrafish, as *zCAR* morphants display phenotypes associated with glomerular dysfunction. Further investigation of the *zCAR* morphants will provide new insights on the role of CAR in podocyte maturation and in the filtering function of the glomerulus. To study the role of CAR during kidney development in mice, one would have to create a conditional knockout, in which CAR specifically is deleted in podocytes, since the direct knockout of CAR results in lethality due to heart failure, which may mask any kidney abnormalities that could present themselves at later time points during development.

Finally, an interesting aspect of the CTX-subfamily members is the accumulating amount of evidence showing that these proteins seem to be involved in leukocyte transmigration across epithelial and endothelial cells in

inflammation (Mandell and Parkos, 2005; Mullin et al., 2005; Wegmann et al., 2006; Zen et al., 2005). (Mandell and Parkos, 2005; Mullin et al., 2005; Wegmann et al., 2006; Zen et al., 2005). The whole process by which cell adhesion molecules mediate leukocyte trafficking across the endothelium, through interstitial tissues and across the epithelium, is not entirely known (Huber et al., 1998; Muller, 2003). It will be important to find out what function the CTX-members have in inflammatory processes in the context of mediating leukocyte transmigration, but also in preserving the tight junction barrier when leukocytes pass through the paracellular pathway.

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Present and former members of the Cell and Biology group

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Inger, what a cool lady you are! I admire your personality and your openness. Thank you for taking care of the most impossible things at work in no time, and for all parties, Lucia-fika and other social events that you have taken care of during all these years!

I would also like to thank **Barbara**, for caring for me all these years! **Per,** you have always being interested in my work, I appreciate that a lot. And how fun we had at Ludwig's parties! OK, THE party is left, are you coming!?! **Ulf**, a special thank to you for always being there for me!

Being a Ph.D. student at Ludwig is combined with a lot of work, but also with even more fun! People that think that scientists are deadly boring people that don't know how to be social are SO wrong! I have been to so many parties, after work get together, conferences and other social events where I had the time of my life! I would first of all like to thank my "Old-Ludwig"-party friends, **Alex, Birgitta O, Diogo, Erika F, Fredrik, Hanna, Jonas, Kristian T** and **Monika M**, for the wildest "after"-parties I have ever been to! And to the new generation of party-Ludwigians, **Ana Sofia, Annelie, Annica, Banafsheh, Carolina, Charlotte, Christian, Claes, Daniel, Jhansi, Joakim, Johan, Kristian P, Hong, Linda, Magdalena, Magnus, Maria, Martin, Mirta, Monika E, Nik, Stina, Thomas and Xun,** I would like to say; Keep having fun! A special thank to Muminbruttan **Carolina**! Although your electrifying Finish personality and my crazy Croatian temper didn't always work well together in sweet harmony, I still like you so much (although I would like you even more if you would come to my "spikning" and my dissertation-party)! I wish you all the best in the future, my friend, you are going to be THE best Ph. D. student ever!

I have always been a pure and totally loyal Ludwigian until last year, when I became a part of Matrix at MBB. It has been a great opportunity for me to work at Matrix and I would like to thank **Karl** for welcoming me as a guest-researcher. You have a great lab, both in turn of all your nice colleagues but also scientifically! I would also like to thank **Arindam** for introducing me into the fascinating world of zebrafish!

My deepest gratitude to the members of Matrix for being so nice to me and for all their help. **Lwaki**, my warrior, you are the wisest man I have ever met. Some of the things you have told me, I will carry with me in my heart for the rest of my life. I admire your calmness and your determination in science. I am happy to have you as a colleague and friend! **Mataleena**, OK baby, its soon time for you to give me back THE Ph.D.-chair! You are my "Croatian sister", a great colleague and party-friend! I hope that we will stay friends even when you and your lovely family, Lyydia and Samuli, move back to Finland. **CalleCalle**, it is so sad that you went from the fabulous academic world at Matrix to the industrial world of Astra. Please, come back! I think you are a great scientist and I am sure that I would enjoy working with you! I miss you and your teasing humour at work every day. Lets meet soon again and have some fun! **Susan** and **Sajila**, you are doing a fantastic job with the zebrafish-facility! I am grateful for your help whenever I needed your expertise. I wish you all luck in the future with the fish-work. **Anne-May** and **Bing**, what a chock it must have been for you when three Fishy-people (Calle, Mataleena and I) moved in to your office all at once! Thank you for your patience, interesting conversations, yoga-lessons and green tea that you provided us with every day! Good luck with your GIGANTIC project! I though that only Ludwig hade crazy party-people, until I came to Matrix! **Asmundur, Eyrun, Jaakko, Juha, Lotta, Mark, Marko, Massa, Maya, Robert, Stefania** and **Yukino**, what good party-friends you are! I hope that we will have a lot of fun together in the future! **Massa**, you are the coolest Japanese man I have ever met! I admire you for being so honest and straightforward! Good luck with work and the secret stuff!

In the grey-zone between work and private life is **Erika,** the most colourful person I have ever met. You are my best friend and I love you so much! You are an amazing woman, a great scientists and a perfect mother! I admire you for these things, and for a million other reasons! Without your concern and caring when my life was a total mess, I would not have gone through it as smoothly and quickly as I did. For that, I am forever grateful. I would also like to express my deepest gratitude to your wonderful husband **Thomas**, for inviting me to be a part of your life!

I also want to thank all my friends for being so patience during this last hectic time at Ludwig, I am sorry that I haven't had time to meet you for so long time. I promise you that it will all change now when I defend my thesis! **Maria** and **Billy**. Thank you for inviting me and Neno to be a part of your wonderful Greek family! And for all of you who doesn't have Greek friends, YES! its just like in the movie "My big fat Greek wedding"! The days we had together at Ko Pha-Ngan were magic! **Carin, Magnus, Amalia, Camilo, Mara** and **Hicham**, I am lucky that I had time to travel during my time at Ludwig, otherwise I would never had met you! Thank you for all nice moments that I have spent with you here at "home". My old friends **Maria**, **Patrik**, **Viktoria** and **Johan**, I miss you so much. **Katarina** and **Lalle**. How lucky I am that you moved back to Huddinge. I never laugh so much as when I am with you two! My dear almost-brother, **Widar**, thank you for always taking care of my body when it decides to go into a strange state of cramp. You are a very good friend! **Wiggo**, you are always so kind to me. Thank you Pappos! And then last but not least, I would like to thank **Heidi** and **Martin** for being such good friends and for all happy and totally relaxing weekends that I have spent at your home in Kalmar! How lucky I am to know you "sjöbusar", otherwise I would never experience and see the Swedish archipelagos as I have done with you two!

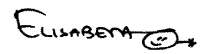
And now to my family, or "THE Croatian mafia" as most people like to call us!

Silvia "Bubico", you are one of my dearest friends. I love you and I am so pride of what you have accomplished in your life. Your crazy humour and e-mails during my time at Ludwig have many times saved me from going nuts here at work. I also want to thank **Diana**, **Ella** and **Darko** for always caring for me and Nenad when we visit you in Croatia. Puno hvala **Dragica** i **Tomo** da sam uvjek dobro dosla kvama, i za sve sta ste za mene napravili u roku ovih godina sto se poznamo. Puno vas volim!

Världens bästa **Mamma** och **Pappa**, tack för att ni älskar mig och att ni alltid har funnits vid min sida och stöttat mig. Tata, sada sam konacno zavrsila skolu! I imas podpuno pravo: u radu je spas! Jag älskar er så otrolig mycket. **Annelie** och min bror **Robert**, tack att ni alltid bryr er om mig och Nenad, och att vi alltid är välkomna hem till er! Jag önskar att vi kunde ses oftare än vad vi gör. Jag älskar er båda.

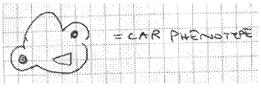
Julia, **Martin** och **Thor**. Jag har älskat er från stunden ni blev till och jag kommer att älska er i all evighet. Fastän ni inte är "mina" så känns det som om ni vore det. Stunderna som jag tillbringar med er är fyllda av total lycka, och ett lugn i själen som jag annars sällan upplever. Tack att ni finns!

Nenad, jag älskar dig av hela mitt hjärta! Du är den mest fantastiska människa jag någonsin har mött. Tack att du alltid finns här för mig, att du älskar mig som jag är, och att du alltid stöttar och tar hand om mig. Tack att du fyller mitt liv med glädje, kärlek och en massa popcorn!



6 Sweet memories

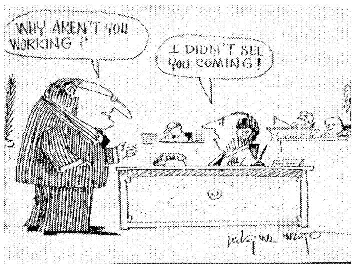
This part is for me, for remembering the good things that made this work worth doing, that made me happy, and that made me laugh! And above all, that me feel loved!



- **In or out.** Your data are good, the P.I. thinks you are good. The data are nonexistent, the P.I. thinks you are nothing. Have a thick skin, and don't rely on results as the sole basis for your feeling of self-worth.

PUNO falite, naravno najvise H&M, pa Ikea, pa Mango, pa Fab5, e ma cekaj sta sam ono jos zaboravila spomenut, a ma da TI- pa naravno Elli Caramelli! /Silvia

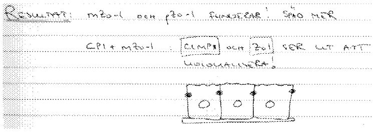
Elisabeth/Etienne
Snnnääääääääääääääääääällllllla, du!
Kan du inte gå ut och äta lite mat med mig???!!!
Jag lovar, det kommer att vara 1000x roligare än att springa.
Yyyyeeeeeeeeezzzzzzzzzzzzzz, please. Ciao baby/Elisabeth
noooooo
PLEASE Etienne!
nooooooooooooooooooo
Please, please, please...
nooooooooooooooooooooooooooooooooooooooooooooooooooo



Elisabeth/Massa
Massa, You know sometimes when we girls are in love, we just can't be alone in the same room as the guy of interest (uh, I almost wrote "gene of interest".... my brain is to occupied with writing about CAR).
Why, well because....
Oh my dear... You made my day completely happy today!!! Wow, your theories were so...beyond description!!!
....
Thanx a lot for entertaining and encouraging me, but don't forget to focus on writing your thesis!!!
Hahaha!! /Massa

Nenad 060810
...OK, köp detta:
En fördigpublicerad artikel som handlar om zCARs enormt viktiga roll under zebrafiskens njurutveckling och en extremt välskriven och intressant avhandling som handlar om tight junctions, CAR och CTX-familjen.
Sen så kan du även köpa mozarella ost, champinjoner, kyckling....

Elli!
Det är nedsatt pris på avhandlingar på RättPris! Vill du att jag köper två på en gång så blir du doktotdoktor!?! /Nenad 080810



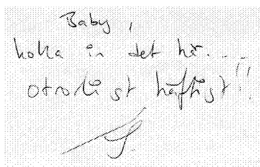
Hej molekylemühle! Lycka till med din poster!/Din Neno

Draga moja mala Elli Carameli,
ma znas i sama, i ja ti uopce ne moram govorit da ces ispast najbolja i najpametnija tamo u NY, i ne bi se uopce cudila da odmah neki trg ili ulicu nazovu po tebi - pa se jednog lijepog dana, za par godina ja i ti nadjemo na trgu Elli Caramelli i NY.
/Silvia

how's the conference? good I'll guess........ let me know how your talk went, I trust that it won't be a problem no sweat baby!! see ya Etienne

So preparing for the big day?? I'm pretty sure that all will go well, I have faith in you (at least this time). Me I just do westerns and westerns and westerns to get some nice blots for one (!!!!!) figure otherwise things are pretty calm over here. Naturally I miss you a lot and things are not the same around here without you...but.... well have fun, good luck and don't eat to much........
Etienne

Hörrdu Fröken ,Fru menar jag, du är ju farlig med en öl i handen! Och så får du cyckla hem nästa gång! Men inte vet jag om jag kan partaja så mycket.. Lördagen blev hård. .men det var det värt ;-) Ses idag antar jag :-) /Calle



TJA ELISABETH!
JAG VAR TVUNGEN ATT TITTA IN EN SNABBIS FÖR ATT FLUKTA PÅ RESULTATEN! SER VÄLDIGT SNYGGT UT!! BRA JOBBAT!
UPPRELLERINGEN AV CAR I TEAR-CELLERNA ÄR JU DRAMATISK!
KANSKE EN LITEN ÖKNING AV CLMP OCKSÅ?
LYCKA TILL MED ALLT. ASSAY IDAG!
JAG HÅLLER TUMMARNA J.

Brian säger of./Susan

Nadi mi tamo (Sri Lanka) nekog deckica,
kada si ga ja neznam sama nac, recimo
nekog sa nekim rudnikom dijamanata....
ili zlata, nisam ti ja izbirljiva.... /Silvia

Tja bruden!
Tusen tack för skjutsen i fredags, det var jätte snällt!
Jag hade jätte jätte kul, nu vet jag att alla vilda historier om dig på Ludwig-festerna är sanna... och troligen lite förmildrade till och med ;-) /Carolina

God morgon! Nä, jag är på rummet också.
Bakis förstås. Ska aldrig dricka mer :-)
Mår ni bra?/Calle

1+4 = OK
Matti

Elisabeth/Erika
... Oh, vad jag saknar våra fm/em fika. ¨
Kan du inte komma hit och besöka mig ibland? Det ska bli härligt att gå ut med dig i em och ta en drink! När ska vi gå? ....

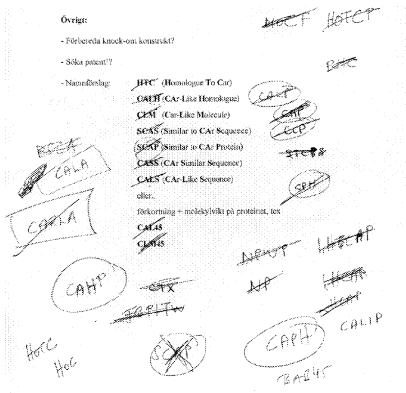
.... Är du hä'r i eftermiddag!!! Då kan vi fika och jag lovar att nästa vecka skall jag komma och fika med dig hos fisk-mannen!!!

Sta se tice tog conference u USA, ja sam se vec polako pocela pakirat – ti njima reci da sam ja zaduzena za slide show, a ako im to nije dovoljno mogu napravit i neki striptease show, iskocit iz neke torte, ili nesto slicno (mislim da bi bilo dosta efektno da iskocim iz neke epruvete, ali cu onda morat malo poradit na liniji!!!). /Silvia

Zao mi je sta tebe tvoje kolege zezaju...to su oni ljubomorni jer si pamtnija, imas bolje ideje, i naravno zgodnija od njih. /Silvia

RESULTAT: SÅÅÅ SNYGGT!
OCH JONAS KOMMER SIMMA NÄR MAN SER ZO-I !!!

Hi, Thanks a lot for the protocol beauty queen. Take good care of yourself as you write your thesis and good luck. /Lwaki



Do tada ce Robbie Williams taman biti muskarac u najboljim godinama, oslobodjen svih poroka i losih navika i takodjer do tada ce zahvaljujuci nekim novim tabletama koje si ti izmislia narasti nekih 20-tak centimetara (meni ce od istih pilula cike narast neka dva tri broja - ne zato sto ja mislim da meni to treba, nego jako cijenim i volim mamu i zelim biti sto vise nalik na nju; uglavnom tu cemo se ja i Robbie ozenit i blaa,blaa,bla... /Silvia

Elisabeth/Etienne
Hello darling!
Hello how's shrilanka???? sun and fun???
Do you miss me???
Yes sure...
I miss you!!!
I have that effect on women!!
So much...
Yes that's understandable
How is the coffee when I'm not around???
Finished.... now nobody is making me a good cupper anymore.
Let us go out an drink some beer when I come back!
Ooooooooohhh yeahhhhhh lot's of it,
see ya and regards to your better (yes much better) half

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