

## Chapter 2

# The IgCAMs CAR, BT-IgSF, and CLMP: Structure, Function, and Diseases

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**Abstract** The coxsackie-adenovirus receptor (CAR) is the prototype of a small subfamily of IgCAMs composed of CAR itself, CLMP, BT-IgSF, ESAM, CTX, and A33. These six proteins are composed of one V-set and one C2-set Ig domains and a single transmembrane helix followed by a cytoplasmic stretch. They are localized in several tissues and organs and - except for ESAM, CTX, and A33 - are expressed in the developing brain. CAR becomes downregulated at early postnatal stages and is absent from the adult brain. CAR, CLMP, and BT-IgSF mediate homotypic aggregation. Interestingly, cell adhesion experiments, binding studies, and crystallographic investigations on the extracellular domain reveal a flexible ectodomain for CAR that mediates homophilic and heterophilic binding.

CAR has been extensively investigated in the context of gene therapy and diseases, while research on BT-IgSF and CLMP is at an early stage. Several mouse models as well as studies on patient tissues revealed an essential role for CAR in (1) the development of cardiac, renal, lymphatic, and intestinal tissue; (2) muscle pathology, remodeling, and regeneration; (3) tumor genesis/suppression and metastatic progression; and (4) in virus-mediated infections and gene therapy. Although the in vivo function of CAR in the brain has not been solved its developmentally regulated expression pattern in the brain as well as its function as CAM suggests that CAR might be implicated in neuronal network formation.

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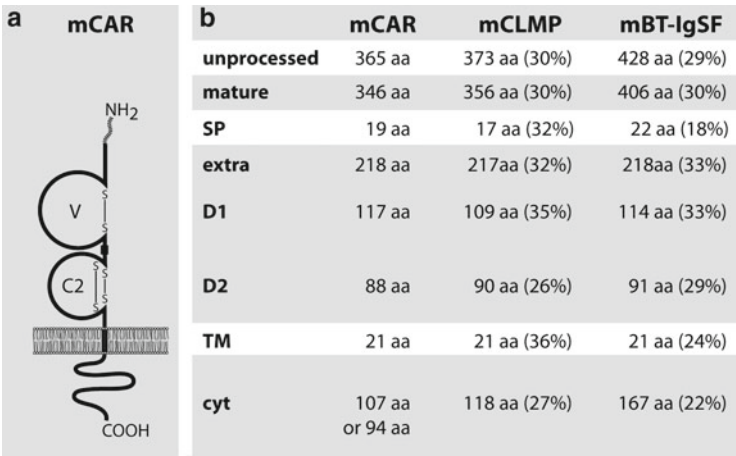
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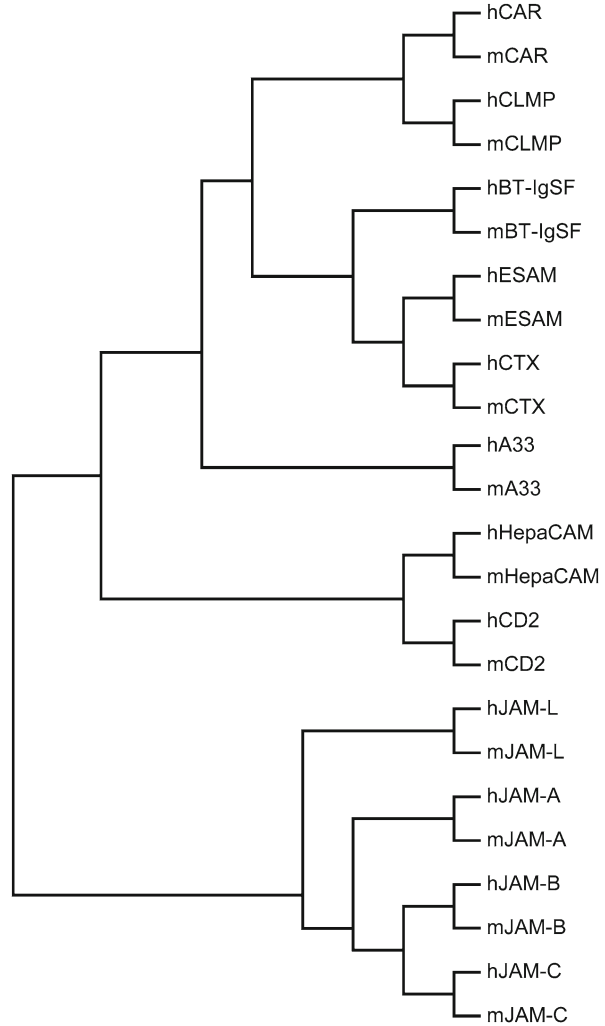
2.1 The CAR Subgroup of IgCAMs

The coxsackievirus-adenovirus receptor (CAR) was initially identified as a receptor protein for group B coxsackieviruses (CVB) and adenoviruses of the groups A, C, D, E, and F (Bergelson et al. 1997; Tomko et al. 1997; Freimuth et al. 2008; Coyne and Bergelson 2005). It is a prototype of a small structural subgroup of transmembrane proteins of the Ig superfamily in vertebrates which was initially founded by the thymocyte protein CTX (Chretien et al. 1996, 1998) and by the small bowel protein A33 antigen (Heath et al. 1997). Further screens testing endothelial adhesion resulted in the identification of a type I transmembrane protein structurally related to CAR, termed ESAM (endothelial cell-selective adhesion molecule), and whose expression is limited to endothelial cells and platelets but is not found on neural cells (Hirata et al. 2001; Nasdala et al. 2002). Bioinformatics searches in databases then resulted in the cloning of BT-IgSF which is highly expressed in brain and testis (therefore named brain- and testis-specific immunoglobulin superfamily, also termed Igsf11) and CLMP (CAR-like membrane protein) (Suzu et al. 2002; Raschperger et al. 2004; Katoh and Katoh 2003). These six IgSF members are highly related in their overall domain organization and in their primary sequence including the combination of a membrane-distal V-type and a C2-type domain, a short junction between both domains, and an extra pair of cysteines in the C2 domain (Fig. 2.1). The junctional adhesion molecules (JAM) whose extracellular



**Fig. 2.1** (a) Schematic representation of CAR and (b) its relationship to CLMP and BT-IgSF. (a) The signal peptide is shaded; Ig domains are shown as loops. The junction between the Ig domains is indicated as a small box. SP, signal peptide; extra, extracellular domain containing Ig domains D1 and D2; TM, transmembrane segment; cyt, cytoplasmic tail. (b) Amino acid identities to CAR are given in percentages. Mouse sequences were used for comparison. ESAM and CTX reveal 29 %, A33 25 %, JAM-A 20 %, JAM-B 21 %, JAM-C 20 %, and JAML 18 % identity to CAR aa, amino acid residues. mCAR, murine Coxsackievirus adenovirus receptor; mCLMP, murine CAR-like membrane protein; mBT-IgSF, murine brain- and testis-specific IgSF member

**Fig. 2.2** The relationship of the CAR and related proteins is shown. Proteins are grouped according to amino acid sequence similarity. Mouse (m) and human (h) sequences were evaluated. Human and murine sequences of IgSF members were aligned using the Gonnet 250 matrix in ClustalW 2.0.12. Gap-rich columns in the multiple sequence alignment were deleted using Jalview 2.7 program and the edited alignment was analyzed in Protdist 3.67 by the Jones–Taylor–Thornton matrix in order to calculate pairwise distances between the sequences. Bootstrapping was performed to test the reliability of the distance matrix. A consensus tree was computed by Neighbor-Joining 3.67 method by means of bootstrapping and the phylogenetic tree was visualized using TreeView 1.6.6



regions are also composed of two Ig domains, in some cases with two V-type domains instead of the V- and C2-type combination, appear less related to CAR (Weber et al. 2007) (Fig. 2.2).

In this review we discuss primarily CAR which has been extensively investigated in the context of adenoviral gene therapy and heart development. Recent findings on the implication of BT-IgSF and CLMP in diseases are included in our overview of this field while ESAM, CTX, and A33 which are not expressed in the nervous system will not be considered further.

Orthologs of human and mouse CAR have been described in a variety of species including bovine, pig, rat, dog (Fechner et al. 1999; Thoelen et al. 2001a), chick (Patzke et al. 2010), and zebrafish (Petrella et al. 2002). High amino acid sequence

identity is observed between mouse and human CAR with 91 % identity in their extracellular domains and 77 % identity within the transmembrane segment and 95 % identity within their cytoplasmic stretch (Wang and Bergelson 1999).

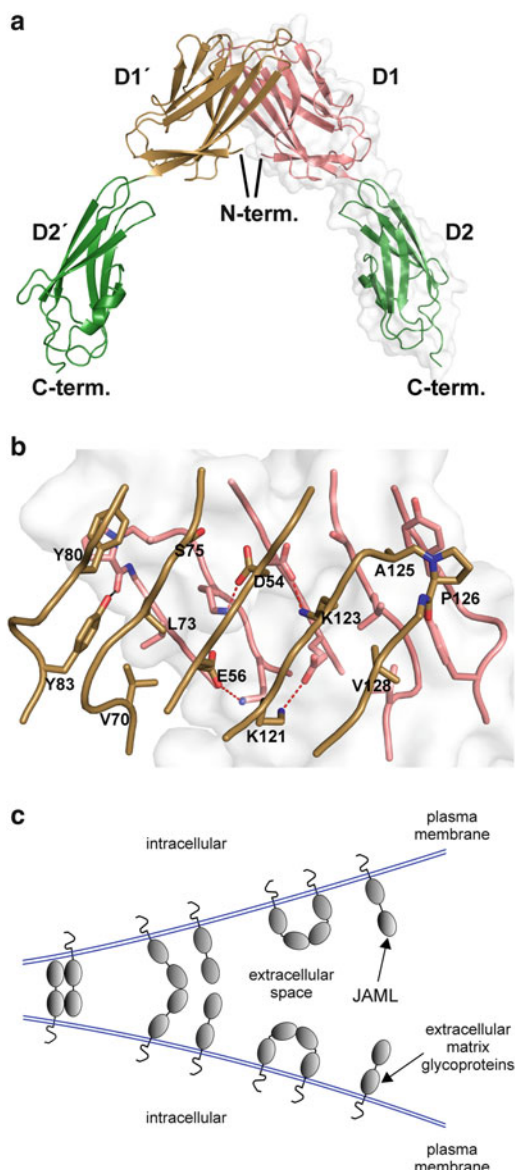
The human *CXADR* gene was initially localized to chromosome 21q11.2 by fluorescence in situ hybridisation analysis (Bowles et al. 1999) and later corrected to 21q21.1 after sequencing of the long arm of chromosome 21 (Hattori et al. 2000). The *CXADR* gene was thought to comprise seven exons, but recent findings revealed the existence of an eighth exon (Excoffon et al. 2010) as found for murine *Cxadr* on chromosome 16 (Chen et al. 2003). Both human and murine genes are transcribed into 6 kb pre-mRNA molecules that are further processed into 2.4 kb and 1.4 kb transcripts, respectively, containing open reading frames of similar size. However, an additional 3' untranslated region of 1.2 kb is found in the human transcript (Tomko et al. 1997). The human gene encoding BT-IgSF is located on chromosome 3q13.32 while its murine ortholog is found on chromosome 16B4. Two isoforms have been described for BT-IgSF generated by alternative splicing (Katoh and Katoh 2003). The gene of human CLMP was identified on chromosome 11q24.1 and is composed of seven exons. A similar genomic organization was found for the murine gene of CLMP which is located on chromosome 9A5.1 (Raschperger et al. 2004).

## 2.2 Structural Features of the Extracellular Region of CAR

After cleavage of the signal peptide, the mature CAR exhibits a 218 amino acid extracellular domain, which represents about two-third of the protein and comprises two Ig-like domains: one membrane-distal V-type Ig domain and one membrane-proximal C2-type Ig domain (termed D1 and D2 in the following). These two domains are separated by a short junction and are followed by a linker of five, a transmembrane segment of 21, and a cytoplasmic tail of either 107 or 94 amino acid residues, respectively. Differential splicing of the CAR encoding pre-mRNA leads to two isoforms which are identical except for the extreme C-terminus, which contain class I PDZ-binding motifs ending either in amino acid residues SIV or TVV (also termed form 1 and 2 of CAR, respectively) (Excoffon et al. 2004). Isoform 2 is 13 residues shorter than mCAR isoform 1 (Chen et al. 2003; Excoffon et al. 2010). Further splice variants lacking the transmembrane segment have been described (termed CAR2/7, CAR3/7, and CAR4/7) (Dorner et al. 2004; Thoelen et al. 2001b; Dietel et al. 2011); however, their existence at the protein level has not been confirmed.

In crystallographic investigations the full extracellular region of CAR revealed a U-shaped homodimer through the binding of their N-terminal domains which is reminiscent of JAM-A homodimers (Fig. 2.3a) (Kostrewa et al. 2001; Protá et al. 2003; Patzke et al. 2010). The D1–D1-binding interface of CAR has a size of 684 Å<sup>2</sup> and is formed by side chains in  $\beta$ -strands GFCC' and C'' as well as the FG-connecting loop. Four salt bridges and two hydrogen bonds as well as two hydrophobic interactions are implicated in binding (Fig. 2.3b). Similar observations on the D1 domain were made for the complex of D1 with the fiber knob of the adenovirus and for the homodimer

**Fig. 2.3** (a) Crystal structure of the extracellular region of CAR reveals a U-shaped dimer which is stabilized by binding of their N-terminal D1 domains. D1 domains are colored in either *red* or *brown* and D2 in *green*. *C-term* C-terminus, *N-term* N-terminus. (b) Detailed view of interactions inside the dimer interface shown in (a). Amino acid residues are given in the *single letter code* and *numbers* indicate their positions. (c) Scheme of putative molecular interactions of CAR on the neural plasma membrane. The D1–D1 association observed by the U-shaped crystal structure most likely occurs between two CAR polypeptides associated with the same plasma membrane. Adhesion and binding experiments suggest that homophilic interactions of two CAR polypeptides between neighboring cells might result from an antiparallel D1–D2 binding. Heterophilic bindings of the D2 domain of CAR to ECM glycoproteins or of D1 to JAML are indicated by *arrows* [adopted from (Patzke et al. 2010)]



of the single D1 domain (Bewley et al. 1999; Seiradake et al. 2006; van Raaij et al. 2000). The D2 domain belongs to the C2 type of Ig domains whose two  $\beta$ -sheets are derived from  $\beta$ -strands ABE and CFG, respectively. Two disulfide bonds link the two sheets together, connecting strand A to G and strand B to F. D1 and D2 associate in a head-to-tail manner in the CAR polypeptide and form a rod-like, dumbbell-shaped structure whose protrusions are formed by the globular Ig domains. The two domains are separated by a junction and a linker segment of five residues tethers the

extracellular Ig domains of CAR to the cell membrane. Both parts, the junction and the linker, might provide some degree of flexibility to the polypeptide which might influence the interactions of CAR with other proteins (Patzke et al. 2010).

Structural information as well as detailed binding data using extracellular domains is not available for BT-IgSF and CLMP.

## 2.3 Molecular Interactions of CAR Subgroup Members

### 2.3.1 *The Extracellular Domain of CAR Is Implicated in Homophilic and Heterophilic Binding*

CAR functions primarily as a homophilic adhesion molecule as demonstrated by aggregation studies using transfected cells (Honda et al. 2000; Patzke et al. 2010). Consistently, the fiber knob of the adenovirus which binds to the N-terminal Ig domain of CAR disrupts the formation of cell–cell contacts (Walters et al. 2002; Patzke et al. 2010). Furthermore, in non-polarized cells and in neurons CAR is diffusely localized over the entire cell surface but accumulates at cell–cell contact sites. Crystallographic studies on the single D1 and the complete extracellular region of CAR suggested that CAR homophilic binding is mediated by interactions of D1 (van Raaij et al. 2000; Verdino et al. 2010; Patzke et al. 2010). However, a detailed analysis of binding activities of single extracellular domains of CAR, combined with adhesion assays, indicated that homophilic interactions of CAR are also mediated by D1–D2 binding (Patzke et al. 2010).

CAR is also implicated in heterophilic interactions with extracellular matrix proteins fibronectin, tenascin-R, and agrin, which appear to be mediated by D2 of CAR (Patzke et al. 2010). In keratinocytes CAR was found to interact with JAML on  $\gamma\delta$  T cells in the skin to induce co-stimulation, cytokine production, and activation of the MAP kinase pathway via recruitment of the phosphoinositide-3-kinase (PI3K) to a JAML intracellular sequence motif. The homophilic binding interface of D1 of CAR overlaps with the structure required for the heterophilic binding to JAML which is mainly based on GFCC'C"-sheets packed face to face, and only the A-strand of CAR interacts with the JAML CC' loop. In contrast to CAR the two V-set domains of JAML associate into a compact assembly, making the extracellular region of JAML more rigid (Verdino et al. 2010). The binding between CAR and JAML is essential for the transmigration of neutrophils across tight junctions (Verdino et al. 2010; Witherden et al. 2010; Zen et al. 2005; Luissint et al. 2008; Guo et al. 2009). In testis CAR interacts with JAM-C (Mirza et al. 2006).

Overall, the binding, structural, and adhesion studies using CAR extracellular domains predict a flexible ectodomain of CAR allowing a conformational shift resulting in either D1–D1 or D1–D2 homophilic binding. A flexible ectodomain has also been observed for CEACAM1 (Klaile et al. 2009) and which might apply to other IgCAMs as well (Volkmer et al. 2013). On the basis of the binding and crystallographic studies a model of molecular interactions of CAR has been

proposed in which CAR self-associates on the same cell via D1–D1 as U-shaped dimer and between opposing cells via antiparallel D1–D2 binding (Fig. 2.3c) (Patzke et al. 2010).

BT-IgSF as well as CLMP—similar to CAR—also mediate homotypic aggregation when expressed in heterologous cells, and expression of CLMP increased the transepithelial resistance in MDCK cells, suggesting that CLMP and BT-IgSF might have related functions to CAR (Harada et al. 2005; Eom et al. 2012; Raschperger et al. 2004).

### 2.3.2 *Molecular Interactions of the Cytoplasmic Segment of CAR*

The two forms of CAR with a cytoplasmic segment differ only in their last 26 or 13 amino acid residues due to alternative splicing. Both isoforms of CAR as well as CLMP and BT-IgSF encode class I PDZ-binding domain sequences. Consequently, several intracellular PDZ domain containing proteins were identified to interact with CAR, suggesting that CAR is part of larger protein complexes. These intracellular binding proteins include ZO-1 (zona occludens 1), MUPP-1 (multi-PDZ domain protein-1), MAGI-1b (membrane-associated guanylate kinase, WW, and PDZ domain containing 1b), and PICK-1 (protein interacting with C kinase 1) and the synaptic scaffolding protein PSD-95 (postsynaptic density protein 95) (Cohen et al. 2001; Raschperger et al. 2006; Coyne et al. 2004; Excoffon et al. 2004; Excoffon et al. 2010). Using a yeast two-hybrid screen, LNX (Ligand-of-Numb protein-X) was found to bind via its second PDZ domain to the cytoplasmic part of CAR. Interestingly, the PDZ-binding segment of CAR was not sufficient for LNX binding, but required a C-terminal upstream sequence (Sollerbrant et al. 2003). Furthermore, LNX2 (Ligand-of-Numb protein-X2) was also discovered as interaction partner by the yeast two-hybrid technique and co-immunoprecipitation. On the basis of truncation and pull-down analyses, the CAR–LNX2 interaction is mediated by the second LNX2 PDZ domain and the CAR PDZ-binding motif, but additional CAR residues 330–339 were also needed for interaction, which is similar to CAR–LNX binding (Mirza et al. 2005). BT-IgSF and CLMP also contain at their C-terminus PDZ-binding motifs and might therefore also interact with several PDZ containing proteins. For example, CLMP was found to associate with ZO-1 on Caco-2, MDCK, and the mouse Sertoli TM4 cells (Raschperger et al. 2004; Sze et al. 2008).

## 2.4 CAR Is Ubiquitously Expressed but Dominates During Developmental Stages

CAR has been found in numerous organs and tissues during embryonic development including epithelia, myocardium, and the nervous system. Its expression is tightly regulated during development and becomes downregulated at early postnatal stages, in particular in the brain (Hotta et al. 2003; Patzke et al. 2010). In non-polarized cells



it is diffusely localized over the entire cell surface but accumulates at cell–cell contact sites. In polarized cells CAR has been found to be associated with tight junctions and co-localizes with the tight junction protein ZO-1 (Cohen et al. 2001; Walters et al. 2002; Nagai et al. 2003; Raschperger et al. 2006). In the developing heart CAR is a component of the intercalated disc (Noutsias et al. 2001; Kashimura et al. 2004), a specialized structure composed of gap junctions, desmosomes, and adherens junctions. CAR is diffusely localized on the plasma membrane of myoblasts and becomes downregulated during skeletal muscle maturation, and in the adult muscle, it is restricted to the sarcolemma of the neuromuscular junction (Shaw et al. 2004; Nalbantoglu et al. 1999). In summary, CAR differs from many IgCAMs in that it is primarily expressed during developmental stages. Its expression is strong on cells with high plasticity. Since CAR is absent from the normal adult nervous system and myocardium it appears to be not a structural protein. It serves as a developmental factor which promotes the formation of cell–cell contacts during development. The strong re-expression of CAR in cardiac diseases associated with heart failure may be considered as an embryonic re-expression (see also below).

Only limited information on the localization of BT-IgSF and CLMP is available. At the transcript level CLMP was detected in several tissues including brain and cell lines (Raschperger et al. 2004; Eguchi et al. 2005; Van Der Werf et al. 2012). BT-IgSF mRNA is highly expressed in the brain, testis, intestinal-type gastric cancers, and melanophores and their precursors of zebrafish (Eom et al. 2012; Suzu et al. 2002; Katoh and Katoh 2003).

## 2.5 CAR Members and Diseases

Although CAR is strongly expressed in the developing nervous system (Patzke et al. 2010; Hotta et al. 2003), its *in vivo* role in the brain or in the peripheral nervous system is currently unknown. Due to the very early embryonic lethality of the constitutive CAR knockout (Dorner et al. 2005; Asher et al. 2005; Chen et al. 2006) so far only investigations on immature cultivated CAR-deficient neurons were possible. *In vitro* experiments revealed that CAR functions in the nervous system as adhesion molecule and might be implicated in the formation of neuronal circuits through homophilic and heterophilic interactions (Patzke et al. 2010). Due to the lack of *in vivo* studies on CAR, BT-IgSF, and CLMP in the developing brain, we concentrate in the following sections of this article on their functions in nonneural tissues and diseases.

### 2.5.1 *The Interactions of CAR with Adeno- and Coxsackieviruses*

The six coxsackie B virus serotypes (CVB1–CVB6) and adenoviruses from subgroups A, C, D, E, and F (but not B) share CAR as a viral receptor; the role of CAR in these viral infections, however, is different. For CVBs, CAR functions for



both attachment and viral infection (Martino et al. 2000), while for adenoviruses the major function of CAR is to mediate initial attachment of the virus to the cell surface. Subsequent entry of the adenovirus into cells is mediated by the penton base protein of the virus and by  $\alpha_v$  integrin receptors on the cell surface (Wickham et al. 1993; Bai et al. 1993). Because these two virus families are unrelated, their binding specificity to CAR must have evolved independently. The CAR-binding sites on CVB are located in deep crevices or canyons on the capsid surface (Muckelbauer et al. 1995). By contrast, the structures of the adenovirus binding to CAR are surface loops and thus are exposed to immunoselective pressure. Interestingly, the JAML and the homophilic D1–D1-binding site on CAR overlap with the binding site required for the interaction with both viruses (Bewley et al. 1999; Roelvink et al. 1999; van Raaij et al. 2000; Patzke et al. 2010; Verdino et al. 2010). This overlap accounts for the competition of these viruses (and CAR itself) on the cell plasma membrane.

The adenoviruses bind to the N-terminal Ig domain of CAR, D1, by means of trimeric fibers emanating from the vertices of their icosahedral capsid, which terminate in a globular knob domain, termed the fiber knob. The D1 domain of CAR is sufficient for binding of the virus (Bewley et al. 1999; Roelvink et al. 1999). Structural studies indicate that up to three CAR D1 monomers are bound per fiber knob trimer. The AB loop, part of the DE loop, and a short segment of the F-strand of one fiber knob monomer and in addition the FG loop of the adjacent knob interact with strands CC'C'' and the second half of  $\beta$ -strand F in the D1 domain of CAR (Bewley et al. 1999). The AB loop contributes over 50 % of interfacial protein–protein interactions, including the three hydrogen bonds involving conserved atoms in Ad12, Ad2, Ad5, and Ad9 knob and thus may be the key anchor for the complex (Law and Davidson 2005).

The cryo-electron microscopic reconstruction of CVB3 complexes with CAR showed that the D1 domain of CAR binds within the canyon of CVB3. The interface between the virus and CAR consists of the BC and FG loops of D1 binding to the north rim and the floor of the canyon, as well as the A and G  $\beta$ -strands interacting with the south rim of the canyon (He et al. 2001). CVB3 canyon walls are formed by viral structural proteins VP1, VP2, and VP3, and although contact residues are contributed by all three subunits, VP1 dominates the interaction with CAR D1. Several charged residues line the binding interface and provide complementary interactions with CAR. These residues in the virus–receptor interface are moderately well conserved among the six CVB serotypes (He et al. 2001).

### 2.5.2 CAR as a Viral Receptor

Since CAR functions as receptor for both viruses it has been therefore mainly related to human diseases on the basis of its role as a viral attachment protein. Adenoviruses are non-enveloped DNA viruses, classified into six subgroups (A–F),

which are further divided into more than 50 different serotypes based on their immunological properties (Bailey and Mautner 1994; Law and Davidson 2005; Rux and Burnett 2004). Serotypes A, C, D, E, and F are able to attach to CAR (Tomko et al. 1997; Roelvink et al. 1998; Bergelson et al. 1997). The most common, and therefore the most extensively studied, human adenoviruses are those that belong to group C (Rux and Burnett 2004), which predominantly infects the upper respiratory tract. Group C, together with groups B and E, which infect the lower respiratory tract, causes clinical symptoms ranging from mild pharyngitis to acute respiratory disease (common cold syndrome, pneumonia, croup, bronchitis) (Brandt et al. 1969; Figueiredo 2009). Besides respiratory tract infections adenoviruses are recognized as etiologic agents of the gastrointestinal, heart and eye infections (Kaufman 2011; Skevaki et al. 2011), hemorrhagic cystitis, hepatitis, hemorrhagic colitis, pancreatitis, nephritis, or encephalitis (Hayashi and Hogg 2007; Martin et al. 1994; Waldman et al. 2008; Hofland et al. 2004; Lynch et al. 2011). More than 80 % of adenovirus infections occur in young children (<4 years) due to their lack of humoral immunity (Mitchell et al. 2000; Lynch et al. 2011). Epidemics of adenovirus infections may occur in healthy children or adults in closed or crowded settings (e.g., military recruits). Additionally adenovirus infections are more severe, and dissemination is more likely in patients with impaired immunity (e.g., organ transplant recipients, human immunodeficiency virus infection, and congenital immunodeficiency syndromes). Although the vast majority of adenovirus infections are mild and self-limiting, the clinical spectrums are broad, and dissemination or pneumonia can be fatal, in both immunocompetent (particularly infants) and immunocompromised patients (Dudding et al. 1972; Zarraga et al. 1992; Ison 2006; Lynch et al. 2011; Horowitz 2001).

Coxsackie B viruses are non-enveloped RNA viruses and belong to human picornaviruses of the enterovirus group (Melnick 1996). CVB causes a wide range of human and animal diseases such as local myositis, myocarditis (Bergelson et al. 1998; Grist et al. 1975), pancreatitis (Yoon et al. 1986; Imrie et al. 1977), and meningitis (Godman et al. 1952; Melnick 1996). Serotype coxsackievirus B3 (CVB3) is one of the most common pathogens of myocarditis (Bowles et al. 1986; Carthy et al. 1997; Grist and Reid 1993) and its persistent infection may lead to dilated cardiomyopathy (DCM) (Carthy et al. 1997; Wessely et al. 1998; D'Ambrosio et al. 2001; Liu and Mason 2001). Especially in children CVB3 accounts for a significant fraction of cases of terminal heart failure (Shi et al. 2009; Feldman and McNamara 2000). A cardiac-inducible CAR knockout (Shi et al. 2009) provided the first genetic evidence that CAR is the receptor for the coxsackievirus in that the heart was protected from virus entry whereas noncardiac tissues were infected. CVB frequently infects the CNS and, together with other enteroviruses, is the most common cause of viral meningitis in humans. Newborn infants are particularly vulnerable. Moreover, CVB also can infect the fetus, causing death, or neurodevelopmental defects in surviving infants (Feuer et al. 2005).

### 2.5.3 *CAR Is Essential for Embryonic Heart Development*

Besides investigations on the role of CAR in viral infections and pathology its function during development as well as its implication in diverse nonviral pathological processes such as cancer or heart arrhythmia has been recently deciphered in loss-of-function and CAR overexpressing mouse mutants. Four constitutive and six conditional CAR knockout mice were generated in different laboratories to gain insight into the physiological function of CAR. These mouse models revealed an essential role of CAR in heart development and in multiple aspects of cardiac function and disease (Fischer et al. 2009). All global CAR knockouts that result in the elimination of all CAR isoforms lead to lethality at midgestation (between embryonic days 11.5 and 13.5) due to cardiac tissue malformation (Asher et al. 2005; Dorner et al. 2005; Chen et al. 2006; Lim et al. 2008; Lisewski et al. 2008; Fischer et al. 2009). Although in all of those mutants the overall outcome was very similar, slightly diverse cardiac phenotypes were reported upon CAR deletion. Dorner et al. (2005) reported that the absence of CAR resulted in enlarged pericards due to edema formation, smaller lumen of the ventricles, and an enlarged cushion. Cardiomyocytes showed reduced density and thickness of myofibrils and their orientation and bundling were disorganized. Neither proliferation nor apoptosis was found to be abnormal in knockout hearts. The authors concluded that the early lethality in these embryos is caused by insufficient heart function and disorganization of Cardiomyocyte structure affecting their contraction capacity. In the study by Asher et al. (2005) CAR-deficient cardiomyocytes exhibited regional apoptosis causing degeneration of the myocardial wall and thoracic hemorrhaging, leading to death at embryonic day 11.5 [see also the reply to (Asher and Finberg 2005)]. Lim et al. (2008) also reported hemorrhage in CAR-deficient embryos and a large pericardial effusion. However, no structural defects, apoptosis, hypertrophy, or ventricular wall thinning in the heart of these CAR-deficient embryos were observed as also described by Dorner et al. (2005). Interestingly, in the study by Chen et al. (2006) CAR deficiency resulted in regional over-proliferation of cardiomyocytes and hyperplasia of the left ventricle. Further analysis revealed that proliferating cardiomyocytes failed to differentiate to form normal trabeculae, which together with poorly organized myofibrils and ill-formed or absent intercellular junctions caused profound heart dysfunction and death at embryonic day 12.5. Surprisingly, when cardiomyocyte-specific deletion occurred not until at E11 [by using an inducible MHC (myosin heavy chain promoter)-Cre recombinase], a significant number (20 %) of conditional mutant animals survived to adulthood and did not reveal cardiac abnormalities. This observation might suggest that CAR is essential during a specific developmental window, and after day 11 when trabeculation is well under way, its loss can be compensated.

In summary, although the phenotype of CAR-deficient embryos appears to be complex, it is clear that CAR is essential for early cardiac development and CAR-mediated signaling is critical for the formation and survival of growing cardiomyocytes (Fechner et al. 2003; Noutsias et al. 2001; Ito et al. 2000).

#### **2.5.4 *CAR Is Implicated in Electrical Conduction in the Developing and Mature Heart***

Conditional ablation of CAR to circumvent embryonic lethality allows the exploration of CAR function at advanced developmental stages and in adult mice. A cardiac-specific CAR knockout (CAR-cKO) showed 70–90 % deletion of CAR at the intercalated discs of cardiomyocytes (Lim et al. 2008). Functional analysis of the heart using electrocardiograms (ECG) of 4- to 5-week-old CAR-cKO mice demonstrated abnormal atrioventricular conduction. While ventricular depolarisations showed no significant difference in the QRS morphology the P-wave was not detectable in the CAR-cKO mice (Lim et al. 2008). A telemetric ECG analysis in awake, conscious mice supported the observation that deletion of CAR leads to an abnormal atrioventricular conduction. In addition, in an embryonic global CAR knockout (CAR-KO) the flow of the blood was assessed by Doppler echocardiography (between E10.5 and 12.5) to study the function of the heart when CAR is absent from the beginning. In CAR-KO embryos the mean PR intervals were significantly enhanced, consistent with a first-degree AV block as shown in CAR-cKO mice (Lim et al. 2008). While the atrial and ventricular action potential generation analyzed by optical mapping studies was not affected, the disruption of CAR leads to a dislocalization of the gap junction protein connexin 45 at the atrioventricular-node cell-cell junctions and a reduced localization of  $\beta$ -catenin and ZO-1 at the ventricular intercalated discs of CAR-cKO at 8 weeks before they developed cardiomyopathy at 21 weeks of age. Similar deficiencies in heart function were uncovered by Lisewski et al. (2008) in a tamoxifen-inducible, cardiac-specific CAR knockout mouse (CAR-cKO). Although the depolarization and repolarization of the CAR-cKO ventricle were normal, the conduction of the electrical activity from the atrium to ventricle was impaired. This is reflected by a prolonged PR interval in CAR-cKO which corresponds with impairments at the level of the atrioventricular node. The changes in electrical conduction are related to a reduced expression and disturbed localization of the gap junction protein connexin 43 which might result in a disturbed communication between cardiomyocytes. Recently, ECG recordings in CAR-cKO mice supported the above findings and revealed a complete atrioventricular block with a temporal dissociation between atrial depolarization (P waves) and ventricular depolarization (QRS complexes) (Pazirandeh et al. 2011). These deficiencies in electrical conductance are associated with a heterogeneous morphology of the intercalated discs in CAR-cKO.

Overall, CAR may be required for the formation of a complex with connexin 45 and 43 at the intercalated discs and might be required for the correct localization of  $\beta$ -catenin and ZO-1 and therefore essential for normal atrioventricular-node conduction. This novel role of CAR in arrhythmia establishes CAR as a potential diagnostic marker for familial cases of atrioventricular block and ventricular dysfunction in genetic and acquired diseases and might also help to explain how, e.g., CVB can cause arrhythmia. Furthermore, arrhythmia could be a potential side effect of therapeutic approaches that target CAR to prevent CVB3-induced myocarditis, pancreatitis, or tumor growth and metastases (see also below) (Fischer et al. 2009).

### ***2.5.5 CAR Re-expression in Diseased Cardiac and Skeletal Muscle***

In contrast to developing mouse tissue expression of CAR in adult tissue is strongly reduced or even absent in some organs (Tomko et al. 2000); in particular in adult rodents and healthy human hearts CAR expression is very low and restricted to the intercalated discs of cardiomyocytes (Kashimura et al. 2004; Sasse et al. 2003; Ito et al. 2000). Interestingly, a strong CAR re-expression was observed in the intercalated discs and sarcolemma in human DCM, in ischemic cardiomyopathy (ICM) (Noutsias et al. 2001; Poller et al. 2002; Mirza et al. 2006; Tatrai et al. 2011), in mitral/aortic valve diseases (Sasse et al. 2003), and in animal models of cardiac inflammation and myocardial infarction (Ito et al. 2000; Fechner et al. 2003). Ito et al. proposed that re-expression of CAR in experimental autoimmune myocarditis in a rat model is induced by inflammatory cytokines such as interferon- $\gamma$ , tumor necrosis factor- $\alpha$ , and interleukin-1 $\beta$  and that CAR upregulation might be required for the regeneration of damaged myocardium (Ito et al. 2000; Noutsias et al. 2001). However, induction of CAR re-expression only by humoral factors seems to be unlikely, since CAR upregulation does not occur in all types of heart failures and is often locally confined to, e.g., infarct zone after myocardial infarction (Fechner et al. 2003). Surprisingly, postnatal overexpression of murine CAR in the cardiomyocyte under the control of a tetracycline-responsive  $\alpha$ -myosin heavy chain ( $\alpha$ MtTA) promoter resulted in inflammatory cardiomyopathy associated with MAPK activation and increased proinflammatory cytokine expression (Yuen et al. 2011). Thus, in contrast to Ito et al. (2000), this study suggests that CAR itself induces inflammation in the heart unrelated to viral infection, rather than just responding to inflammation and injury with upregulation. Therefore, CAR might have a dual function in the pathogenesis of myocarditis: as viral receptor and in addition induction of signals that activate components characteristic of innate immunity. Interestingly, increased CAR expression in the adult heart caused a cardiac phenotype distinct from that observed following embryonic overexpression of CAR which resulted in disrupted cardiomyocyte junctions (Caruso et al. 2010).

An active role of CAR re-expression in diseased cardiac tissue is supported by a similar observation made in regenerating skeletal muscle. CAR is highly expressed in myoblasts and is diffusely distributed on the plasma membrane of immature myofibers. In adult skeletal muscle fibers CAR is confined to the sarcolemma at the neuromuscular junction (Nalbantoglu et al. 1999; Shaw et al. 2004) whereas in diseased muscle with necrosis and regeneration (polymyositis and Duchenne muscular dystrophy, DMD) extrasynaptic sarcolemmal and cytoplasmic CAR is found to be co-expressed with regeneration markers such as desmin and utrophin (Sinnreich et al. 2005). Additionally, similar to cardiac overexpression of CAR, homozygous transgenic mice, in which CAR is regulated by the muscle creatine kinase (MCK) promoter, showed a severe myopathy with a large numbers of necrotic and regenerating fibers and premature death that was associated with an upregulation of caveolin-3 levels and deficiencies in dystrophin and dysferlin (Shaw et al. 2006).

In summary, the general view that emerges from these studies on cardiac and skeletal muscles is that CAR serves as a factor that is transiently expressed during development to establish cell–cell contact-mediated signaling. It is strongly down-regulated in normal adult human tissue and becomes re-expressed in certain diseases or damaged tissues to induce complex processes of tissue remodeling and regeneration (Noutsias et al. 2001). CAR-overexpression studies suggest that there is a threshold level above which CAR expression is detrimental to the muscle tissue. Since CAR expression is strictly regulated during development, it might be not surprising that its persistent, non-physiological overexpression may be deleterious in other tissues too.

### ***2.5.6 CAR in Renal and Intestinal Tissues and in the Lymphatic System***

Knockdown of CAR gene function by morpholino antisense oligonucleotides in zebrafish resulted in specific ultrastructural defects in pronephric glomerular maturation and terminal epithelial differentiation. Although podocytes differentiate in CAR morphants they were not able to elaborate a regularly patterned architecture of foot processes. In the tubules loss of CAR resulted in a clear increase in distance between the neighboring membranes of epithelial cells but did not influence the formation of tight junctions. Additionally, in tubular epithelia lacking CAR apical microvilli were very much reduced in number and appear disorganized. These findings established a new role of CAR in the terminal differentiation of renal glomerular and tubular cell types (Raschperger et al. 2008).

Detailed analysis of internal organ morphology and physiology revealed several abnormalities in a recently developed conditional mouse model of CAR (Pazirandeh et al. 2011). CAR-depleted mice demonstrated a striking atrophy of the exocrine part of the pancreas and small but significant increase in the total number of thymocytes in the thymus. Furthermore, CAR-deficient mice also displayed dilated intestines along the whole intestinal system which showed normal length. This could be either due to exocrine dysfunction of the pancreas or due to an altered neuro/hormonal regulation of the gut motility.

CAR is expressed in neonatal lymphatic endothelial cells where it is found at cell–cell junctions (Vigl et al. 2009). Deletion of CAR by tamoxifen injections in mice containing a conditional allele of CAR at embryonic day 12.5—a developmental stage when CAR is no longer essential for cardiac development—resulted in dilated lymphatic vessels. These vessels were filled with erythrocytes and revealed gaps at lymphatic endothelial cell–cell junctions, indicating that CAR is also essential during the development of the lymphatic vasculature (Mirza et al. 2012).

### ***2.5.7 Loss of CAR Expression Correlates with the Aggressiveness of Tumors***

The application of adenoviral vectors in gene therapy depends strongly on the level of CAR expression in targeted tissue (Kim et al. 2002; Hemmi et al. 1998; Li et al. 1999). In this context many tumor samples have been examined for CAR expression. In addition, a number of cell adhesion proteins have been linked to cancer progression in that loss of cell–cell contacts allows malignant cells to detach from their neighbors and to escape (Okegawa et al. 2002). Interestingly, in several human cancer tissues CAR expression was significantly downregulated during the progression of the malignancy leading to the hypothesis of a tumor-suppressive role for CAR in human cancers. Furthermore, re-expression of CAR in highly tumorigenic CAR-deficient human prostate and bladder cancer cells suppressed tumor growth (Okegawa et al. 2000, 2001; Rauen et al. 2002). Similar results have been reported for glioblastoma cell line (Kim et al. 2003), malignant glioma, high-grade primary astrocytoma (Huang et al. 2005), thyroid tumor (Marsee et al. 2005), gastrointestinal cancer (esophageal, pancreatic, colorectal, and liver cancer) (Anders et al. 2003b, 2009; Korn et al. 2006), human endometrial adenocarcinoma (Anders et al. 2003b), skin cancer cell lines (Anders et al. 2003b), colon cancer cell line adenomas, primary colon cancers, and colon cancer metastases (Stecker et al. 2011). These observations suggest that loss of CAR expression increases migration and invasion of cancer cells and therefore leads to disease progression with an unfavorable clinical outcome (Korn et al. 2006; Rauen et al. 2002; Matsumoto et al. 2005; Buscarini et al. 2007; Okegawa et al. 2007; Anders et al. 2009; Sachs et al. 2002), whereas forced expression of CAR protein inhibits tumor growth *in vitro* and *in vivo*. A possible mechanism for the reduced expression of CAR in malignant cells is the activity of the Raf-MEK-ERK pathway, which is frequently deregulated in cancer. A series of studies have shown that activation of Raf-1 decreases CAR expression, and conversely, inhibition of ERK leads to increased accumulation of cell surface CAR (Huang et al. 2005; Korn et al. 2006; Anders et al. 2003a, 2009). Since CAR is a cell adhesion molecule, loss of CAR weakens intercellular adhesion and might increase proliferation and migration as well as invasion of cancer cells (Okegawa et al. 2000, 2001; Bruning and Runnebaum 2003, 2004; Huang et al. 2005; Wang et al. 2005). An impaired adhesion of cancer cells is considered as a crucial prerequisite for both invasion and metastatic spread (Buda and Pignatelli 2004; Kimura et al. 1997; Resnick et al. 2005; Grone et al. 2007). Moreover, CAR upregulation was associated with an accumulation of the cell cycle regulators p21 and hypophosphorylated retinoblastoma (pRb) protein (Okegawa et al. 2001) suggesting that CAR can inhibit cancer growth by behaving as a membrane receptor, which conveys its signal into the nucleus, suppressing the proliferative mechanisms (Okegawa et al. 2000, 2002; Kim et al. 2003; Huang et al. 2005).



## 2.6 CLMP Is Implicated in Congenital Short-Bowel Syndrome

Several loss-of-function mutations in the CLMP gene were found in patients with congenital short-bowel syndrome (CSBS) (Van Der Werf et al. 2012). Patients born with CSBS have substantially shorter small intestine, with an average length of 50 cm, compared to a normal length at birth of 190–280 cm. They also reveal intestinal malrotation. Because CSBS occurs in many consanguineous families, it is considered to be an autosomal-recessive disorder. Knockdown of CLMP in the zebrafish resulted in a similar but more severe phenotype including a significant reduction in intestinal length and lack of goblets cells in the mid-intestine. The discrete phenotype observed in human CSBS compared to the zebrafish CSBS model together with the broad expression of CLMP observed in many other tissues argues for functional redundancy of CLMP in human beings. Nevertheless, CLMP function is required for normal small intestine development in both fish and human beings, suggesting a potential evolutionary conservation in this gene's function and its loss of function has a pathological consequence in human beings causing CSBS (Van Der Werf et al. 2012). In addition, CLMP (known also as ACAM, adipocyte adhesion molecule, or ASAM, adipocyte-specific adhesion molecule) has been suggested to be involved in adipocyte differentiation and development of obesity (Eguchi et al. 2005). CLMP mRNA was upregulated in white adipose tissues (WATs) of Otsuka Long–Evans Tokushima fatty (OLETF) rats (an animal model for Type II diabetes and obesity) (Kawano et al. 1992) and in WATs of genetically obese db/db mice (Koya et al. 2000), diet-induced obese ICR mice, and human obese subjects. Also in primary cultured mouse and human adipocytes, CLMP mRNA expression was progressively upregulated during differentiation. These results indicated that CLMP mRNA expression is strongly correlated with accumulation of WATs in human and animal obesity state and that the intervening CLMP expression or activities may alter the adipocyte differentiation status and contribute to therapy of obesity and metabolic syndrome (Eguchi et al. 2005). A provisional analysis of a CLMP knockout mouse indicated that its absence causes hydronephrosis at adult stages (Tang et al. 2010).

## 2.7 BT-IgSF (Igsf11) Is Implicated in the Migration of Melanophores

BT-IgSF gene expression is highly restricted to testis and brain. In the brain BT-IgSF transcripts are found in both neurons and glial cells, with abundant expression especially in pyramidal cell layers of the dentate gyrus and hippocampus and in commissure fibers of the corpus callosum (Suzu et al. 2002). This expression pattern of BT-IgSF might suggest a role in spermatogenesis and the development or function of the nervous system. Interestingly, so far, three human

patients have been reported to have an interstitial deletion of chromosomal 3q spanning the region 3q11–3q21—representing the location of the IgSF gene—and to have an agenesis of the corpus callosum (Genuardi et al. 1994; Mackie et al. 1998). Although the gene responsible for the corpus callosum agenesis in these patients has yet to be identified, the overlay of the BT-IgSF gene localization with the deleted region in these patients together with an abundant expression of BT-IgSF in the corpus callosum makes BT-IgSF a candidate gene responsible for this anomaly. Recent studies using zebrafish mutants indicated that BT-IgSF is implicated in pigment cell development and patterning (Eom et al. 2012). In the wild type melanophores form horizontal stripes during the larval-to-adult transformation while in the absence of BT-IgSF melanophores form an irregularly spotted pattern. BT-IgSF mediates cell–cell contact formation and promotes migration and survival of melanophores.

## 2.8 Perspectives

CAR and the CAR-related proteins CLMP and BT-IgSF have recently received increased attention due to their role in specific disease states. In particular, CAR has been shown to be essential during the development of the heart, renal, lymphatic, and intestine tissues. Although these three adhesion proteins are expressed in the developing nervous system their functions on neural cells are currently not fully understood. In vitro experiments suggest that CAR might be implicated in the formation of neuronal circuits. In contrast to many IgCAMs, CAR is preferentially expressed in the developing nervous system and becomes downregulated at early postnatal stages. This unusual timing and pattern of expression suggest that CAR is most likely not a structural protein; in contrast it appears to be a developmental factor that might be essential for the development of the brain. Mouse models that allow specific inactivation of CAR within the complete or in parts of the brain might help to understand the function of CAR on neural cells.

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