Chapter 8
Carbonic Anhydrase Related Proteins: Molecular Biology and Evolution

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Abstract The catalytically inactive isoforms of α-carbonic anhydrases are known as carbonic anhydrase related proteins (CARPs). The CARPs occur independently or as domains of other proteins in animals (both vertebrates and invertebrates) and viruses. The catalytic inactivity of CARPs is due to the lack of histidine residues required for the coordination of the zinc atom. The phylogenetic analysis shows that these proteins are highly conserved across the species. The three CARPs in vertebrates are known as CARP VIII, X and XI. CARPs orthologous to CARP VIII are found in deuterostome invertebrates, whereas protostomes only possess orthologs of CARP X. The CA-like domains of receptor-type protein tyrosine phosphatases (PTPR) are found only in PTPRG and PTPRZ. Most of these CARPs are predominantly expressed in central nervous system. Among the three vertebrate CA isoforms, CARP VIII is functionally associated with motor coordination in human, mouse and zebrafish and certain types of cancers in humans. Vertebrate expression studies show that CARP X is exclusively expressed in the brain. CARP XI is only found in tetrapods and is highly expressed in the central nervous system (CNS) of humans and mice and is also associated with several cancers. CARP VIII, PTPRZ and PTPRG have been shown to coordinate the function of other proteins by protein-protein interaction, and viral CARPs participate in attachment to host

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cells, but the precise biological function of CARPs X and XI is still unknown. The findings so far suggest many novel functions for the CARP subfamily, most likely related to binding to other proteins.

Keywords Expression • Cerebellar-ataxia • Phylogeny • Cancer • Bioinformatics • Cell adhesion

1 Introduction

The α-carbonic anhydrases (CAs), EC 4.2.1.1, are zinc containing metalloenzymes that catalyze the reversible hydration of CO_2, which is relevant in many important biological functions, such as photosynthesis, respiration, renal tubular acidification and bone resorption [1–4]. With the progress in genome sequencing of different organisms the number of CA and CA like proteins has been increasing constantly. Recently, we have reported several duplications in zebrafish CAs, and consequently the number of α-CAs has increased up to 17 [5]. The CA isozymes are categorized into different groups depending on the subcellular localization and enzymatic activity.

Apart from the enzymatically active CAs, there are CA isoforms which are evolutionarily well conserved with a sequence and structural similarity to active CAs, but which lack the classical enzymatic activity of active CAs. The inactive CA isoforms either occur independently as carbonic anhydrase related proteins (CARPs) or as domains of other proteins. The catalytic inactivity is due to lack of one or more of the three histidine residues which are necessary for co-ordination of zinc metal ion in the active site. The enzymatic activity of mammalian CARP isoforms (CARP VIII, X, and XI) can be regained by restoration of these histidines [6, 7]. In the family of protein tyrosine phosphatases (PTPs), there are two receptor-type protein tyrosine phosphatases, PTPR zeta (ζ) and PTPR gamma (γ), that contain an N-terminal CA-like domain [8, 9], termed "CARP XVI domain" [5, 10].

In addition to the above acatalytic CAs and CA-like domains found in vertebrates, CARP X like sequences have been identified in invertebrates, including groups as primitive and as distinct as insects and nematodes [10]. Furthermore, invertebrate genomes contain other, unrelated seemingly acatalytic CAs (Ortutay C and Tolvanen MEE, unpublished), and the D8 surface antigen in vaccinia viruses has been known to contain a CA-like domain since long time [11, 12].

The biological functions of CARPs and CA domains have started emerging recently. However, the precise physiological roles of these proteins are poorly understood till date. In the present chapter, we chronicle recent data on CARPs and CARP-like domains.

2 Carbonic Anhydrase Related Protein VIII

The CARP VIII is sequentially and structurally highly similar to catalytically active CAs. The catalytic inactivity of the CARP VIII is due to substitution of the first of the three histidine residues required for co-ordination of zinc atom in the active site (by Arg in all vertebrates). CARP VIII seems universal in vertebrates, and is also found in a number of deuterostome invertebrate species, and the sequence is highly conserved across the species [13]. A recent report [14] describes a CARP VIII sequence in pearl oyster, *Pinctada fucata*, which is a protostome, indicating that the evolutionary origin of CARP VIII might be much earlier than previously thought.

Among the catalytically inactive proteins of α-CA gene family, CARP VIII was the first to be reported based on its expression pattern in mouse brain in 1990s by Kato [15]. Subsequently, several studies have been done on CARP VIII, which include expression pattern during development and in adult tissues both in human and mouse and in pathological conditions [16–20] The structure of CARP VIII has been resolved and the basis of its inactivity has been studied [13, 21]. Its association with ataxia and neurodegeneration both in human and mouse has started emerging recently from several independent studies [22–25]. The previously reported interaction of CARP VIII with ITPR1 [26] provides a plausible mechanism for these cerebellar disorders. CARP VIII is known to be upregulated particularly in lung cancer and several others cancers [27–29]. However, the precise mechanism of how the regulation of ITPR1 leads to the biological effects is still not known. Very recently, we have developed an ataxic zebrafish model by knocking down *CA8* gene using antisense morpholino oligonucleotides which may help in elucidating the function of CARP VIII [30].

2.1 Molecular Properties and Bioinformatics of CARP VIII

Human CARP VIII is encoded by nine exons, and the exon boundaries in *CA8* have been maintained in the same locations through deuterostome evolution . The gene product consists of 290 amino acids with about 40 % of amino acid identity with other mammalian CA paralogs [31]. The N terminus of CARP VIII contains a unique feature among CAs, an acidic region. In mammals, the acidic region is considerably longer than in the orthologs in non-mammalian vertebrates. With the exclusion of glutamic acid stretch in mammalian *CA8*, the amino acid identity between human and zebrafish CARP VIII is 84 %. Such a high degree conservation between CARP VIII sequences of distant vertebrate species speaks for a conserved, essential function. An exhaustive review on CARP VIII has been published recently,

describing the data related to molecular biology, expression in healthy tissues and disease conditions and role in human diseases [32]. The data related to basis of inactivity and sequence and phylogenetic analysis has also been published in the recent past [13, 33].

Interaction of CARP VIII with inositol 1,4,5-trisphosphate receptor type 1 (ITPR1), an ion channel protein that regulates internal Ca^{2+} ion release is well known [26]. The binding of CARP VIII to ITPR1 is believed to reduce the sensitivity of ITPR1 to inositol trisphosphate (IP3) and control the release of Ca^{2+} ions from the internal stores of the endoplasmic reticulum [34]. Deletion mutagenesis experiments in a yeast two-hybrid system showed that almost the entire CA domain of CARP VIII is required for ITPR1 binding [14]. We studied the functional association of ITPR1 and CARP VIII throughout the vertebrate evolution; two coevolution analyses of genes encoding these two proteins from 31 species were performed. In fish species ITPR1 gene has been duplicated into genes which are named *itpr1a* and *itpr1b* in zebrafish. The analysis of *itpr1a* orthologs show more sites that appear to coevolve with CARP VIII compared to the analysis of *itpr1b* [30]. This indicates that the gene product of itpr1a is more likely to have retained the interaction with CARP VIII in fish.

2.2 Catalytic Activity and Inhibition Studies

Replacement of Arg117 to His and Glu115 to Gln in CARP VIII restored its catalytic activity, which was significantly higher than that of the mammalian CA III isozyme [7]. The inhibition studies on this protein showed its activity could be strongly inhibited by acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide). The replacement of Gln92 to Glu in human CA II has only slight effect on zinc affinity suggesting that the single mutation of Arg117 to His is enough to change inactive CARP VIII to an active CA [7, 35, 36]. Indeed, later studies by the same group showed that replacement of single arginine residue with histidine was able to restore the catalytically inactive mouse CARP VIII to active carbonic anhydrase with CO_2 hydration turnover number of $1.2 \times 104\ s^{-1}$. Further mutations in CARP VIII of residues His94, Gln92, Val121, Val143 and Thr200 (numbering system of human CA I) greatly increases CA activity similar to CA I with a kcat value of 4×104 s-1 [36].

Recently, another site-directed mutagenesis study was performed to restore the catalytic activity of CARP VIII [6], resulting to a kcat/km value which was 67.3 % of the value for human CA II, one of the most highly active enzymes in nature [3]. The mutant CARP VIII was effectively inhibited by acetazolamide, with inhibition constants in the range of 12–35 nM (the KI of this sulfonamide against human CA I is of 250 nM). Interestingly, the catalytic activity of human CA VIII mutant was higher than was reported for the mouse CA VIII mutant reported earlier, although there is only difference of three amino acid residues between these two mutant

enzymes [6]. The inhibition study done with inorganic anions showed much weaker inhibition compared to CA X and CA XI. However, inhibitory effect of thiocyanate to restored CA VIII was stronger than to other restored CARPs.

2.3 Ataxic Zebrafish Model Knocked Down for CA8

Many types of congenital ataxia and mental retardation are caused by alterations in genes that affect the development of the brain. Studies done in the past in mouse showed that spontaneously occurring mutation in *CA8* gene leads to ataxia and lifelong gait disorder [24]. Similarly, recent reports from human studies also show that mutations in *CA8* gene cause mental retardation and ataxia with a reduction in the cerebellar volume [22, 23]. Recently, we have developed a zebrafish model by knocking down the *CA8* gene using antisense morpholinos against *CA8*, which imitated the human disease [30].

2.3.1 Expression of *CA8* in Zebrafish

The developmental expression studies using RT-qPCR showed that *CA8* mRNA is expressed throughout the embryonic development period. The expression of *CA8* was particularly high at 0 hpf, 24 hpf and 120 hpf. The presence of *CA8* at 0 hpf suggests that *CA8* is of maternal origin and that it is required early in the development. The expression analysis of *CA8* in a panel of tissues showed that *CA8* is expressed in almost all the tissues of zebrafish at mRNA level. The *CA8* mRNA was predominantly high in the brain, similar to the expression in mouse and human [33, 37]. The high level of expression was also seen in kidney, eye, gills and heart. The overall expression pattern zebrafish tissues was similar to the expression pattern in mouse tissues [33]. Immunohistochemistry of cerebellar region showed intense signal in cerebellar Purkinje cells, which is similar to the expression in mouse and human brain [33, 37]. Similarly, developmental expression analysis of *itpr1a* showed an expression pattern similar to *CA8* mRNA during the early embryonic development, suggesting the need of their interaction during development.

2.3.2 Morphant Zebrafish with Ataxia

The zebrafish larvae injected with 125 μM translation-blocking antisense morpholinos (MOs) against *CA8* completely suppressed its expression, which was confirmed by western blotting at 3 dpf in zebrafish larvae. So, this fairly low concentration mimics a null mutation. The larvae lacking the *CA8* gene product showed a variety of phenotypic defects, including a curved and fragile body, pericardial edema, absence of otolith sacs, and incomplete utilization of yolk. Swim pattern of the

5 dpf morphant larvae injected with 2 μM and 6 μM *CA8*-MOs, which had an otherwise normal phenotype, showed difficulty in balancing the body and increased turning angle, suggesting an ataxic phenotype similar to mice and humans with a mutation in *CA8* gene [22–24]. Histological examination showed reduction in cerebellar volume similar to the human patients from Saudi Arabian family [23]. Similarly, electron microscopy and TUNEL assay showed increased neuronal cell death. The results obtained from zebrafish model suggest an important role for CARP VIII during embryonic development. Lack of CARP VIII leads to defects in motor and coordination functions, mimicking the ataxic human phenotype. Zebrafish knockdown work also reveals an evolutionarily conserved function of CARP VIII in brain development. The novel zebrafish model will be helpful in investigating the mechanisms of CARP VIII-related ataxia and mental retardation in humans.

2.4 Distribution of CARP VIII in Neuronal Cells Expressing Mutant Ataxin-3

In a recent study, changes in the gene expression profile of human neuroblastoma cells expressing a repeat expansion mutant ataxin 3 were studied using microarray technology [38]. This microarray study showed a nearly 9-fold increase in *CA8* expression in the presence of mutant ataxin 3 compared to cells expressing normal ataxin 3. Further analysis of distribution of CARP VIII in these cells showed significant increase in the expression of CARP VIII in the neuroblastoma cells (SK-N-SH) expressing mutant ataxin 3 [39]. Perinuclear and cytoplasmic localization study using monoclonal antibodies showed intense and even distribution of CARP VIII. The expression of CARP VIII in the perinuclear region was consistent with its co-localization with ITPR1, which is expressed on endoplasmic reticulum membrane [26]. It was shown earlier that defective ataxin 3 disturbs neuronal calcium signaling by binding to ITPR1 [40]. In vitro and cultured cell studies showed that the sensitivity of ITPR1 to IP3 is increased when it is bound to mutant ataxin 3. The increased expression and altered localization of CARP VIII in the cells expressing mutant ataxin 3 might be linked to the disruption of ITPR1-mediated calcium signaling in neurons by mutant ataxin-3 [39].

3 Carbonic Anhydrase X-like Sequences

CARP X-like sequences seem to be found in all bilaterian animal genomes, both in deuterostomes and protostomes (Tolvanen et al., unpublished results), unlike CARP VIII orthologs, which have been observed only in some deuterostome invertebrates

[33] and in a single protostome species [14], in addition to their ubiquitous presence in vertebrates. In protostomes many species harbor several copies of CARP X-like genes. For example, in the nematode *C. elegans* the α-CA genes *cah-1* and *cah-2* code for CARP X-like proteins, and in most insects we see a system of two CARPs which are one-to-one orthologs of *D. melanogaster* genes *CG1402* and *CG32698*, for which we have used the names CARP-A and CARP-B [10].

The data related to mammalian CARP X and XI expression studies and role in diseases has been reviewed recently [32]. In this chapter, we review recent data related to catalytic activity of mutated human CARP X and XI. We also discuss the information related to distribution of CARP XI in human brain with spinocerebellar ataxia 3 (SCA3) and in a transgenic mouse model with Machado-Joseph disease (MJD). Finally, we present evolutionary data related to CARP X-like sequences.

3.1 Catalytic and Inhibition Studies of Engineered CARP X and CARP XI

The CARP X is catalytically inactive due to the absence of two out of the three histidine residues necessary for coordination of the zinc atom in the active site. Similarly, mammalian CARP XI lacks all the three histidine residues and is enzymatically inactive [33]. In the past, studies on catalytic activity of mutated CARP VIII have been described, but no information was available on restoring the catalytic activity of CARP X and CARP XI till very recently [6]. The mutated CARP X and CARP XI demonstrated high catalytic activity. Human CARP X had the highest activity among the mutated CARPs, showing 92 % catalytic activity compared to human CA II, which is considered as one of the fastest enzymes in nature [3]. The catalytic activity of mutated CARP XI was only slightly lower than that of CARP X, at 82.6 % of the activity of human CA II.

These enzymes were effectively inhibited by acetazolamide, with inhibition constants ranging from 12 to 35 nM [6]. Inorganic anions represent another well-known group of CA inhibitors. They bind metal ions in solution or they bind within the active site of the metalloenzyme [41]. The inorganic anions showed higher inhibition of these enzymes than of CARP VIII. The anion thiocyanate which inhibits CARP VIII strongly was less effective against CARP X. Nitrate, nitrite, hydrogen sulfide, cyanide, and azide were effective CARP X inhibitors. The best CARP X anion inhibitors were sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid.

The inhibition rate of mutated CARP XI was intermediate between CARP VIII and CARP X, similar to its catalytic activity. The effective inhibitors against CARP XI were cyanide, hydrogen sulfide, sulfamide, sulfamate, phenylboronic acid and phenylarsonic acid, with K_I values in the range of 5.1–88 μM [6].

3.2 Altered Expression of Mammalian CARP X and XI

Expansion mutations in CAG trinucleotide repeats of ataxin 3 lead to spinocerebellar ataxia 3/Machado-Joseph disease (SCA3/MJD), a neurodegenerative disorder in humans and mice [42]. Microarray studies on mRNAs isolated from human neuroblastoma cells expressing mutant ataxin-3 demonstrated that *CA11* is highly upregulated and *CA10* slightly upregulated in these cells [39]. As a follow-up of these results, the authors studied the cellular distribution of CARP X and CARP XI in cultured neuronal cells, in the brain tissues of transgenic MJD mice and in the brain tissues of a human patient with SCA3 and of a normal human. There was no significant change in the cellular localization of CARP X in the cultured MJD-positive or -negative cells. However, localization of CARP XI was changed in the cells expressing mutant ataxin-3 compared to the cells expressing normal ataxin-3. Namely, cellular localization studies in normal and MJD84.2 mouse and in human SCA3 patients showed that the cerebellar Purkinje cells in MJD mouse stained strongly especially in the nucleus and cytoplasm compared to the normal mouse. The neural cells in the cortex also showed a stronger staining in MJD mouse compared to normal mouse. The immunostaining of cerebellar tissue of SCA3 mutation-positive human patients confirmed the results of the staining obtained for MJD mouse. The cerebellar white matter in the cytoplasmic region and Purkinje cells showed increased immunoreactivity compared to the staining of age-matched control brains. The results on the distribution of CARP XI suggest that in the presence of mutant ataxin-3, CARP XI changes its expression level and cellular relocalization to nuclei of neuronal cells. It remains an interesting question whether this plays a role in the progression of the disease and dysfunction of nervous system.

3.3 Evolutionary Aspects of CARP X-like Sequences

The expression of mammalian CARP X and CARP XI is most abundant in the central nervous system (CNS). In Drosophila, *CG1402* (CARP-A) is similar in being most highly expressed in larval CNS and in adult fly brain (http://flybase.org/reports/FBgn0029962.html). In *C. elegans*, both of the CARP X-like genes *cah-1* and *cah-2* are expressed in several types of neurons (http://gbrowse.raetschlab.org/cgi-bin/gbrowse/ce200/) [43]. So, neural expression of CARP X-like genes seems to have been conserved since the earliest bilaterian animals. We can find orthologs of CARP X even in flatworms (Tolvanen et al., unpublished), but there is no data on their expression pattern.

It is remarkable how well the overall structure of CARP X and CARP XI has withstood the changes during its history. Despite having been acatalytic since very early metazoan evolution, these proteins can still reach nearly the same reaction rates as the very highly active CA II when the critical zinc-binding histidines are restored in their sequence [6].

4 Receptor-Type Protein Tyrosine Phosphatases (PTPR) Gamma and Zeta

The phosphorylation of tyrosine residues in proteins is a basic regulatory mechanism which controls proliferation, differentiation, communication, and adhesion of cells. Receptor-type protein tyrosine phosphatases (PTPRs) are a family of integral cell surface proteins that possess intracellular protein tyrosine phosphatase (PTP) activity, and contain extracellular domains that have sequence homology to cell adhesion molecules. The function of PTPRs is less well known compared to receptor-type protein tyrosine kinases (RPTKs), especially as regards to their substrate specificities, regulatory mechanisms, biological functions, and their roles in human diseases. The PTPRs are divided into eight subfamilies (R1/R6; R2A; R2B; R3; R4; R5; R7; and R8) based on structure of their extracellular domains [44, 45]. In the context of the present chapter, PTPR-zeta (ζ) which is also known as PTPR-beta (β), (henceforth, it will be referred as PTPRZ) and PTPR-gamma (γ) (PTPRG) are of particular interest. PTPRZ and PTPRG contain carbonic anhydrase (CA) domains and belong to the R5 sub-family.

The PTPRZ/G proteins are encoded by the genes *PTPRZ1/PTPRG*, and possess an N-terminal CA-like domain and a single Fibronectin type III domain in their extracellular domains (ECD), and an intracellular domain with tandem PTP domains [8, 46]. The CA domains of PTPRs lack the CA enzymatic activity due to absence of two of the possible three histidine residue required for co-ordination of zinc atom at active site. The CA like domain at N-terminal end is comprised of seven exons and six introns, and the positions of introns are similar to the introns found in mammalian *CA1, 2, 3, 5 and 7* genes [47]. The CA domains of these proteins show up to 50 % similarity with the other CA-related domains and active CAs [8, 9]. The two of the histidine residues in human PTPRZ and PTPRG are replaced by threonine and glutamine and therefore, these proteins are catalytically inactive [6, 13, 14]. However, the CA domains in these proteins can serve as a hydrophobic binding pocket for contactin molecules [48]. In this chapter, we mainly present the data on structure of CA domains of PTPRZ and PTPRG and their interaction with contactin molecules emerged recently and the role of these proteins in diseases.

4.1 Molecular Biology and Bioinformatics

The CA domain of PTPRZ binds to contactin 1 (CNTN-1) and promotes cell adhesion and neurite outgrowth of primary neurons [48]. The other ligands that can bind to ECD are tenascin, N-CAM, pleitrophin and midkine [49, 50]. The PTPRZ is expressed by astrocytes and oligodendrocytes in the developing and adult nervous system and it binds to contactin-1 (CNTN1) which is expressed on the surface of oligodendrocyte precursor cells and is involved in proliferation

and differentiation of these cells [50–52]. Similarly, PTPRZ is also involved in oligodendrogenesis, suggesting that the interaction between PTPRZ and CNTN1 is required for maturation of oligodendrocytes.

A full length PTPRZ contains, an N-terminal carbonic anhydrase-like (CA-like) domain followed by a fibronectin type III (FNIII) repeat, a spacer region, a large insert with attachment sites for chondroitin sulfate proteoglycan and two tyrosine phosphatase domains (D1 and D2) in the cytoplasmic region [53]. It is expressed in soluble form, phosphacan, which is one of the most abundant proteoglycans in the brain and is expressed during myelination [51]. CNTN1 is a glycophosphatidyl-anchored protein composed of six Ig repeats and four FNIII domains and is the first identified member of the CNTN family of neural cell adhesion molecules, which are involved in the construction of neural networks [54]. It is shown that Ig repeats two and three of CNTN1 interact with the CA-domain of PTPRZ [55]. Recently, the structural basis of interaction between PTPRZ and CNTN1 has been shown to demonstrate that this interaction regulates the proliferation and differentiation of oligodendrocyte precursor cells OPCs [56].

4.2 Crystal Structures of PTPRZ-CNTN-1 Complex and of CA Domains of PTPRs

The crystal structure of CNTN1 fragment Ig2-Ig3 bound to CA-domain of PTPRZ shows that the Ig2-Ig3 repeats in CNTN1 adopt a horseshoe-like conformation in which apolar contacts between Ig2 and Ig3 bury 1,355 $Å^2$ with a shape complementarity coefficient of 0.77 [57]. The interface between the CA domain and Ig2-Ig3 repeats of CNTN1 occludes 1,658 $Å^2$ with a shape complementarity coefficient of 0.68, both of which compare favorably to those of known biological interfaces and are similar to the values obtained for the complex between PTPRG and CNTN4 [55]. The crystal structure complex shows that the interface between PTPRZ and CNTN1 consists of a β-hairpin loop contacting repeats of Ig2 and Ig3 domains of CNTN1 (PTPRZ residues 265–280) and a short loop (PTPRZ residues 205–208) that contacts the Ig3 domain of CNTN1 exclusively.

The PTPRZ and CNTN1 are known to be involved in development of oligodendrocytes [52, 58]. Studies on the interaction of PTPRZ and CNTN1 at cell surface of oligodendrocytes showed that PTPRZ co-localizes with CNTN1 [56]. Similarly, binding studies between different CNTN molecules and PTPRZ confirmed that PTPRZ binds specifically to CNTN1 and not other CNTN molecules [55]. Further examination of proliferation of oligodendrocyte precursor cells (OPC) in $PTPRZ^{-/-}$ mice during early postnatal development showed that the lack of PTPRZ increases the proliferation of OPCs [56]. Taken together these results suggest that formation of PTPRZ and CNTN1 complex represses proliferation of OPCs and promotes oligodendrocyte maturation.

Recently, crystal structure of CNTN4-Ig1-4 and PTPRG-CA complex has also been resolved and structural basis of interaction of CNTN4-Ig1-4 with PTPRG-CA was studied [55]. In PTPRG-CA, the flexible β-hairpin (residues 288–301) accounts for almost 80 % of the interaction with CNTN4; rest of the contacts are mediated by residues 225–229 and the contacts between CNTN4 and PTPRG 4 are restricted to Ig domains 2 and 3. The two strands of the β-hairpin in PTPRG-CA complement the 3-strand antiparallel β-sheet in CNTN4 Ig 2 to form a 5-strand antiparallel β-sheet with the main chain atoms of residues 295–299 of PTPRG, forming hydrogen bonds with the main chain atoms of residues 139–143 in CNTN4. All the CNTN4 residues that interact with PTPRG are conserved in CNTN3, 5, and 6, thus explaining why PTPRG binds specifically to these four CNTN family members. Similarly, In-vitro affinity-isolation assay showed binding of PTPRG-CA to CNTN3,4,5, and 6, and further binding studies of CNTN3,4,5 and 6 lacking different Ig domains showed that the PTPRG binds to 2 and 3 Ig domains of CNTN3,4,5, and 6 forming 1:1 complex [55].

Similarly, the crystal structures of mouse and human PTPRG-CA domain and CA domain of human PTPRZ have been resolved at a resolution of 1.7 Å. The CA domain of all three receptors are similar and have central 8 strand antiparallel β-sheet surrounded by three to four α-helices and extensive loop regions [55]. Mouse and human PTPRG-CA domains superimpose with a rmsd of 0.5 Å for 257 C-α positions, whereas mouse PTPRG-CA and human PTPRZ-CA superimpose with a rmsd of 1.4 Å for 257 C-α positions. However, unlike PTPRG-CA, PTPRZ-CA includes an additional disulfide bond between C133 and C264, which is conserved in all known orthologs of PTPRZ. The presence of this additional disulfide bridge has little effect on the structure of PTPRZ-CA when compared to PTPRG-CA and its biological significance is currently unknown [55].

Structural studies show that the CA domains of PTPRG and PTPRZ resemble α-CAs. Mouse PTPRG-CA, human PTPRG-CA, and human PTPRZ-CA superimpose with mouse CA-XIV with rmsd of 1.5–1.7 Å for 253–255 C-α positions and most of the differences reside in the orientation of an extended β-hairpin loop in type V PTPRs [59]. This hairpin loop is disordered in human PTPRG-CA and in three of the four molecules found in the asymmetric unit of mouse PTPRG-CA-crystals, indicating that this region is flexible.

4.3 Role in Diseases

The PTPRZ plays critical role in oligodendrocyte survival, in recovery from demyelinating disease, and in memory formation [58, 60]. The up-regulation of pleiotrophin is associated with the repair of injured nervous system, which suggests that pleiotrophin may have protective effects in Parkinson's disease [61]. Binding of pleiotrophin to PTPRZ modulates the PTP activity by increasing β-catenine, Fyn

and β-adducin tyrosine phosphorylation by inhibiting PTP activity of PTPRZ [49, 62]. The activation of these substrates is needed for proliferation of dopaminergic progenitors and survival and differentiation of dopaminergic neurons [63]. The inhibition of phosphatase activity of PTPRZ using specific inhibitor may be useful in treatment of Parkinson's disease.

PTPRZ plays a role in pathogenesis of gastric ulcers by acting as a cell surface receptor for VacA, a protein toxin produced by *H. pylori* [64]. The VacA protein toxin inhibits the PTP activity of PTPRZ and induces the detachment of gastric epithelial cells by increasing tyrosine phosphorylation of its substrate Git-1 a protein involved in cell adhesion and cytoskeleton [65]. Git-1 tyrosine phosphorylation accompanies epithelial detachment, leading to gastric ulcers with direct action of gastric acid. The defect in signaling of PTPRZ is a basic mechanism of gastric ulcers caused by *H. pylori*. The mice which are knocked out for PTPRZ are resistant for gastric ulcers caused by *H. pylori* also support the above conclusions [66].

The neurogulin-1 (NGR) is a signaling molecule and functions through the interaction of epidermal growth factor receptor (ErbB4) receptor. Interestingly, PTPRZ is known to inhibit its role in signaling [67]. The expression studies showed that the gene encoding PTPRZ is up-regulated in the brain of patients with schizophrenia [68]. Similarly, over expression of PTPRZ in transgenic mouse model reduced the NRG1 signaling, leading to molecular and cellular changes such as, altered glutamatergic, GABAergic and dopaminergic activity and delayed oligodendrocyte developments, which occur during the pathogenesis of schizophrenia. These mice also showed reduced sensory motor gating, hyperactivity and working memory deficit which are typical symptoms of schizophrenia [68].

The PTPRZ is also implicated in remyelination, as it is upregulated in human oligodendrocytes during the repair of lesions to myelin sheath [58]. The experimental allergic encephalomyelitis-induced PTPRZ$^{-/-}$ mice have an impairment of repair of demylinating lesions. These findings suggest that the formation of PTPRZ/CNTN1 complex is necessary for the differentiation of OPCs leading to remyelination. Involvement of PTPRG in different diseases is less well known. However, it has been implicated in renal and lung cancers as it was found to be frequently deleted in these cancers in humans [69]. The PTPRG is also associated with motor coordination function, and the mice deficient in this protein exhibit impaired motor coordination during rod walking and string tests [70].

The PTPRZ and RTPRG play different roles in cellular processes and can be potential drug targets for treating certain diseases. More importantly, the discovery of secondary substrate-binding sites in these proteins provides an opportunity for development of highly specific inhibitors. Similarly, small molecules with high affinity targeting ECDs can up-regulate the activity of these proteins. The deeper understanding of physiological function and regulatory mechanisms of these proteins will help us to design specific inhibitory molecules for balancing of activity of these proteins.

5 Carbonic Anhydrase Related Proteins in Poxviruses

The vaccinia virus transmembrane protein D8 [11, 12, 71] was reported to contain a CA-like N-terminal domain in 1990 [11, 12, 71]. The D8 protein was observed to participate in attachment to the host cell [11, 12, 71], and later the ligand was discovered to be chondroitin sulfate [72] Now we know that altogether four proteins mediate attachment of vaccinia virus, including A27 and H3, which bind heparin, and laminin-binding A26 [73].

5.1 Structure

The vaccinia virus *D8L* gene codes for a 304-residue protein. Figure 8.1 presents a multiple sequence alignment of 14 viral sequences, detailed in Table 8.1, together with selected human CA and CARP sequences. In the N-terminal ectodomain the first 236 residues are clearly homologous with the catalytic domain of carbonic anhydrases. There is no counterpart for the first 24 residues of CA II in the viral sequences. The truncated CA domain is followed by a spacer of almost 40 residues, and residues 275–294 form the predicted transmembrane domain (indicated in Fig. 8.1), leaving just 11 C-terminal residues on the other side of the membrane.

The three-dimensional structure of vaccinia D8 binding to an antibody has been determined using 225-residue and 269-residue constructs of the ectodomain [74]. In the longer construct, the last 60 residues following the CA domain are not visible. Figure 8.2a shows that despite missing the first 24 residues of the CA fold, the backbone of vaccinia D8 follows very closely that of other CAs and CARPs. Of the 236 visible residues, 214 are superimposable (within 5.0 Å) on all of the other four structures in Fig. 8.2a with RMSD values ranging from 1.16 to 1.53 Å. This is similar to the RMSD values between all other structures shown: 1.10–1.54 Å, except for PTPRG to PTPRZ which gives an RMSD of 0.86 Å. Figure 8.2b–d show the surfaces of D8 (2B), CARP VIII (2C), and PTPRZ (2D), demonstrating that the missing 24 residues leave a large dent at the upper right.

The binding site for the negatively charged chondroitin sulfate chain is predicted to be at the bottom of the cavity created by the N-terminal deletion (arrow in Fig. 8.2b), lined with positively charged residues [74]. The sequence alignment of Fig. 8.1 shows all Lys and Arg residues on grey background. Residues K41, R44, K108, and R220, on black background in the bottom sequence, are the residues implicated in binding in a docking experiment with a short chondroitin sulfate fragment [74]. The large amount of additional conserved Lys and Arg residues (Figs. 8.1 and 8.2b) indicates that even a longer CS chain could bind efficiently.

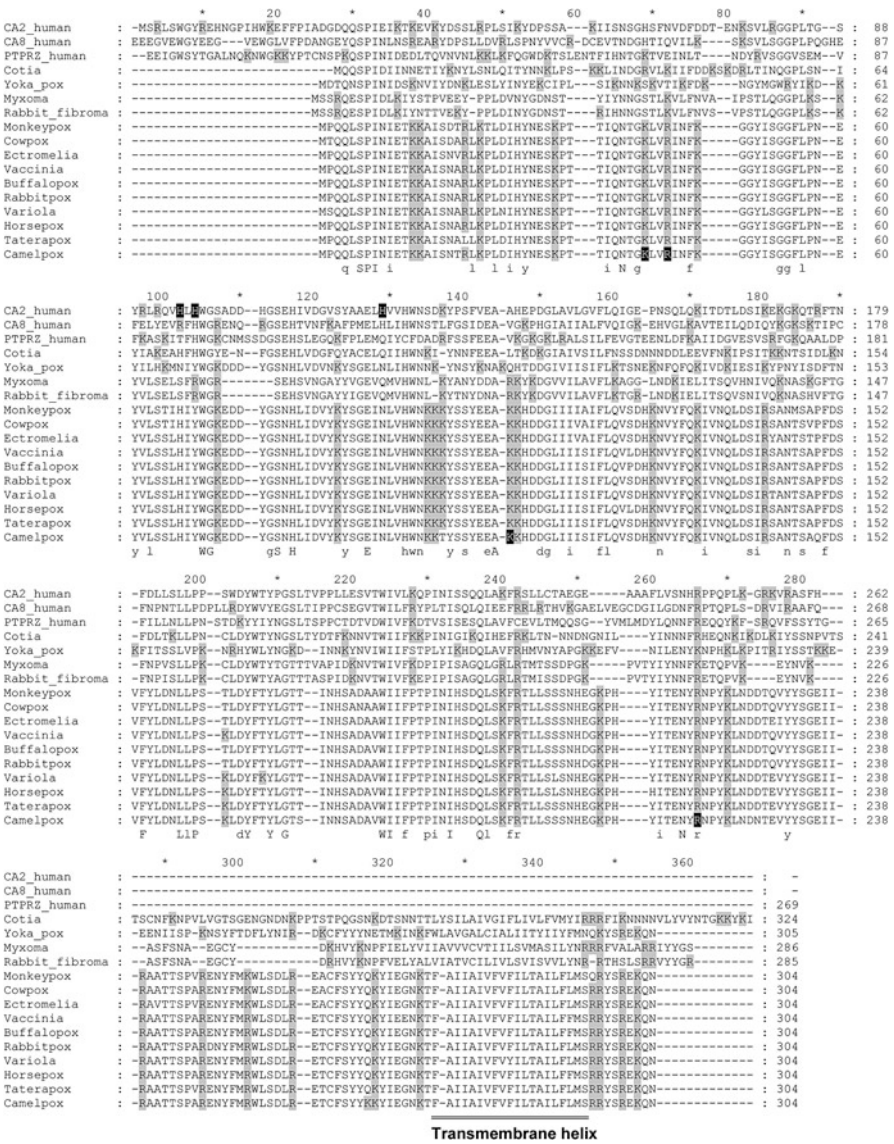

Fig. 8.1 Multiple sequence alignment of viral CARPs with selected human CAs. CA8_human includes only the visible part of structure 2W2J, and PTPRZ_human the visible part of 3JXF. Alignment with Clustal Omega iterated four times and manually edited to match a structure-based alignment near the end of the CA domain. Grey highlighting for all K and R residues. Black highlighting in the bottom row for residues K41, R44, K108, and R220 in the predicted chondroitin sulfate binding site. Black highlighting in the top row for for the three Zn-binding residues in CA II

Table 8.1 List of virus proteins homologous to carbonic anhydrases included in the alignment of Fig. 1

Probable chondroitin sulfate binding 32 kDa proteins	Name in GenBank	GenBank protein accession number
Buffalopox virus	immunogenic envelope protein	ADW84807.1
Camelpox virus	CMLV111	NP_570501.1
Cowpox virus	CPXV125 protein	NP_619909.1
Ectromelia virus	EVM097	NP_671615.1
Horsepox virus	HSPV114	ABH08220.1
Monkeypox virus Zaire-96-I-16	E8L	NP_536532.1
Rabbitpox virus	RPXV102	AAS49815.1
Taterapox virus	IMV membrane protein	YP_717422.1
Vaccinia virus	IMV membrane protein	YP_232995.1
Variola virus	hypothetical protein VARVgp098	NP_042142.1
Other CA-like viral proteins		
Cotia virus SPAn232	carbonic anhydrase-like protein	YP_005296290.1
Myxoma virus	m83L	NP_051797.1
Rabbit fibroma virus	gp083L	NP_051972.1
Yoka poxvirus	IMV membrane protein	YP_004821447.1

5.2 Evolution and Function of Viral CARP Sequences

We have constructed a phylogenetic tree of selected viral CA-like sequences for this review, together with all human CA isoforms (Fig. 8.3). The tree shows that the viral CA-like proteins are most likely to have been derived from cytoplasmic CA isozyme genes of the group CA1/CA2/CA3/CA13. Even if the tree indicates human CA13 as the most closely related gene, it remains uncertain, since we do not know from which host animal the gene was originally captured in a poxvirus.

The Orthopoxvirus sequences (bottom 10 sequences in Fig. 8.1, and the tightly linked group indicated in Fig. 8.3) are highly similar to each other, with protein sequence identities of at least 93 % to the prototype vaccinia D8 sequence. It is highly probable that they all retain the same function of CS binding. The other four virus sequences are less than 40 % identical to the vaccinia sequence, so they may or may not share this function. In case of myxoma and rabbit fibroma viruses, some of the assumed CS-binding site residues are conserved, so they might retain the function, but for Yoka pox virus and Cotia virus, this seems less likely. However, this is highly speculative since the binding site has been defined only by a docking simulation.

The zinc-binding His residues of CA II are shown with black highlight in Fig. 8.1. In all of the viral sequences some of these His residues are missing, and they are predicted to be devoid of CA enzymatic activity. It was verified that the vaccinia D8

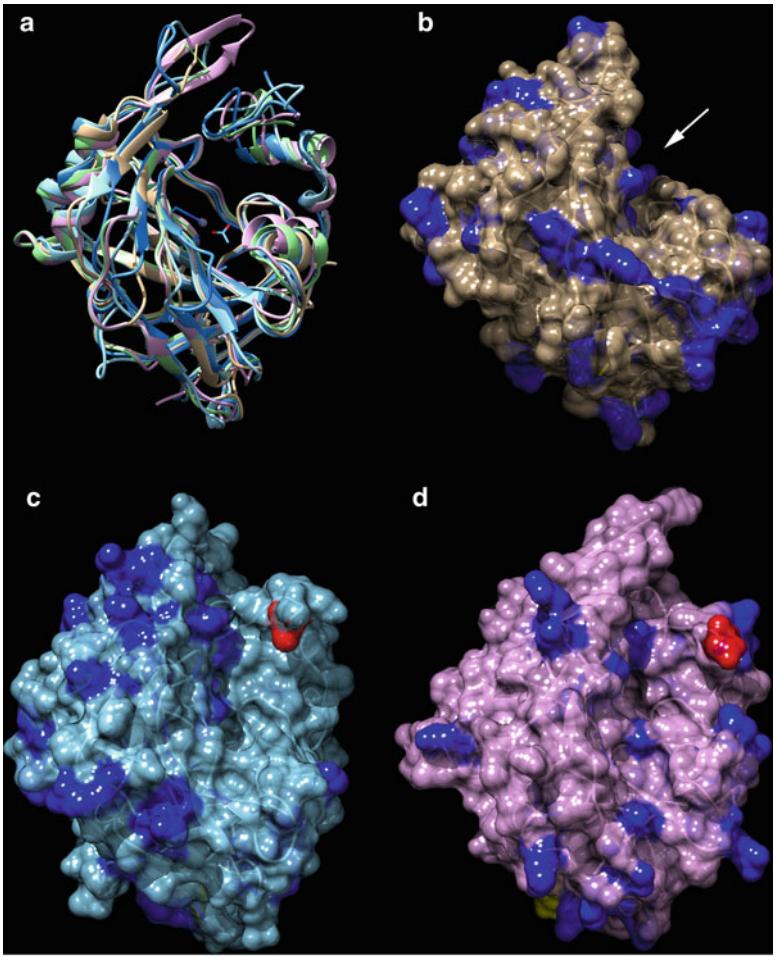

Fig. 8.2 Structures of CARPs. (**a**): four CARPs and CA II superimposed. Protein backbones of vaccinia D8 ectodomain (4E9O, *beige*), CARP VIII (2W2J, *cyan*), CA-like domain of human PTPRZ (3JXF, *lilac*), CA-like domain of human PTPRG (3JXH, *light green*), and human CA II (2D0N, *darker blue*). Note the small beige stump at 3 o'clock which is the N-terminus of 4E9O, residue 2 of the viral sequence. (**b**): Vaccinia D8 ectodomain (4E9O). (**c**): CARP VIII (2W2J). (**d**): CA-like domain of human PTPRZ (3JXF). In all of 2B to 2D, blue shows positively charged Lys and Arg residues on the surface, *yellow* (*at the bottom*) indicates the C-terminus, and *red* the N-terminus of each protein

protein does not have catalytic activity, but when the two histidines missing from the catalytic site are returned, the activity and acetazolamide inhibition sensitivity is restored [75]. This proves the surprising robustness of the CA fold—despite the lack of the N-terminal 24 residues, the reconstituted enzyme worked at the

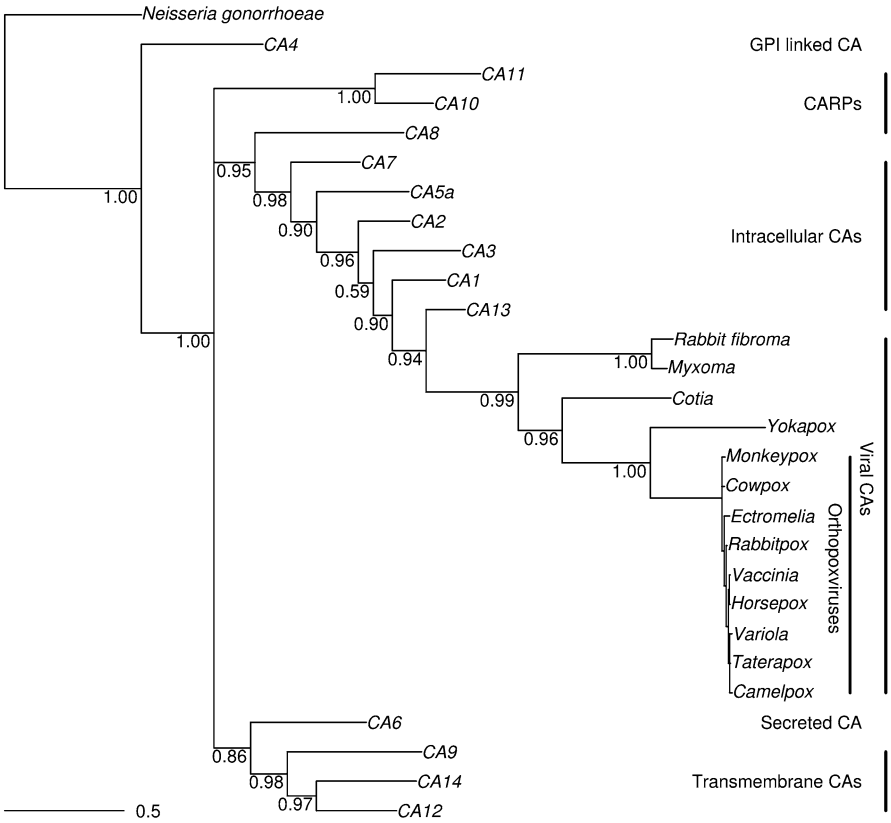

Fig. 8.3 Phylogenetic tree of viral CA-like sequences together with human CA isoforms. Abbreviated virus species names are indicated, for full names and strains and database accession numbers, see Table 8.1. Protein sequences were aligned with Clustal Omega, then replaced with their corresponding cDNA sequences using PAL2NALweb service [78]. This alignment was used with MrBayes v 3.2 [79] to estimate the phylogeny of the sequences using Bayesian inference. Bayesian estimation was run for 40,000 generations, with flat *a priori* distribution of base frequencies, substitution rates, proportion of invariable sites, and gamma shape parameter. The arithmetic mean of the estimated marginal likelihoods for runs sampled was −12386.95. The 50 % majority rule consensus tree was saved and visualized using the APE R package [80]. The tree was rooted using the *Neisseria gonorrhoeae* alpha CA gene as outgroup

rate of medium-active human CA isoforms. Incidentally, the phylogenetic analysis presented in the above paper suggested the closest relatedness with CARP X and XI, but we believe that the methods used in our analysis are more robust, especially if we take into account the approximately two folds substitution rates observed in pox viruses compared to the humans [76, 77]. Therefore, we suggest that viral CARPs are related more closely to intracellular CAs than to genomic CARPs.

6 Conclusions

We have reviewed above the major groups of acatalytic α-CAs, a.k.a CARPs, namely CARP VIII; CARP X-like, including CARP X, CARP XI, and their invertebrate orthologs; CARP XVI domains in PTPRG and PTPRZ; and viral CARPs. With the exception of CARP X and XI, we now know ligands for all of these CARPs, and in some cases even the consequences of binding. The study of CARPs has made it evident that the CA fold can serve as a scaffold for developing various binding functions instead of the original catalytic activity. This can be taken as evidence for the robustness of the fold, a robustness which is also highlighted by the experiments which have restored the catalytic activity of CARP VIII, CARP X, CARP XI, and a viral CARP, still after tens or hundreds of millions of years of evolution in acatalytic functions.

CARP X and XI are the isoforms which remain the most mysterious, without any published information on their actual functions. The very high degree of sequence conservation of the CARP X-like proteins and their presence in neural tissues in all animals which have a distinct brain (triploblasts) would open much room for speculation of very essential functions, but we need to wait for more experimental data before any conclusions can be made.

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