HHS Public Access

Author manuscript

Gene. Author manuscript; available in PMC 2018 August 05.

Published in final edited form as:

Gene. 2017 August 05; 623: 33–40. doi:10.1016/j.gene.2017.04.027.

Carbonic Anhydrase XII Functions in Health and Disease

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Abstract

Human CAXII was initially identified as a cancer marker in different cancers and tumors. Expression of CAXII is regulated by hypoxia and estrogen receptors. CAXII expression has been also detected in several tissues, whereas in cancer and tumor tissues its expression is several fold higher. In brain tumors, an alternatively spliced form of CAXII is expressed. Higher expression of CAXII in breast cancer is indicative of lower grade disease. CAXII plays a key role in several physiological functions. Mutation in the CAXII gene causes cystic fibrosis-like syndrome and salt wasting disease. CAXII is also seen in nuclear pulposus cells of the vertebrae. Aging dependent stiffness or degeneration of backbone correlates with CAXII expression level. This finding suggests a possible implication of CAXII as a biomarker for chronic back pain and a pharmacological target for possible treatment of chronic back pain.

INTRODUCTION

Carbonic anhydrases (CAs) catalyze a reversible reaction of carbon dioxide hydration and dehydration (1; 2). Based on cellular and sub-cellular location, CAs are classified into four different groups: cytosolic (CA I, II, III, VII, XIII); mitochondrial (CA VA, VB); secretory (CAVI), and membrane associated (CA IV, IX, XII, XIV, XV) (1–6). These isoforms differ with respect to enzyme activity and extent of inhibition with CA-inhibitors (2; 3). All membrane-associated CAs are high activity enzymes and have all essential histidine residues for enzyme activity (3; 5). With the exception of human CAIV, all membrane associated CAs are glycoproteins (7–10). Oligosaccharide moieties of the enzyme do not affect structure and function of the protein (9). Membrane associated CAs contain one or two disulfide bonds that are essential for the native structure and optimal function of the enzyme (10). Reduction of the disulfide bridge with reducing agents results in loss of enzyme activity (10). CA-related proteins have been identified, which include structural homologues of the enzyme without anhydrase activity due to lack of essential histidine residues at the active site (4). Several CA-isoforms play important roles in different physiological functions (4; 5; 7–15).

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Carbonic anhydrase IV was the first membrane associated, GPI-anchored carbonic anhydrase that has been extensively characterized for its structure and function (16). Mutations in the leader sequence or in the gene body cause retinitis pigmentosa-17 (RP-17), a protein folding disease in humans (17). Progressive renal injuries have also been observed in transgenic mice expressing folding mutants of CAIV (18). Recently, it has been reported that mouse CAIV is also a tumor suppresser protein as well (19), and it may have some role in wound healing (Altonen M., Barker H., Pan P., unpublished observations, 2016).

CAXII and CAIX, both membrane-associated enzymes, have been characterized as tumor markers in human cancers (20). Expression of both membrane-associated CAs was induced by hypoxia in breast tumors and in several cancer cell lines (21–23). In breast tumors, only CAXII expression, and not CAIX, is under ER regulation (24; 25). CAXII expression in breast tumors is indicative of lower grade disease, lower relapse rates, and better overall patient survival (26; 27). An alternatively spliced form of CAXII is expressed in brain tumors, where its expression is associated with poorer prognosis (28). Mutation in CAXII has also been associated with the cystic fibrosis-like syndrome, including hyponatremia (29–32).

CAXII was initially identified from human renal clear cell carcinoma using the serological expression screening method (21). Simultaneously, CAXII was also cloned from renal carcinoma cells as a target of the von Hippel Lindau (VHL) gene using the RNA differential display method (22). Overexpression of wild type VHL in cancer cell lines reduced the overexpression of the CAXII gene, while overexpression of mutant VHL enhanced the expression of CAXII protein (22). CAXII expression, like CAIX, is induced by hypoxia in different cell lines (22). Unlike CAIX, CAXII is expressed in breast and renal tissues (23). The expression of CAXII is under estrogen receptor regulation (25–27; 33) and expression in breast tumors is associated with positive ER alpha receptor status (26; 27).

To understand the role of CAXII in renal physiology (34), carcinogenesis (35; 36), astrocytic gliomas (28), and hyponatremia (29; 30), CAXII cDNA has been cloned from human and mouse kidney tissues (21; 22). The cDNAs were expressed in heterologous mammalian cell lines (21) and in *E. coli* to produce recombinant CAXII proteins (8; 9). Recombinant CAXII has been used to study the structure and function of the enzyme by crystallography (9) and enzyme kinetics (8). Affinity purified enzymes have been used to produce rabbit specific CAXII antibodies and the antibodies were characterized by immunological procedures and used for immunohistochemistry of CAXII (8; 9; 21–23; 28; 32; 35; 36).

Carbonic anhydrase XII regulates pH and CO_2 homeostasis in different tissues and in cancer cells and tumors. CAXII is widely expressed (22; 23; 37).

GENOMIC ORGANIZATION AND CHROMOSOMAL LOCALIZATION OF CAXII

The mouse CAXII cDNA is 3,716 bp long and contains 11 coding exons that encode a protein of 354 amino acid residues (21; 35). The human CAXII gene locus has been mapped to 15q22, a band sensitive to amplification in some human cancers (21). The genomic organization of the CAXII gene is similar to other human carbonic anhydrase genes (2).

Genomic organization of CAXII has been compared between humans and mice. The results are summarized in Figure 1. The amino acid sequences are depicted in Figure 2 (22).

GENOMIC CLONING OF CAXII

Full-length human CAXII cDNA was isolated from cDNA expression libraries developed from renal clear cell carcinoma (RCC) of a 69-year-old woman (21). Immunoreactive clones against autologous serum of RCC patients was isolated and characterized as described (21). Full-length cDNA was also cloned from human kidney (21). Initially, a 4.5-kb transcript that was overexpressed in RCC and not in surrounding tissues was characterized. Sequence analysis of the transcript suggested that it is a partial transcript of 1,670 bp. Therefore, a partial transcript was cloned into a 1,032-bp fragment using the technique of rapid amplification of cDNA ends. This analysis gave rise to a complete human CAXII cDNA clone encoding 7 bp of 5' untranslated sequence containing a classical initiation codon, followed by a 1,062-bp open reading frame and 1,584 bp of 3' untranslated sequence (21).

MECHANISM OF CAXII FUNCTION

CAXII, like many alpha-CAs, shows great homology of amino acid sequences near the active site, folding of polypeptides, and three-dimensional structure (9). The active site amino acids are occupied with a Zn^+ ion that is coordinated with three histidine residues, His94, His96, and His119, and a water molecule or hydroxide ion (9). In summary, Zn^+ with a hydroxyl ion binds to a CO_2 molecule and catalyzes the formation of bicarbonate. The bicarbonate ion is displaced by a water molecule by releasing into water or buffer solution. This reaction can be described using the following equations:

$$\mathrm{Ezn{-}OH^- + CO_2 = Ezn{-}HCO_3^-}$$
$$\mathrm{Enz{-}HCO_3^- + H_2O = Enz^+{-}OH_2 + HCO_3^-} \quad \text{Equation (1)}$$

$$\mathrm{EZn^+{-}OH_2 = EZn^+{-}OH^- + H^+} \quad \text{Equation (2)}$$

The rate-limiting step is the proton transfer reaction that is carried by proton transfer histidine residue 64 (His64).

MOLECULAR CHARACTERIZATION OF CAXII

The open reading frame of CAXII predicted a protein of 354 amino acids with apparent molecular weight of 39.4 kDa (21). A homology search of CAXII showed homology to a conserved region of human carbonic anhydrases and CA-related proteins (21). Deduced amino acid residues from CAXII cDNA predicted 29 amino acids at the N-terminal as a signal sequence preceding the predicted cleavage site between Gly-1 and Ser-1. A 261-amino acid carbonic anhydrase homology domain of CAXII contains all conserved amino acid residues near the active site, a common feature among all carbonic anhydrases (21). CAXII also contains all critical histidine residues, His-94, His-96, and His-119, that are

necessary for zinc binding, and also His-64, which contributes to the efficiency of the enzyme by acting as proton shuttle between the Zn-bound water molecule and surrounding buffer molecules (21). The extracellular CA-domain contains two potential asparagine residues for N-linked glycosylation. The N-linked glycosylation of the enzyme was confirmed by the treatment of CAXII with endoglycosydases.

The removal of oligosaccharides by Endo F from CAXII results in a decrease in the apparent molecular mass of the protein (21). The extracellular CA domain of the enzyme also contains four cystine residues, two of which, Cys-23 and Cys-203, might form an important disulfide bond that is conserved among other membrane associated carbonic anhydrases to provide structural stability and function to the enzyme molecule (21). The hydropathy plot of the CA domain suggests a 26-amino acid hydrophobic segment of the protein that serves as a transmembrane domain for anchoring the enzyme to the plasma membrane (9; 21). The transmembrane domain of CAXII contains two distinct motifs, GXXXG and GXXXS, which are involved in dimerization of the protein polypeptides (9; 21). Beyond the transmembrane domain, a 29-amino acid hydrophilic cytoplasmic domain at the C-terminus contains two potential sites for phosphorylation (9; 21). It has been suggested that phosphorylation of these sites might play a role in metabolic signaling by recruiting kinases to the cytosolic tail of the CAXII (9). The biological significance of the cytosolic tail was further suggested when an alternate spliced isoform of CAXII was identified in astrocytic gliomas (28). The mutant CAXII in astrocytomas was 11 amino acids shorter than the wild type enzyme. The shorter alternatively spliced isoform of CAXII lacks the unique GXXXG sequence, resulting in a loss of quaternary structure and metabolic signaling function (21; 28). Deletion of the hydrophobic, membrane spanning domain and cytosolic tail allows expression of a fully active secretory form of CAXII.

PURIFICATION OF RECOMBINANT CAXII

The secretory form of CAXII cDNA was cloned into a mammalian (21) or bacterial expression system (9) to produce and purify recombinant CAXII enzyme for specific antibody production (8; 9; 21; 23), enzyme kinetic measurements (8), and for crystallographic studies to determine the 3D structure of the CAXII enzyme (9). Using CA-inhibitor affinity chromatography (p-aminobenzene sulfonamide linked Sepharose resin), CAXII was purified from secretion medium of CHO cells or *E. coli* extracts expressing CAXII (8; 9; 21; 23).

STRUCTURE/FUNCTION OF CAXII

The affinity purified CAXII produced from *E. coli* extracts migrates as a 30-kDa protein; however, CAXII purified from CHO cell secretions showed a 35-kDa peptide on SDS-PAGE under reducing conditions, suggesting that CAXII is a glycoprotein (9). CAXII isolated from CHO cell secretions was also eluted as a dimeric protein on a sizing column. These results suggested that the secretory form of CAXII enzyme is a dimeric glycoprotein (9). This finding is a very striking difference between CAXII and all other soluble or membrane associated carbonic anhydrases (7; 10; 11). Purified human CAXII is a dimeric enzyme in solution as well as in crystal form (9). Both glycosylated and non-glycosylated CAXII

showed similar crystal structures, suggesting that the oligosaccharide chain does not affect protein folding (9). The polypeptide fold of CAXII is similar to the beta-fold observed for other CA-isozymes (9). The amino acid sequence of CAXII derived from cDNA showed two glycosylation sites at Asn-52 and Asp-136 and a single disulfide bond between Cys-23 and Cys-203 (21). This disulfide bond is also present in CAIV, where it helps to stabilize the structure and function of the enzyme (8; 9; 21).

Further molecular characterization was carried out by determining the high resolution, 3D structure of the recombinant enzyme by x-ray crystallographic methods at 1.55 Å resolution. The structure of CAXII showed that two CAXII domains associate to form an isologous dimer that was also seen by gel-filtration studies of the enzyme in solution. The dimer interface is located such that the active site of each monomer is fully exposed for optimum function of the enzyme. A high-resolution structure of CAXII without and with CA-inhibitor provides a further tool for possible use in chemotherapy (9). The crystal structure is shown in Figure 3.

The active site structure of CAXII is nearly identical to that of CAII and CAIV (9). The catalysis of CO_2 hydration by CAXII shows a maximum K_{cat}/K_m value of 34 μM per second that is similar to membrane associated CAIV (16). The catalytic proton shuttle, His-64, is conserved in CAXII (8; 9; 16; 21). Substitution of His-64 suggested that it plays an important function in proton shuttling (8) Inhibition of CAXII by a 10 μM concentration of acetazolamide was found to inhibit invasion of renal carcinoma cells *in vitro* (39). In a separate study, CAXII knockdown has shown an effect on invasion, migration, and cell growth of breast cancer cells (40).

EXPRESSION OF CAXII IN DIFFERENT TISSUES

Expression of CAXII protein has been observed in different tissues (21; 22), including the basolateral membrane of epithelial cells of endometrium (41) and the basolateral surface of the epithelial cells in male efferent ducts (42). From these results, it has been concluded that CAXII may play an important role in bicarbonate and proton homeostasis in reproductive tissues (41; 42).

CAXII has been detected in the human gut, including all segments of the large intestine; however, it is absent from the small intestine (36). Immunostaining of CAXII has also been observed at the basolateral plasma membrane of enterocytes (36). Therefore, CAXII seems to play an important role in acidification and concentration of extracellular fluids in the human gut (36). CAXII is also weakly expressed in the gastric mucosa (43).

In human renal tissues, CAXII is expressed at the basolateral plasma membrane of epithelial cells in the thick ascending limb of the loop of Henle, distal convoluted tubules, and principal cells of the collecting ducts (35; 44). A weak signal of immunostaining of CAXII is also seen in the epithelium of the proximal convoluted tubules.

CAXII expression in the pancreatic epithelium at the basolateral plasma membrane of acinar and duct cells was seen (44).

In the human eye, CAXII is expressed in the non-pigmented ciliary epithelial cells, suggesting a role in humor production (45). Expression of CAXII in human eyes has been shown to increase in patients with glaucoma (45).

CAXII is also expressed in sweat glands and the enzyme might be responsible for salt homeostasis in sweat (29–32).

CAXII is expressed in different tissues, including mouse and rat kidney (46), gastrointestinal tract (47), and endometrium (48). Staining of CAXII has also been observed in the corpus and proximal caudal regions of the epididymis (49). The wide-spread expression of CAXII in mammalian tissues is a testament to its physiological significance (46–49).

EXPRESSION OF CAXII IN TUMOR AND CANCER CELLS

Following initial CAXII discovery as tumor marker (21; 22), expression of CAXII has been reported in different cancers (22) with higher expression in tumors than tissues (35). It has also been reported that the level of CAXII expression in cancer may be correlated with the outcome of the disease from poor to favorable prognosis (35).

In kidney cancer, CAXII expression has been found mostly in clear cell carcinomas and oncocytomas (35). In clear cell carcinomas, the level of CAXII expression correlates with the histological grade of the tumor (35).

In colorectal tumors, CAXII expression is very distinct from tissues (36). The extent of positive staining of CAXII was found to increase with a high grade of dysplasia (36).

Overexpression of CAXII has been detected in brain tumors, gliomas, hemangioblastomas, and meningiomas (50). CAXII expression has also been detected in diffuse astrocytomas (28).

CAXII expression has been detected in many other tumors and cancers, such as breast cancer (39), non-small cell lung cancer (51), and cervical cancer (52). In breast cancer, the degree of CAXII expression predicts a favorable outcome of the disease (27).

EXPRESSION OF CAXII DURING EMBRYONIC DEVELOPMENT

CAXII expression has been detected in a variety of mouse embryonic tissues, including the central nervous system, heart, lung, stomach, pancreas, liver, GI-tract, and urogenital tissues (53).

Expression of CAXII in human embryonic tissues is limited to cells involved in secretion and absorption of water (54).

MOLECULAR MECHANISM OF CAXII EXPRESSION

Expression of both CAXII and CAIX as tumor markers is regulated by hypoxia and they are known as hypoxia inducible genes (23; 37). Until recently, only CAXII, and not CAIX, showed a strong positive correlation with estrogen receptor (ER) expression in breast tumors

(24; 25). It has been shown that CAXII is upregulated by estrogen using ER-α in breast cancer cells. This regulation involves ER binding to a distal estrogen-responsive enhancer region of CAXII (33). This study suggests an important role of ER in CAXII expression (33).

PHYSIOLOGICAL FUNCTIONS OF CAXII

RENAL FUNCTION

Approximately 95% of the total CA activity in the kidney comes from the cytosolic fraction of the enzyme and about 5% of the remaining activity is due to the membrane-associated enzyme that comprises CAIV, CAXII, and CAXIV (6; 7; 55; 56). CAIX is not expressed in kidney; however, its expression has been observed in renal cancer tissues (21; 22; 35; 46). In luminal membranes of the kidney, membrane associated CAs catalyze the dehydration of carbonic acid (H_2CO_3) to CO_2 and water for removal of bicarbonate (57). CAXII is localized at the basolateral surface of the nephrons where efflux of bicarbonate is enhanced by CAXII (31; 32). CAXII also plays an important role for fluid and bicarbonate homeostasis in epithelial cells (31; 34), suggesting an important role of CAXII in kidney physiology (31; 34). CAXII may also function in the kidney as a metabolan of the membrane-associated CAXII and the chloride/bicarbonate exchanger (AE1) (31; 32) or sodium/bicarbonate co-transporter (NBCe) (32), and proton anti-porter (34; 58).

NEURONAL FUNCTION

Several observations have been reported for extracellular CA function in the neuronal activity of rat cerebellum (59). Initially, extracellular CA activity was identified as CAIV. CAIV was the only known CA isozyme that associated with the extracellular surface of neuronal endothelial and epithelial cells (60). Based on EST data, two more membrane associated CAs, CAXII and CAXIV, are also expressed in the brain (61; 62). Using CAIV and CAXIV knockout mice, the physiological function of both membrane associated CAs has been analyzed (38). The buffering efficiency of the extracellular space of the brain and response to light in mouse eyes have been studied, and both CAIV and CAXIV showed their respective roles (38). However, there has been no study to implicate CAXII in brain physiology.

EYE FUNCTION

Membrane associated carbonic anhydrases are important for eye function (63; 64). Mutations in the signal sequence of CAIV are associated with an autosomal dominant eye disease, retinitis pigmentosa or RP-17 (63; 64). CAIV and CAXIV double knockout mice showed abnormality in eye physiology (65). Expression of CAIX and CAXII transcripts in non-pigmented epithelial cells (NPE) has been reported (45), and the NPE from glaucoma patients showed increased CAXII gene expression. From these results, it has been concluded that CAXII is expressed in ciliary cells, and thus, may be involved in aqueous humor production (45).

SKELETAL AND CARDIAC MUSCLE FUNCTION

Both the sodium-proton exchanger (NHE1) and the chloride-bicarbonate exchanger are functionally linked to cytosolic CA and membrane associated CAs (66). CAXII in mouse heart has not been detected. However, membrane associated CAIV, CAIX, and CAXIV in mouse skeletal muscle play an important role (67; 68). It is uncertain whether human skeletal and cardiac muscle fibers may also require membrane associated CAXII for their function.

CAXII FUNCTIONS IN METABOLONS

After it was discovered that the association of CAII with AE proteins enhances the transport of bicarbonate ions (69), CAIV and CAIX (70) have also been shown to associate with AE proteins and enhance bicarbonate transport. Most recently, it was reported that CAXII regulates the function of the chloride-bicarbonate exchanger (AE2) by associating with the proteins (31; 34). Mutations in bicarbonate transporters such as CFTR, AE2, and NBCe1 cause defects in fluid homeostasis and bicarbonate secretion (71; 72), which results in pathological diseases like cystic fibrosis (73), pancreatitis (71; 74), and Sjogren's syndrome (71; 72).

LOSS OF FUNCTION MUTATIONS IN CAXII RESULT IN CYSTIC FIBROSIS-LIKE DISEASE

Mutations in CAXII have been associated with an autosomal recessive form of salt wasting disease that results in hyponatremia in two consanguineous Bedouin kindred (29; 30). Clinical symptoms include high sweat chloride concentration, dehydration, and failure to thrive in infancy (30). In both pedigrees, similar missense mutations were observed. The effect of mutation on the enzyme activity was analyzed and a modest reduction (30%) in enzyme activity was observed. This result was unexpected for an autosomal recessive disorder where mutations generally result in complete loss of function. Thus, these authors suggested that the partial reduction in CAXII function results in a pathological phenotype limited to the sweat glands (29; 30).

In a separate study, Lee et al. (32) have shown that CAXII, with either a His121Gln or Glu143Lys mutation, localized to the basolateral membranes of MDCK cells, similar to wild type CAXII. However, the enzyme activity of the CAXII mutants was completely diminished at physiological concentrations of sodium chloride. Thus, loss of function of CAXII in sweat glands and lungs is the molecular basis in Cystic Fibrosis-like patients with normal CFTR. Since CAXII and AE2 are part of the metabolan (31; 34), mutant CAXII might compromise epithelial AE2 function, resulting in a decrease of fluid secretion and causing dry mouth syndrome in patients with a mutation in the CAXII enzyme (31; 32; 34).

CARBONIC ANHYDRASE XII IS A FUNCTIONAL BIO-MARKER OF NUCLEUS PULPOSUS

Degeneration of inter-vertebral discs (IVD) is associated with chronic neck and lower back pain (75). This degeneration results in a major disability causing many health issues. The IVD contains two distinct interdependent tissues: a gelatinous center, known as the nucleus pulposus (NP), and the surrounding coaxial lamellae that generate the inner and outer annulus fibrosus (AF). Due to natural aging, the gelatinous NP region of the disc becomes a more rigid and fibrous disc. This results in chronic back pain (76).

In order to develop specific therapeutic drugs, NP cell specific proteins were studied by DNA microarray analyses (77). Several hundred genes are upregulated in NP cells of which 90 contained trans-membrane domains. Twenty-eight were further characterized by RT-PCR. The most prominent signal was observed in CAXII genes of NP cells from young subjects and in degenerative tissues.

From these results, it was concluded that for the diagnosis of degenerative disc causing back pain, CAXII may be a suitable extracellular biomarker and may be applicable for therapeutic targets. The presence of CAXII in NP cells further suggests a role for CAXII in intracellular homeostasis of proton and bicarbonate ions. Thus, CAXII may function to normalize the cellular acidosis in the hypoxic NP cells under chronic back pain syndrome.

Acknowledgments

This review and the corresponding Gene Wiki article are written as part of the Gene Wiki Review series--a series resulting from a collaboration between the journal GENE and the Gene Wiki Initiative. The Gene Wiki Initiative is supported by National Institutes of Health (GM089820). Additional support for Gene Wiki Reviews is provided by Elsevier, the publisher of GENE. The authors would like to thank Tracey Baird and Medina Hasanagic for editorial assistance in the preparation of this manuscript.

The corresponding Gene Wiki entry for this review can be found here: ≪https://en.wikipedia.org/wiki/CA12≫.

List of Abbreviations

CA	carbonic anhydrase
GPI	glycophoshatidylinositol lipid anchor
VHL	von Hippel Lindau
RCC	renal clear carcinoma
CHO cells	Chinese hamster ovary cells
E. coli	Escherichia coli
GI-tract	gastrointestinal tract
ER	estrogen receptor
AE1	anion exchanger isoform 1

NBCe	sodium bicarbonate cotransporter isoform e
NPE	non-pigmented epithelial cells
NHE1	sodium proton exchanger
AE2	chloride-bicarbonate exchanger 2
CFTR	cystic fibrosis transporter receptor

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Highlights

- CAXII is a cancer marker
- CAXII gene is a target of VHL gene
- CAXII expression is under estrogen receptor regulation
- CAXII expression in breast is indicative of lower grade disease and better patient survival
- Alternatively spliced form of CAXII is expressed in brain tumors
- Mutant form of CAXII is associated with cystic fibrosis-like syndrome
- CAXII is a functional biomarker of nuclear pulposus

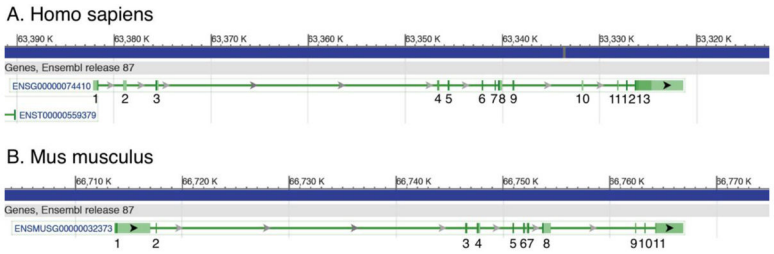

Figure 1.

Genomic organization of CAXII gene. A. The Homo sapiens gene contains 13 exons whereas the mouse gene (B) has 11 exons (38). Other mouse CA genes contain different numbers of exons; however, the functional domains are highly conserved (2). The amino acid sequences for Homo sapiens (Gene ID 771) and Mus musculus (Gene ID 76459) shown below were obtained from the Gene database in NCBI using the Ensembl Genome Browser (release 87).

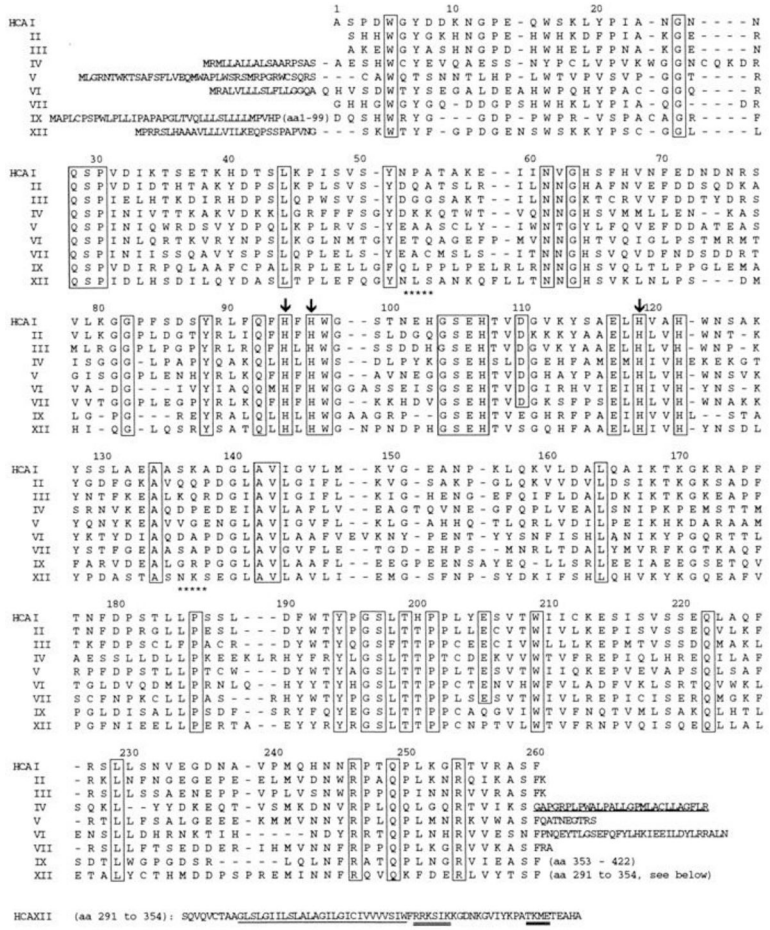

Figure 2.

Amino acid sequence homology of human carbonic anhydrases. Sequence homology alignment of human CAs show highly conserved residues among all enzymes. Conserved amino acids near the active site, including important histidine residues, are boxed. Metal binding histidine residues are marked by an arrow. Glycosylation sites are not conserved among all CAs and are indicated for CAXII by stars. Trans-membrane domains and phosphorylation sites are underlined (21), Copyright 1998.

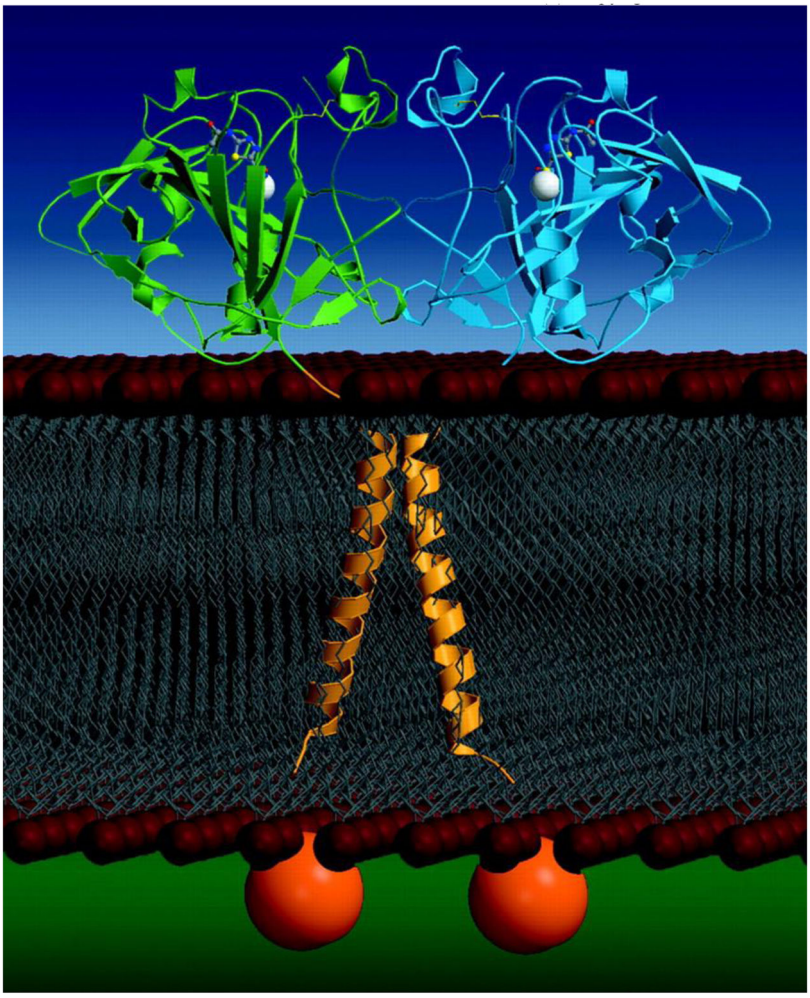

Figure 3.

Crystal structure of CAXII. Human carbonic anhydrase XII was crystallized at pH 7.5. Diffraction results of CAXII at 1.55 Å resolution suggested that the crystals belong to space group C2 with unit dimensions a=1.46.7 Å, b=44.6 Å, c=85.2 Å, and B=94.1 Å. One homodimer occupied the asymmetric unit. The native structure of CAXII consists of a dimer containing two zinc atoms, two Zn bound acetate ions, and 537 water molecules (9), Copyright 2001.