Localization and expression of carbonic anhydrase isozyme XV in mouse tissues

Master's Thesis

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PRO GRADU -TUTKIELMA

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Hiilihappoanhydraasi XV (CA XV) on α-hiilihappoanhydraasi-entsyymiperheen viimeisin jäsen. Kädellisissä sitä koodaavasta geenistä on tullut pseudogeeni ja sen fysiologinen merkitys muissa lajeissa on vielä tuntematon. Tutkimuksen tavoitteena oli selvittää CA XV:n sijainti hiiren kudoksissa ja selvittää sen mahdollisia kompensoivia ominaisuuksia muiden hiilihappoanhydraasien suhteen poistogeenisissä kudoksissa.

Tutkimusmenetelmät: CA XV paikannettiin hiiren kudoksista immunohistokemian avulla ja sen sijaintia vertailtiin muiden isoentsyymien esiintymiseen. CA XV:n kompensoivaa merkitystä CA IV ja CA XIV poistogeenisissä kudoksissa tutkittiin immunohistokemian lisäksi myös geeni-ilmentymisen tasolla kvantitatiivisella reaaliaikaisella PCR:llä (qRT-PCR).

Tutkimustulokset: CA XV:n esiintyminen hiiren kudoksissa on hiilihappoanhydraasi-isoentsyymeistä rajoittuneinta. Entsyymiä esiintyy vain munuaisissa, jossa sitä havaittiin eniten kuorikerroksen alueella mutta myös jonkin verran ytimen ulommassa osassa. Munuaistiehyessä entsyymiä esiintyy Henlen lingon paksussa nousevassa osassa sekä kokoojatiehyessä. Vertailevat värjäykset osoittivat, että tämä esiintymiskaava on ainutlaatuinen munuaisissa esiintyvien isoentsyymien keskuudessa. Immunohistokemialla oli havaittavissa pieniä muutoksia CA XV:n esiintymisessä poistogeenisissä kudoksissa, mutta transkriptionaalisella tasolla CA IV tai CA XIV puuttumisella ei ollut vaikutusta *Car15*:n geenin ilmentymiseen.

Johtopäätökset: Immunohistokemialla havaittu voimakas reaktio munuaisissa viittaa siihen, että CA XV:llä on oleellinen merkitys munuaisten fysiologiassa ja isoentsyymi tulisi ottaa huomioon tulevissa eläinmalleja hyödyntävissä inhibiittoritutkimuksissa.

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Abstract

Background and aims: Carbonic anhydrase XV (CA XV) is the most recently found member of the α-CA family. The isoform has become a pseudogene in primates and its physiological role in other species is yet unknown. The aim of the study was to localize CA XV in mouse tissue and study its possible compensatory role relative to other CA isozymes in knock-out tissues.

Methods: Immunohistochemistry was used in localization of CA XV in mouse tissues as well as in co-localization studies with other CA isoforms. In addition to immunohistochemistry, quantitative real-time PCR (qRT-PCR) was used to detect compensatory effects on *Car15* mRNA levels in CA IV and CA XIV knock-out tissues.

Results: Localization studies showed that the distribution of CA XV is most limited of all CA isozymes as it was only expressed in the kidney where the most abundant expression was detected in the cortex region, although, some activity was also found in the outer medulla. Along the nephron, the isozyme was present in the thick ascending limbs of Henle and the collecting ducts. Parallel stainings for other CA isozymes showed this to be a unique distribution pattern among all other isoforms found in kidney. Some changes in distribution were detected in CA IV and CA XIV knock-out sections in the immunohistochemical stainings, but there were no compensating changes in *Car15* expression at the transcriptional level.

Conclusions: The intense reaction for CA XV in rodent kidney along with moderate activity suggests a substantial role in renal physiology, and the presence of this isoform should be taken into account in future studies on CA inhibitors when using animal models.

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ABBREVIATIONS

AE	anion exchanger
BSA	bovine serum albumin
CA	carbonic anhydrase
CA15	carbonic anhydrase 15 (refers to the human gene)
Car15	carbonic anhydrase 15 (refers to the mouse gene)
cDNA	complementary deoxyribonucleic acid
DAB	3,3'-diaminobenzidine
ER	endoplasmic reticulum
GABA	gamma-aminobutyric acid
GI	gastrointestinal
GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
HIF-1	hypoxia inducible factor 1
HRE	hypoxia-responsive element
HRP	horseradish peroxidase
IgG	immunoglobulin G
kAE	kidney anion exchanger
mGAPDH	mouse glyceraldehyde-6-phosphate dehydrogenase
mRNA	messenger ribonucleic acid
NBC	sodium (Na) bicarbonate co-transporter
NHE	Na+/H+ exchanger
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase-antiperoxidase method
PBS	phosphate-buffered saline
PG	proteoglycan
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
PI-PLC	phosphatidylinositolspecific phospholipase C
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative real time polymerase chain reaction
RP	retinis pigmentosa
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TBST	tris-buffered saline with tween
THP	Tamm-Horsfall glycoprotein
VHL	von Hippel-Lindau

1. INTRODUCTION

Carbonic anhydrases (CAs) are zinc-metalloenzymes that contribute to the maintenance of acid-base homeostasis by catalyzing the reverse hydration of carbon dioxide and facilitating transport of bicarbonate. Of the mammalian α-CA family, 13 active members have been discovered and they are expressed ubiquitously in different cell types and tissues. The physiological roles of these isozymes vary due to differences in their kinetic properties as well as the type of cells and tissues where they are localized (Chegwidden & Carter, 2000).

Immunohistochemistry has made a considerable contribution to the localization of α-CAs beyond the erythrocyte where the enzyme was first discovered. Now, the distribution of each known isozyme is quite well known in normal physiological states as well as in some diseases and they all seem to possess a unique pattern of distribution. CA II, as the most active isozyme, is present in all major mammalian tissues including blood cells, kidney, liver, bone and intestine, while for example CA IX shows a much more restricted distribution pattern (Chegwidden & Carter, 2000, Saarnio et al., 1998). However, in carcinomas CA IX has been localized also in other tissues, including the kidney, where it enhances the survival and growth of tumor cells. These isozyme-specific features as well as the vital role of CAs have aroused a need for isozyme-specific inhibitors in drug development. Despite individual inhibition profiles the task is not simple and the differences in distribution and kinetic properties of CAs between mammalian species only scale up the level of difficulty (Supuran, 2008).

The most recently discovered member of the α-CA family is CA XV whose physiological role is unclear. The isozyme has many interesting characteristics as it is the first α-CA that is present in rodents but has become a pseudogene in primates including humans. The structure resembles that of CA IV as both are attached to the cell membrane through a glycosylphosphatidylinositol (GPI)-anchor and resistant to denaturation by SDS (Hilvo et al., 2008b). The activity of rodent CA IV is only one fifth of that of human CA IV and another isoform, CA XIV, has been found to compensate this loss of activity at least in brain. CA XV has been suggested to play a similar role which would explain why the enzyme has become redundant in primates

(Hilvo et al., 2008b, Shah et al., 2005). In a previous study, RT-PCR and *in situ* hybridization on mouse tissue suggested localization in the kidney, testis and brain (Hilvo et al., 2005). Aims of this thesis were to localize CA XV in mouse tissues by immunohistochemistry as well as study possible changes in the distribution pattern and *Car15* expression levels in *Car4*$^{-/-}$ and *Car14*$^{-/-}$ tissues.

2. REVIEW OF THE LITERATURE

2.1. ACID-BASE BALANCE

Maintenance of the acid-base homeostasis is one of the most critical physiological features that enable the function of biological housekeeping processes. Although most proteins are modestly sensitive for changes in pH, for some, even a slight shift in the balance may have a substantial effect on the conformation and biological activity. The alterations in protein function will lead to changes in a variety of biochemical reactions, channel/transporter function, and protein-protein interactions, and therefore, also cellular processes such as cell proliferation, cell cycle progression and apoptosis. In mammals, the pH range of extracellular fluid and most cytosolic compartments is from 6.9 to 7.4. Although the intracellular acid-base homeostasis is tightly regulated, it is highly affected by changes in the extracellular pH which, in turn, is strictly regulated by several buffer systems of the body. The main organs to adjust the pH of the extracellular fluid and prevent the body from entering acidosis or alkalosis are kidneys and lungs. The lungs act within a few minutes while the kidneys are responsible for pH regulation over longer periods of time (Boron, 2005).

The regulation systems that react fastest to changes in pH are the chemical buffer systems. In human physiology, there are several buffer systems including bicarbonate, phosphate and ammonia buffer systems as well as cytosolic proteins that contain functional groups, such as phosphate groups, acting as weak acids or bases. One well known protein involved in pH regulation is hemoglobin in the blood. Of the three main buffer systems, ammonia system buffers urine while the phosphate system acts in the cytoplasm of the cells using $H_2PO_4^-$ as a proton donor and HPO_4^{2-} as a proton acceptor: $H_2PO_4^- \leftrightarrow H^+ + HPO_4^{2-}$. However, the bicarbonate buffer system is considered to be the most important of the buffer systems. It is the main buffer system in blood plasma and consists of carbonic acid, H_2CO_3, as proton donor and bicarbonate, HCO_3^-, as a proton acceptor: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$ (Nelson & Cox, 2005). It is an open system as the concentration of carbonic acid is determined by the concentration of dissolved carbon dioxide and water. The concentration of dissolved CO_2, in turn, depends on the partial pressure of gaseous CO_2 in the air space of the lungs. By inhaling and exhaling CO_2, the lungs are able to maintain a stable CO_2 concentration in the blood

plasma despite the physiological conditions (Boron, 2005). For example, when H+ is induced into the blood plasma from muscle tissue during vigorous exercise, it fuses with bicarbonate and the concentration of H_2CO_3 is increased. This increase causes a raise in the concentration of dissolved CO_2 which, in turn, increases the pressure of gaseous CO_2 in the lungs. The excess CO_2 is finally exhaled (Nelson & Cox, 2005).

The regulation of intracellular and tissue pH is further affected by the transport of HCO_3^- -related species. This is especially important in renal tubules of the kidney where the pH is regulated by reabsorbing and returning bicarbonate to the blood circulation as well as in the gastrointestinal tract in promoting the digestion of the foodstuffs and in male reproductive tract to render spermatozoa quiescent. The transport is able to affect the pH in response to changing demands of a single cell as well as the whole body (Boron, 2005).

In the reaction $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$, the dissociation of carbonic acid to form bicarbonate and a proton is a fast reaction but the hydration of carbon dioxide is too slow to respond to physiological needs (Supuran, 2008). This problem has been solved by generating an enzyme family today known as carbonic anhydrases. These enzymes catalyze the reversible hydration reaction in which carbon dioxide is directly hydrated to form a proton and bicarbonate: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$ (Supuran, 2008, Boron, 2005).

2.2. CARBONIC ANHYDRASES

2.2.1. General aspects

Until 70 years ago, when the enzyme known as carbonic anhydrase was discovered, no one really knew how CO_2 was carried in the blood and released in the lung capillaries. One hypothesis was that CO_2 was carried as HCO_3^- but the major problem was that HCO_3^- did not dissociate to CO_2 rapidly enough for the hypothesis to be true. The discovery of carbonic anhydrase in erythrocytes gave the reaction the catalyst it required. Later these enzymes were found to consist of several different enzyme families that are involved in numerous physiological processes including acid-base balance, respiration, bone resorption, calcification as well as ion, gas and fluid transport.

Recently, they have also been found to be involved in cell growth and therefore also play a role in oncogenesis and cancer (Chegwidden & Carter, 2000).

Table 1. The subcellular locations and tissue distribution of the active α-CA isozymes. Mammalian CAs are ubiquitous in their subcellular localization and tissue distribution (Supuran, 2008).

CA isozyme	Subcellular location	Tissue distribution
hCA I	Cytosol	Erythrocytes, GI tract
hCA II	Cytosol	Erythrocytes, eye, GI tract, bone osteoclasts, kidney, lung, testis, brain
hCA III	Cytosol	Skeletal muscle, adipocytes
hCA IV	Membrane-bound	Kidney, lung, pancreas, brain capillaries, colon, heart muscle
hCA VA	Mitochondria	Liver
hCA VB	Mitochondria	Heart and skeletal muscle, pancreas, kidney, spinal cord, GI tract
hCA VI	Secreted	Salivary and mammary glands
hCA VII	Cytosol	Central nervous system, liver, skeletal muscle
hCA IX	Transmembrane	Tumours, GI mucosa
hCA XII	Transmembrane	Renal, intestinal, and reproductive epithelia, eye, tumours
hCA XIII	Cytosol	Kidney, brain, lung, gut, reproductive tract
hCA XIV	Transmembrane	Kidney, brain, liver
mCA XV	Membrane-bound	-

Carbonic anhydrases (CAs) catalyze the reversible hydration of carbon dioxide forming bicarbonate: $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$. Carbonic anhydrases exist in at least five families of isoforms known as α, β, γ, δ, and ζ, and they are present in all living organisms. All mammalian CAs are members of the α-CA family which have also been

found in bacteria, algae and cytoplasm of green plants. In mammals, 16 isoforms of α-CAs have been discovered of which 13 have been found to be active: CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV and XV. Of these, CA I, II, III, VII and XIII are cytosolic, CA VA and VB are located in mitochondria, CA IV, IX, XII, XIV and XV are membrane-bound and CA VI is secreted. α-CAs are zinc-metalloenzymes whose CA-domain is usually around 29 kDa and they are produced ubiquitously in most tissues of mammals. Several different isoforms are often in the same tissue but in different compartments and in different types of cells (Table 1.). The spectrum of activities among the isoforms is wide as CA II is one of the fastest enzymes known, while the activity of CA III is very low (Table 2.). They all possess structural homology and the activation site is highly conserved (Chegwidden & Carter, 2000, Supuran, 2009).

In the active site of the enzyme, the zinc ion is situated at the bottom of a deep cleft where it is coordinated by three histidine residues (His94, His96, His119) and a hydroxide ion/water molecule. Through hydrogen bond interactions, so called gate-keeping residues Thr-199 and Glu-106 enhance the nucleophilicity of the zinc-bound water molecule and orient CO_2 in a favorable location for the nucleophilic attack (Supuran, 2009).

In the active site, the hydration of carbon dioxide happens in two phases:

1. $EZn\text{-}OH^- + CO_2 \leftrightarrow EZn\text{-}HCO_3^- + H_2O \leftrightarrow EZn\text{-}OH_2 + HCO_3^-$

In the first phase of the catalysis the CO_2 molecule is directed to a hydrophobic pocket close to the active site and the zinc-bound hydroxide-group attacks CO_2 by nucleophilic addition forming bicarbonate. The water molecule then displaces the bicarbonate that is released into the solution and with water bound to the zinc the enzyme is in inactive form (Supuran, 2009).

2. $His64\text{-}EZn\text{-}OH_2 \leftrightarrow H^+\text{-}His64\text{-}EZn\text{-}OH^- + B \leftrightarrow His64\text{-}EZn\text{-}OH^- + BH^+$

The second reaction is the rate-limiting step of the catalysis when the enzyme is regenerated into active hydroxide-binding form. In the reaction, a proton is transferred

from zinc-bound water through His-64 that functions as a proton shuttle to a buffer molecule and a hydroxide molecule is again bound to the zinc ion. The proton shuttle formed by His-64 makes CAs effective catalysts that can increase the rate of the reaction up to 6,000 fold (Supuran, 2009).

Table 2. The catalytic activities and affinities for the sulfonamides of the active α-CA isozymes. The activities between the different isozymes vary greatly as well as their affinity for inhibitors (Hilvo et al., 2008a, Supuran, 2008, Supuran, 2009, Supuran & Winum, 2009).

CA Isozyme	**k_{cat} (s^{-1})**	**k_{cat}/K_m (M^{-1} s^{-1})**	**CO_2 Hydration Activity**	**Affinity for Sulfonamides**
hCA I	2.0×10^5	5.0×10^7	Moderate	Medium
hCA II	1.4×10^6	1.5×10^8	High	Very high
hCA III	1.0×10^6	3.0×10^5	Very low	Very low
hCA IV	1.1×10^6	5.1×10^7	High	High
hCA VA	2.9×10^5	2.9×10^7	Low-moderate	High
hCA VB	9.5×10^5	9.8×10^7	High	High
hCA VI	3.4×10^5	4.9×10^7	Moderate	High
hCA VII	9.5×10^5	8.3×10^7	High	Very high
hCA IX	1.1×10^6	1.5×10^8	High	High
hCA XII	4.2×10^5	3.5×10^7	Low	Very high
hCA XIII	1.5×10^5	1.1×10^7	Moderate	Medium-high
hCA XIV	3.1×10^5	3.9×10^7	Moderate	High
mCA XV	4.7×10^5	3.3×10^7	Moderate	-

2.2.2. CA inhibition

There are two main classes of CA inhibitors: metal complexing anions and aromatic and heterocyclic sulfonamides. They bind to the zinc ion by either substituting the non-protein zinc ligand or by adding to the metal coordination sphere (Supuran, 2009). CAs are involved in many physiological processes and therefore represent important therapeutic targets. When first discovered as CA inhibitors, sulfonamides were developed and used as diuretics and antiepileptics (Chegwidden & Carter, 2000). Nowadays, CA inhibitors are extensively used in treatment of glaucoma and they have also shown to be promising anticancer and antiobesity agents (Chegwidden & Carter, 2000, Supuran, 2009). As CAs are widely produced also in protozoa, fungi and bacteria, the potential use of CA inhibitors to fight infections has emerged as a new direction of research (Supuran 2008). Around 30 clinically used drugs or their agents have been reported to possess CA inhibitory properties, but inhibitors targeting solely CAs are used only for treating glaucoma. At the moment the problem with most known CA inhibitors is that they inhibit more than one of the isozymes and their effects range to different tissues and organs. Also, their affinity to their target is often weak. Future aim is to attain more efficient inhibition by designing more selective and specific inhibitors for individual isozymes (Supuran, 2009). One way is to design membrane-impermeant CA inhibitors that would then only inhibit the extracellular and membrane-associated isozymes without having an effect on the cytosolic and mitochondrial isoforms (Supuran 2008).

2.3. CYTOSOLIC CAs

2.3.1. CA I

After the carbonic anhydrases were first found in erythrocytes it took 30 years till the enzyme was discovered to be two isozymes, CA I and CA II, and it was not until 1970's that other isozymes were found. Both are produced as cytosolic enzymes but although CA II is produced in lower concentrations than CA I it shows greater affinity for CO_2 and is, in fact, one of the fastest enzymes known (Chegwidden & Carter, 2000). Sequence similarity between these two isozymes is high but differs in amino acids lining the active site which causes differences in their activities. In CA I at position 200

and 67 there are histidines as CA II has threonine and asparagine residues, respectively. By affecting the binding, the bulkier histidine residues lower the activity of CA I but they also make it less prone to inhibitors and activators (Temperini et al., 2009). In addition to red blood cells where CA I concentration is about 10 μM, high amounts of the isoform has also been found in the gastrointestinal tract and smaller amounts in the human eye (Wistrand, 1999, Temperini et al., 2009). The physiological function of CA I has remained unclear. Some mammals have been found to express CA I and lack CA II but the role of CA I in the presence of CA II is not understood (Temperini et al., 2009).

2.3.2. CA II

CA II is the most widely distributed of mammalian CA isoforms and produced in all major mammalian tissues including blood cells, kidney, liver, bone and intestine (Chegwidden & Carter, 2000). The importance of CA II in erythrocytes is evident as the process of blood CO_2 transport and excretion is largely dependent upon the rapid catalysis of the CO_2 reactions by CAs. CA II forms an important link between carbon dioxide transport and oxygen transport, and inhibition of the enzyme leads to a respiratory acidosis in the blood. Also, in the human alimentary tract, CA II is the most widely distributed isoform and its role is thought to supply the alimentary tract canal secretions with bicarbonate. In immunohistochemical stainings, the isoform was present in surface epithelia throughout the alimentary tract as well as in the different salivary glands, hepatocytes and also in the epithelia of the bile and pancreatic ducts and gall bladder (Parkkila et al., 1994). Together with CA I, CA III and the membrane-bound CA IV, CA II is thought to participate in protection of the mucosa against corrosive gastric acid by augmenting bicarbonate in saliva and esophageal submucosal gland secretions. It can also enhance CO_2 and H_2O elimination and ion exchange during growth (Christie et al., 1997).

In kidney, CA II accounts for 95 % of all CA activity and is most widely distributed along the nephron. The isoform has been located in proximal tubule, distal tubule, thin descending limb of Henle and intercalated cells of both cortical and medullary collecting ducts (Purkerson & Schwartz, 2007). In duodenum, CA II has been associated with alkaline secretion and is mainly located in the villi. A study by

Leppilampi et al. (2005) showed that CA II enables PGE_2-stimulated mucosal bicarbonate secretion in the murine duodenum and therefore protects the duodenal mucosa against ulcerations. Mild pit-cell hyperplasia, mild glandular atrophy, and gastric cysts found in mice lacking CA II also suggests a possible role in normal gastric morphogenesis (Leppilampi et al., 2005). CA II has been found in human submandibular and parotid glands as well as in human lens of the eye along with CA I, CA III and the membrane-bound CA IV (Wistrand, 1999, Parkkila et al., 1990). Human lens seems to have the same composition of CA isoforms as found in erythrocytes, only in different proportions and smaller amounts. No CA activity has been found in senile cataract lenses, however, patients suffering from CA II deficiency have not been reported to develop cataract (Wistrand, 1999). Nevertheless, due to its high activity, ubiquitous expression and role in many physiological processes, CA II is the only isoform the lack of which causes a defined clinical phenotype known as Guibaud-Vainsel syndrome or marble brain disease that includes osteopetrosis and cerebral calcification as well as impaired renal acidification leading to renal tubular acidosis (Chegwidden & Carter, 2000, Kaunisto et al., 2002, Sly et al., 1983, Kim et al., 2004).

As a base, bicarbonate has a significant role in the regulation of cellular and physiological pH but it is membrane-impermeant and therefore not able to diffuse through the cell membrane. Cells express several different bicarbonate transport proteins that assist and regulate transportation of bicarbonate through the membrane. These transporter proteins have been found to closely interact with both cytosolic and membrane-bound CAs (McMurtrie et al., 2004). In the cytosol, CA II is thought to interact or even weakly bind to different transporters, mostly anion exchangers like AE1 and kNBC1, and facilitate bicarbonate transport by forming so called transport metabolons. This interaction seems to be pH-dependent as the affinity of the enzyme for the transporter proteins has been found to decrease in pH values above 7. Most bicarbonate transporter family members contain a consensus CA II binding motif that consists of at least one hydrophobic residue followed by two acid residues as well as two basic residues, lysine and histidine. The pH dependence of CA II binding is probably caused by protonation of these basic residues (Purkerson & Schwartz, 2007). In a study by McMurtrie et al. (2004), cells where treated with acetazolamide, a CA-

inhibitor, transport of bicarbonate was also nearly completely inhibited which demonstrates the importance of the metabolon for the transporter to function efficiently. The complex formed by transporter protein and CA facilitates the transportation by decreasing the diffusion distance of the substrates through the membrane. The active sites of the enzyme and the transporter are located in the proximity of each other enabling the bicarbonate to immediately attach to the next site when liberated from the first one. This close association allows formation of a strictly controlled bicarbonate concentration gradient (McMurtrie et al., 2004).

Transporter proteins have a central role in regulating the acid-base homeostasis of the kidney. AE1 (anion exchanger 1) is 911 amino acids long glycoprotein that in exchange for chloride transports bicarbonate through the membrane. In human kidney, cells express a shorter form of AE1 known as kAE1 and it has been thought to function in close proximity with CA II. When bicarbonate is transported out of the cell, the enzyme forms a high local concentration gradient to the transportation site. During transportation of bicarbonate into the cell, the enzyme on the other hand lowers the cytosolic bicarbonate concentration by catalyzing its conversion to carbon dioxide (Sterling et al., 2002). CA II has been found to function similarly with other exchangers, AE2 and AE3. Anion exchangers are mainly located at the basolateral membrane of the kidney tubules and are therefore responsible for the return of the reabsorbed bicarbonate into the blood circulation (Purkerson & Schwartz, 2007). CA II interacts also with many apical transporter proteins including Na^+/HCO_3^- transporter NBC3 and Cl^-/HCO_3^- exchangers involved in bicarbonate secretion in the gastrointestinal tract (Purkerson & Schwartz, 2007, Leppilampi et al., 2005). In addition to anion exchangers, CA II has been associated with NHE1, a member of the NHE family of cation transporters. NHE1 is a glycoprotein that protects the epithelial cells from intracellular acidification by exchanging protons to extracellular sodium and therefore, the role of CA II is not just to facilitate bicarbonate transport but also to participate in proton efflux by interacting with at least one member of the cation transporter family (Purkerson & Schwartz, 2007).

The interaction between CAs and transport proteins is evident but whether the enzyme binds directly to the transporter has been a subject of a lot of debate. According to earlier studies, CA II binds directly to the transporter protein by recognizing a LDADD

consensus sequence at the C-terminal of the transporter. However, these studies have been performed with S-transferase (GST) fusion proteins and according to Piermarini et al. (2007) CA II is not able to bind pure transporter proteins and therefore does not interact with them through direct binding. Nonetheless, there is strong evidence for the formation of a metabolon between the enzyme and the transporter and this can happen through several other mechanisms. The LDADD sequence of the transporter protein might form a unique structure combined with other parts of the protein or the enzyme can simply bind to another sequence. It is also possible that the binding is mediated by a yet unknown intermediary protein (Piermarini et al., 2007). However, CA II has an important role in the regulation of renal acid-base homeostasis, and lack of it leads to proximal renal tubular acidosis (Purkerson & Schwartz, 2007).

2.3.3. CA III

The third isoform found in 1970's was also cytosolic and named CA III. It was first located in skeletal muscle and later also in adipocytes and liver (Chegwidden & Carter, 2000). CA III shows the lowest activity of α-CAs as it possesses only 2 % of the activity of CA II (Gailly et al., 2008). This is thought to be due to replacement of His-64 by a lysine which notably reduces the efficiency of the proton shuttle. In addition, a phenylalanine at position 198 that in CA II is occupied by a leucine, is thought to cause the steric constriction of the active side (Purkerson & Schwartz, 2007). However, unlike most CA isozymes, CA III is resistant to acetazolamide inhibition (Chegwidden & Carter, 2000). Instead of pH regulation and hydration of CO_2, CA III has been found to be expressed in oxidizing environment and it may function as a member of the defense system against oxidative stress and therefore protect cells from oxidative damage. The isoform has two reactive sulfhydryl groups that are able to bind glutathione through disulfide bonds in oxidizing conditions and one possibility is that it removes oxygen radicals (Chegwidden & Carter, 2000, Gailly et al., 2008). CA III may also protect cells from H_2O_2-induced apoptosis as CA III-overexpressing NIH/3T3 cells have been found to grow faster and have improved tolerance for cytotoxic levels of percarbonic acid in comparison to normal cells. CA III is thought to function as a percarbonic acid anhydrase by catalyzing the detoxification reaction $H_2O_2+CO_2 \leftrightarrow H_2CO_4$ (Kim et al., 2004).

The proposed function of CA III as an antioxidant protein may suggest a role in handling oxygen radicals in skeletal muscle cells, but the function in adipocytes and possibly also fatty acid metabolism remains unknown (Chegwidden & Carter, 2000, Kim et al., 2004). CA III-activity has been found in the kidney during proximal tubule dysfunction, also known as the Fanconi syndrome, and it has been suggested as a potential biomarker for the disease as it has no defined function in the normal kidney. Proximal tubule dysfunction is caused by mutation in *CLCN5* gene coding for endosomal Cl^-/H^+-exchanger ClC-5 leading to oxidative damage in the cells. The dysfunction leads to solute wasting and proteinuria leading to clinical manifestations like growth retardation, rickets, nephrocalcinosis and renal failure (Gailly et al., 2008).

2.3.4. CA VII

The gene encoding cytosolic CA VII was isolated from a human genomic library in 1991. Despite the early discovery the isozyme is one of the less studied and understood of the CAs. It shows activity that is approximately 70 % of human CA II activity and is therefore the second most active of the cytosolic isoforms (Vullo et al., 2005). The active site of CA VII seems to be the most highly conserved of α-CAs as the active sites of other known isoforms resemble it more closely (Hewett-Emmett & Tashian, 1996). CA VII has been localized in brain where it is thought to participate as a molecular switch for GABAergic neuronal transmission. In mouse, the isozyme has also been localized in several other organs including stomach, duodenum, colon, liver and skeletal muscle. The wide distribution and conservation of the active site suggest a significant and versatile physiological role that yet remains unknown (Hewett-Emmett & Tashian, 1996, Bootorabi et al., 2010).

2.3.5. CA XIII

According to phylogenetic studies, the cytosolic isoform CA XIII is most closely related to CA I, II and III. In human and mouse, it has been found in the alimentary tract and kidney, and in mouse, also in the brain and lung (Lehtonen et al., 2004). However, the expression of this enzyme seems to differ in the alimentary tract between mice and humans as in humans it is present in enterocytes mainly in the jejunum and ileum while in rodents it was found in the colon. In human, the isoform is also produced in the

submandibular gland (Pan et al., 2007). In addition, CA XIII is the only isoform that is expressed in the reproductive tissues of both mice and humans, including the uterus and in humans also in the testis (Hilvo et al., 2008b, Lehtonen et al., 2004). The enzyme is produced in all stages of developing sperm cells and it is thought to have a significant role in the fertilization process. CA XIII has been also found in the collecting ducts of kidney, in oligodendrocytes of brain and alveolar cells of lung (Hilvo et al., 2008b). The activity of CA XIII is rather low and similarly to CA II, it shows strong affinity to sulfonamide inhibitors but is resistant to bicarbonate and chloride inhibition. The resistance may indicate a possible interaction with transporter proteins such as AE1 and NBC1 that have been found to interact with CA II as well (Hilvo et al. 2007).

2.4. MITOCHONDRIAL AND SECRETED CAs

2.4.1. CA VA and VB

CA VA and VB are the two isoforms expressed in mitochondria where they have been suggested to provide HCO_3^- for several biosynthetic processes like gluconeogenesis, ureagenesis and lipogenesis. Both are low activity enzymes but CA VA is produced only in the liver, while CA VB has been found in the heart, skeletal muscle, pancreas, kidney, salivary gland, and spinal cord. The differences in distribution suggest also differences in physiological properties, and CA VA has been thought to have arisen from CA VB to perform a specific function in the liver. However, these differences are yet unknown (Fujikawa-Adachi et al., 1999b). In the liver, CA VA participates in ureagenesis and gluconeogenesis by providing bicarbonate ions for mitochondrial enzymes carbamyl phosphate synthetase I and pyruvate carboxylase and therefore, facilitating the conversion of ammonia to citrulline. Also, mitochondrial CAs have been found to be essential for rapid uptake of calcium by mitochondria in hepatocytes (Ghandour et al., 2000). The role of the isozyme in the metabolic processes of extrahepatic tissues is supported by the finding that the main sources of CA VB in the alimentary tract tissues has been found to be the epithelia and endocrine cells such as the parietal cells and endocrine G-cells of the stomach and enterocytes of the large intestine. The findings suggest that the main physiological role of both CA V isoforms seems to be the detoxification and disposal of ammonia (Saarnio et al., 1999). In pancreas, CA VB has been found in isolated rat islets where it was co-localized in beta

cells with insulin and the isoform was thought to be involved in the regulation of glucose-stimulated insulin secretion (Parkkila et al., 1998). In the rodent nervous system, CA VB has been localized in both the astrocytes and neurons. Expression of CA VB in the neurons that lack pyruvate carboxylase expression suggests a possible role in the neuronal transmission and/or maintenance of ion homeostasis in the nervous system (Ghandour et al., 2000).

An anti-epileptic drug known as topiramate has been found to efficiently inhibit both human mitochondrial isoforms as well as CA II. As these enzymes are involved in lipogenesis, a side effect of topiramate observed in obese patients is loss of weight and as same has been detected in mice, the inhibitor has been considered also as a potential anti-obesity drug (Supuran, 2008).

2.4.2. CA VI

CA VI was initially discovered as a zinc-binding salivary protein named gustin and was linked functionally to taste bud growth. It is the only known secreted isoform of the α-CA family and has been found in the saliva secreted by the serous acinar cells of mammalian parotid and submandibular glands, lacrimal and nasal glands, as well as in mouse fibroblasts where it is produced as an intracellular, stress-inducible form whose role has remained unknown. The secretory form is thought to participate in protecting the teeth from caries by neutralizing plaque acid and it also participates in neutralizing excess acid in the mucous layer of the esophageal and gastric epithelium. Small amount of salivary CA VI is also produced in the lacrimal gland and secreted into the tear fluid and it is presumed to be involved in the maintenance of acid-base homeostasis on the eye surface. The enzyme is also present in both the alveolar milk and the glandular epithelia but the concentration is far lower in mature milk compared to the one found in human colostrum. The concentration of CA VI in saliva of newborn babies has been reported to be low compared to adults, and milk CA VI has been suggested to compensate this difference, especially during the early postnatal period (Karhumaa et al., 2001).

Secreted from nasal glands, CA VI is thought to increase the buffering capacity of the nasal mucus and thus protect the underlying tissues by maintaining the optimal pH for

proteins involved in elimination of environmental toxins and microbes. The enzyme is also presumed to be involved in CO_2 sensation by facilitating CO_2 diffusion into the mucus and enhance detection of CO_2 changes in the atmosphere by the mucosa. CA VI also causes a balanced ion concentration that is needed for depolarization of the olfactory receptor neurons during olfactory transduction. Also, CA VI is suggested to participate as a growth factor in maturation of olfactory epithelial cells. Decreased amounts of the enzyme in the nasal mucus and the saliva as well as apoptotic degeneration of the taste bud cells have been reported in patients with gustatory and olfactory dysfunction after an influenza-like illness (Kimoto et al., 2004). CA VI has also been found to have a mucosa protective role in both the gastrointestinal tract and the respiratory tract where it also acts as a pH neutralizer and potential growth factor. In the lung, the enzyme is secreted by the seromucous tracheobronchial glands, the serous epithelial cells of the trachea and bronchi, and by the Clara cells of the bronchioli. CA VI seems to be a multifunctional protein that in addition to the CA activity also possesses growth factor functions. This is supported by the findings that CA VI is expressed in the submandibular gland at birth, whereas bicarbonate producing CA II is not expressed until 2 weeks after birth (Leinonen et al., 2004).

2.5. MEMBRANE-BOUND CAs

2.5.1. CA IV

CA IV was originally purified from bovine lung and it shows high-activity similar to CA II. It is the most widely distributed of membrane-associated isoforms by being expressed in several tissues including lung, kidney, brain, heart and pancreas, and functions in tandem with CA II (Chegwidden & Carter, 2000, Tamai et al., 1996). The enzyme is a 52 kDa glycoprotein that contains two disulfide linkages which makes it resistant to denaturation by SDS that inactivates most CA isoforms (Purkerson & Schwartz, 2007, Tamai et al., 1996). CA IV is also the second CA isoform that is attached to the membrane through a GPI-anchor except in pancreas where it has been found as a transmembrane protein. However, the role of the transmembrane form remains unclear as the lack of the GPI-anchor leads to a reduction in activity (Purkerson et al., 2007).

The activity of CA IV shows wide variation among different mammalian species. As the activity of CA IV is considered high in species like human, bovine and rabbit, the activity in rodents is markedly lower. The lower activity of the rodent form is considered to be due to replacement of Gly-63 to a bulkier Gln residue which causes impairment of the proton shuttle that leads to reduced activity. This has been demonstrated by replacing Gln-63 of mouse CA IV to Gly which increased the activity 2- to 3-fold (Tamai et al., 1996).

CA IV has been found in the capillary endothelium of the lung where it dehydrates HCO_3^- to CO_2 that diffuses through endothelium and is exhaled out of the lung (Zhu & Sly, 1990). In the gastrointestinal tract of both human and rat, the isozyme has been localized in the esophageal epithelium and in the apical plasma membrane of the mucosal epithelium in the distal small intestine and large intestine where most intense signal was detected in the colon. In addition, CA IV has been found in the submucosal capillary endothelium of all regions of the gastrointestinal tract. In both the ileum and large intestine, CAs are thought to participate in ion and water transport (Christie et al., 1997). In the pancreas, CA IV has been detected in epithelial ductal cells where it is anchored to the luminal plasma membrane via a hydrophobic segment at the C-terminal. The hypothesis is that the enzyme stimulates HCO_3^- secretion by forming a channel structure that allows passage of HCO_3^- ions and back up the activity of Cl^-/ HCO_3^- exchanger in the regulation of luminal pH (Fanjul et al., 2004). In both the skeletal and heart muscle, CA IV has been detected in the sarcoplasmic reticulum and sarcolemma as well as in the capillary endothelium. In the heart, CA IV is located in specialized sarcolemmal structures such as intercalated discs and T-tubules. In the sarcoplasmic reticulum, H+ is moved across the membrane in exchange for Ca++ and it has been suggested that the role of CA activity is to rapidly produce and buffer this H+ (Sender et al., 1998).

CA IV has been located in the eye of several species including human. Along with CA II, the isoform promotes transepithelial movement of ions and fluid and is involved in intracellular buffering in the lens, but based on inhibition studies their activity is not essential for lens function and can be compensated to some extent by the small amounts

of CA I and CA III present (Wistrand, 1999). Missense mutations in CA IV have also been associated with an autosomal dominant form of retinis pigmentosa (RP17), a progressive eye disease characterized by the deaths of retinal photoreceptors. CA IV has been found to be highly expressed in the endothelial cells of the choriocapillaries in the eye but not in the retina itself. The mutations seem to interfere with the trafficking of CA IV to the cell surface leading to accumulation of misfolded proteins in the cytosol and cause endoplasmic reticulum (ER) stress-induced apoptosis of endothelial cells. The capillaries provide the retina with nourishments, and therefore, apoptosis of the endothelial cells leads to secondary damage in the overlying retina and finally RP. The apoptosis can be at least partially prevented by using CA inhibitors, such as dorzolamide, as pharmacological chaperones and the treatment has been suggested as a potential therapy to delay or prevent the onset of blindness in RP17 (Datta et al., 2009).

Most of the interstitial CA activity in the brain was first attributed to CA IV. However, some of the activity originally thought to be CA IV has been found to be provided by another membrane CA, CA XIV (Shah et al., 2005). The significance of CA IV in the extracellular buffering of the interstitial space in hippocampal slices was demonstrated by a phosphatidylinositol-specific phospholipase C (PI-PLC) treatment that abolished most of the extracellular CA activity (Tong et al. 2000). The residual activity has been attributed to CA XIV. The enzyme is involved in the regulation of activity-dependent pH shifts including rapid alkaline transients. However, studies on knock-out mice have shown that, although, CA IV seems to have a greater effect on pH regulation in hippocampus than CA XIV, both isoforms are able to compensate for the lack of the other (Shah et al., 2005). In the human gallbladder, CA IV has been localized in the subepithelial capillary endothelium where, together with CA II and ion transporters, it has been suggested to be involved in the concentration and acidification of alkaline hepatic bile (Parkkila et al., 1996). The isoform has also been found to participate in the epididymal fluid acidification in the epididymal ducts preventing premature sperm activation as well as urinary acidification in the kidney (Parkkila et al., 1993, Parkkila et al., 1996).

In the kidney, CA IV plays a major role in urinary acidification and pH-regulation of tubule lumen where it facilitates fluid and bicarbonate reabsorption. This is demonstrated by the fact that, although, only 5 % of CA activity in the kidney is membrane-associated, inhibition of luminal CA activity blocks bicarbonate absorption in proximal tubule. Unlike most GPI-anchored proteins that are usually found in apical membranes, CA IV was the first GPI-anchored protein found on both apical and basolateral membrane in the proximal tubule of the kidney. The isoform is mainly expressed in the S2 segment of the proximal tubules but also in the distal tubules as well as outer and inner medullary collecting ducts and α-intercalated cells of the cortical collecting duct (Purkerson et al., 2007).

Like CA II, CA IV has also been found to form bicarbonate metabolons with transporter proteins. Binding sites for CA IV have been found in anion exporters AE1, AE2 and AE3 as well as NBC1 (Purkerson & Schwartz, 2007). With the help of CAs, a high concentration of bicarbonate is formed near the active sites of transporters whereas on the opposite side of the membrane the concentration is kept low. CA IV is attached into the vicinity of the transporter on the luminal side and it is involved in regulating of the concentration gradient depending on the direction of the transportation (Casey et al., 2009). On the membrane, CA IV is usually located at lipid rafts but together with AE1 it has also been localized elsewhere. Together CA IV and cytosolic CA II are thought to ”push n’ pull” anions through the transporter (Purkerson & Schwartz, 2007). In the heart, no cytosolic CA is expressed but two extracellular CAs are expressed, including CA IV as well as transporters AE1, AE2 and AE3 which all have been shown to need CA II for maximal activity. However, in the heart, CA IV is able to completely replace the lack of CA II and this was also thought to be the case in kidney. In a study by Sterling et al. (2002), the rate of transport remained at almost normal level in the absence of CA II and the activity was abolished by inhibition with acetazolamide which indicates that the rescue is due to increased catalytic activity of CA IV that compensates reduced activity of CA II (Sterling et al. 2002). However, the compensation is not enough to prevent renal tubular acidosis caused by insufficient bicarbonate reabsorption in patients suffering from CA II-deficiency (Kaunisto et al., 2002, Sterling et al., 2002).

2.5.2. CA IX

Due to its strong association with tumor progression in various cancers, including renal, colorectal and ovarian cancers, CA IX has been the main interest in CA research during the recent years. In normal physiological state, CA IX is expressed in the alimentary tract where it is mainly produced in the stomach but is also present in the duodenum and jejunum of the small intestine as well as to some extent in the ileum and colon. The subcellular location of the enzyme is highly restricted to the basolateral surfaces of proliferating crypt enterocytes, demonstrating a unique distribution in comparison to any other CA. The enzyme seems to be involved in cell proliferation as the expression is highest in the proximal segments of the intestine where the rate of cell proliferation is highest, which also makes it a useful marker for cell proliferation in the intestinal epithelium. However, the physiological role of CA IX in pH and ion homeostasis is unclear. Localization of the isozyme to the basolateral surfaces suggests that it might be involved in intercellular regulation, possibly as a ligand or a receptor (Saarnio et al., 1998). CA IX is also thought to be involved in Cl^-/HCO_3^- exchange as well as in cell adhesion in crypts where high proliferative capacity is required. The isoform has been found to form transport metabolons and increase activity of AE1, AE2, AE3 and SLC4 Cl^-/HCO_3-transporters (Purkerson & Schwartz, 2007).

CA IX is the only member of α-CA gene family that has a proteoglycan-like (PG) domain in its structure. Through the PG-domain, the enzyme has been thought to reduce cell-cell adhesion by interfering with the function of β-catenin, and therefore, enable induction of new cells into the epithelium. The PG domain has also been suggested as the potential moiety involved in tumor invasion due to the effects it has on cell-cell adhesion. The crystal structure of the CA IX catalytic domain was recently published by Alterio et al. (2009). In hypoxic cells, the catalytic domain converts CO_2 that is diffused through the plasma membrane, into bicarbonate and protons and therefore, contributes to extracellular acidosis. In addition, the presence of the PG domain seems to give catalytic domain a better activity at acidic pH values and improves the catalytic features of the enzymes, especially in the hypoxic circumstances of tumors. CA IX might also be involved in intracellular signal transduction through its intracellular tail. The tail contains a tyrosine residue that is able to interact with the regulatory subunit of PI3K

when phosphorylated. PI3K activates the Ser/Thr protein kinase Akt which is involved in activating several cancer-related signaling cascades including one mediated by a transcription factor known as hypoxia inducible factor 1 (HIF-1) (Alterio et al., 2009).

CA IX is thought to promote cell growth and survival in hypoxic and acidic environment of tumors by facilitating acid/base transport and sustaining physiological intracellular pH and, at the same time, increase acidosis of the environment (Purkerson & Schwartz, 2007). The development of this tumor hypoxia is mediated by HIF-1. *CA9* promoter includes a hypoxia-responsive element (HRE) region that is recognized by HIF-1. At normal oxygen levels, hydroxylated HIF-1 is bound by von Hippel-Lindau (VHL) tumor suppressor protein and directed to the ubiquitin-proteosome for degradation. However, in hypoxia, HIF-1 is not hydroxylated in which case VHL is not bound and HIF-1 is transferred into the nucleus to activate transcription. The expression of *CA9* has been found to be associated with mutations in the VHL gene or suppression of the promoter by methylation which leads to loss of VHL protein function or expression (Supuran, 2008, Dorai et al., 2005).

In addition to CA IX, another CA isoform, CA XII, has been found to enhance tumor growth and survival in hypoxic environment by maintaining the intracellular pH neutral. In a study by Chiche et al. (2009), combined silencing of *CA9* and *CA12* significantly decreased growth rate of xenograft tumors which was greater than inhibition of HIF-1. Until recently, main focus in developing new cancer treatments has been on inhibitors of HIF-1 but based on recent findings on both CA IX and CA XII the focus seems to be better directed to the inhibition of target gene products downstream of HIF-1. Especially over-expression of CA IX has been found to correlate with poor patient survival and some therapeutic applications focused on CA IX are already in clinical trials. Also, CA inhibition is considered as a potentially effective anticancer treatment (Chiche et al., 2009). The first step in developing inhibitors is the design of membrane-impermeant inhibitors that will not interact with the cytosolic and mitochondrial isoforms. The crystal structure of the catalytic domain will enable structure-based drug design of selective CA IX inhibitors and potential anticancer drugs (Alterio et al., 2009).

2.5.3. CA XII

CA XII is a type I membrane protein that has been found in the kidney and colon of human, mouse and rabbit (Purkerson & Schwartz, 2007). In human, the isoform has also been localized in the epididymis and endometrial epithelium where it is thought to contribute to production of bicarbonate that is considered as key factor in maintenance of sperm motility both in the male and female reproductive tract. CA XII has also been hypothesized to regulate extracellular pH in the proximity of the epithelium during morphological changes of the menstrual cycle (Karhumaa et al., 2000). The expression of CA XII in the kidney varies among different species, but it is mainly located in the cells secreting protons. In human, CA XII has been found in the basolateral membrane of thin descending limb of the loop of Henle, in distal convoluted tubules and principal cells of collecting ducts. Some activity has also been detected in the proximal tubules. In rabbit, the isoform is produced in the proximal tubules, both cortical and medullary collecting ducts and thick ascending limb of Henle as well as papillary epithelium. In mouse, CA XII functions on the basolateral side of the proximal tubules and proton secreting α-intercalated cells of cortical and medullary collecting ducts (Purkerson & Schwartz, 2007). The role of basolateral CAs in kidney epithelial cells is unclear but in the proximal tubule they are assumed to facilitate Na^+/HCO_3^- co-transport by preventing formation of alkaline disequilibrium pH in the interstitium. The principal cells of the collecting duct, where CA XII is produced, are involved in absorption of sodium and water. The isoform has been found to be expressed also in other tissues with high absorption capacity of water e.g. the colon, and it is suggested that CA XII has a role in the process (Parkkila et al., 2000). In the alimentary tract, the isoform is only present in the large intestine and the cellular distribution is limited to basolateral surface of the enterocytes in the surface epithelial cuff. The enzyme is thought to be involved in transcellular water transport (Kivelä et al., 2000).

CA XII has not been reported to form metabolons with transporter proteins, but the activity of the isoform has been found to be increased in cancer cells during hypoxia, and the expression is upregulated in tumors of renal carcinoma where the production of VHL protein is decreased. Unlike CA IX, another isoform involved in cancer, the presence of CA XII has not been found to be related to expression of HIF-1 and it lacks

the extracellular PG-like domain involved in cell adhesion and therefore the meaning of the isozyme in tumors is unclear. However, the activity of CA XII seems to be increased in tumors that lack CA IX activity (Chiche et al., 2009).

2.5.4. CA XIV

Both the human and mouse CA XIV isozymes were characterized in 1999 by two research groups. These studies showed that the isoform is widely expressed in different tissues of both species (Fujikawa-Adachi et al., 1999a, Mori et al., 1999). In human, CA XIV was found in the brain, spinal cord, colon, small intestine, liver, heart and also to some extent in the skeletal muscle, urinary bladder and kidney (Fujikawa-Adachi et al., 1999a). In mouse, the isoform was detected in the kidney and heart, followed by the skeletal muscle, liver, brain, and lung, but unlike in human, no expression was found in the intestine tissues (Mori et al., 1999). Since then, the expression of CA XIV has been widely studied in several of these organs.

CA XIV is a type I transmembrane protein with a predicted molecular mass of 37.5 kDa and is most closely related in terms of amino acid similarity to CA XII. The hydratase activity of the isoform is inhibited by acetazolamide. The human form seems to be much less active than CA II, whereas the mouse form appears to have activity that is comparable to CA II. Especially in mouse kidney, CA XIV has been found to be responsible for activity that was long thought to belong to CA IV and discovery of CA XIV also explains CA activity in segments were membrane activity has been found but where CA IV is not present (Purkerson & Schwartz, 2007). In the rodent kidney, CA XIV has been detected in apical plasma membranes of S1 and S2 segments in the proximal tubules in both the cortex and medulla regions. The isoform has also been found in the initial portion of the thin descending limb that is known for water permeability and sodium transport. CA IV is present mainly in S2 segment while bicarbonate reabsorption is thought to be greater in the proximal tubular cells of S1 segment. This, along with the high activity of murine CA XIV, suggests that the isoform may play an even more important role than CA IV (Kaunisto et al., 2002). An interesting discovery by Premkumar et al. (2005) was the unexpected halo-tolerance of CA XIV, a physiological property that allows the isozyme to stay active at a lower pH than CA IV. It is possible that the local pH at the brush border where the protons are

secreted is even lower than in the lumen itself. Therefore the functional significance of CA XIV may not only be to compensate for the lower activity of murine CA IV compared to its human counterpart as expected but to also adapt to a more challenging environment. It is, however, clear that CA XIV and CA IV most likely account for the majority of the membrane-CA activity required for the acidification of urine (Premkumar et al., 2005).

Another interesting tissue found to express CA XIV in both human and mouse is the brain where the isoform has been localized on neuronal membranes and axons by Parkkila et al. (2001). Earlier, all interstitial CA activity in brain had been principally attributed to CA IV (Tong et al., 2000). The highest expression of CA XIV was observed on large neuronal bodies and axons in the anterolateral part of the pons and medulla oblongata. Other tissues showing positive reaction to CA XIV included the hippocampus, corpus callosum, cerebellar white matter and peduncles, pyramidal tract, and choroid plexus. In the mouse brain, the molecular layer of the cerebral cortex and granular layer of the cerebellum were also positive for CA XIV. Based on these findings, CA XIV was suggested to contribute to modulation of excitatory synaptic transmission in the brain (Parkkila et al., 2001). In a later study by Shah et al (2005) both CA IV and CA XIV were found to contribute to extracellular buffering in the central nervous system, but CA IV seemed to have a more important role in the hippocampus. However, in the study, single knock-out mice showed no change in phenotype, while mice lacking both isoforms displayed a delay in the alkaline shift. It is therefore suggested that the importance of the individual isoforms may differ between different regions of the brain (Shah et al. 2005).

CA XIV is also present on the plasma membranes of hepatocytes in the mouse liver where the enzyme participates in the regulation of ion and pH homeostasis at both apical and basolateral membrane (Parkkila et al., 2002). The isoform has also been localized in the rat epididymis and in the longitudinal sarcoplasmic reticulum and the sarcolemmal membrane of both the skeletal and cardiac muscle (Hermo et al., 2005, Wetzel et al., 2007, Scheibe et al., 2006). In the mouse retina, the expression of CA XIV was highest on the apical surface of the pigmented epithelial cells. Some expression was

also found on the basal surface as well as in the Muller glial cells. Studies on knockout mice by Ogilvie et al. (2007) demonstrated that lack of CA XIV causes a functional defect in the retinal light response, which CA IV can partly compensate for (Ogilvie et al., 2007).

2.6. CARBONIC ANHYDRASE XV

Carbonic anhydrase isoform XV is the most novel member of the mammalian α-CA gene family and the first member that is expressed in most mammals but not in humans as the corresponding gene seems to have become a pseudogene in primates. A genome-wide analysis was made by Hilvo et al. (2005) to see which species might possess an active form of CA XV. The analyses showed well maintained exon structures in several species including humans that showed 3 potential *CA15* genes in 22q11.21. Chimpanzees were also suspected to have several copies but only one copy was included in all other genomes. There seemed to be many possible reasons why *CA15* has been lost in humans. The analysis showed several frameshifts resulting in loss of conserved hallmark residues as well as an AluY repeat that splits exon 8. Also, an insertion was found in the active site and, in addition to several point-mutations, one intron seemed to have lost an essential GT dinucleotide in the beginning. The findings suggest that the activity of CA XV have been lost in humans and chimps not so long ago. The isoform was found to be a pseudogene also in rhesus monkeys which refers to the fact that the activity has probably been lost in all primates. From evolutionary point of view, this is an interesting finding as only 27 genes expressed in rodents have been found to have become pseudogenes in human (Hilvo et al., 2005).

CA XV is the 13th member of the α-CA gene family that has been found to be active. As highly conserved enzymes, all members catalyze the same reaction, and therefore, it is likely that CA XV has become redundant in some species during evolution. However, the isoform has remained active in several species including rodents and as the gene is a pseudogene in humans, mouse CA XV has been used to study its functions. According to the phylogenetic studies, CA XV is most closely related to CA IV and they seem to possess several similar properties. The structure of CA XV has been predicted using CA IV as a template. Biochemical characterizations performed by Hilvo et al. (2005)

showed that, like CA IV, CA XV is attached to the plasma membrane with a GPI-anchor and it contains more than one N-linked glycosylation. In addition, both isozymes seem to include intramolecular disulphide bonds critical to the stabilization of the structure (Hilvo et al. 2005). CA XV is susceptible to inhibition by sulfonamides and sulfamates, and the best inhibitors include celecoxib, sulfanilyl-sulfonamides, methazolamide and ethoxzolamide (Hilvo et al., 2009).

In the study, recombinant mouse CA XV was produced in COS-7 cells to study the kinetic properties. The activity of CA XV showed out to be moderate and comparable to those of human CA XII and CA XIV which strongly suggests that the isoform is important for species expressing it (Hilvo et al., 2005). The inhibition constant of acetazolamide for CA XV is nearly identical to human CA IV. Interestingly, the activity of murine CA IV is only 20% of human CA IV, which might suggest a compensating role for CA XV in rodents (Hilvo et al., 2008b, Hilvo et al., 2005). In the same study, the expression of *CA15* was studied in both human and mouse tissue by RT-PCR and also by *in situ* hybridization for mouse tissue. In RT-PCR, a strong band was shown for the mouse kidney and a weaker band for the testis, brain and 7- and 17-day-old embryos. CA XV seems to also have a 104 nt longer splicing variant. This variant, however, does not produce a functional enzyme due to an addition that causes a frameshift, resulting in a stop codon. The human tissues remained negative as expected. In *in situ* hybridization, the strongest signal was detected in the kidney, specifically in the renal cortex and in embryos it was found in the dorsal root ganglia, dorsal spinal cord, pons, tegmentum and inferior colliculus. According to these results, CA XV seems to be the most limited in distribution among all α-CA isoforms (Hilvo et al., 2005).

2.7. THEORY BEHIND THE METHODS

2.7.1. Immunohistochemistry

A novel immunohistochemical detection system that utilizes horseradish peroxidase (HRP) polymers has been recently introduced. In comparison to the traditional peroxidase-antiperoxidase (PAP) method, the polymer detection system protocol includes fewer steps which minimizes binding conflicts and results in decreased cross-

reactivity and increased antibody sensitivity. Unlike the PAP method, that uses three-layered immunological sandwich amplification, the polymer system has only two steps (Figure 1.). First, the sample is incubated with a primary antibody that recognizes the tissue antigen of interest. The primary antibody is then recognized by a secondary antibody raised against it in another animal species e.g. if the primary antibody is produced in mouse, the secondary antibody is produced against mouse antibodies in rabbit. A HRP enzyme polymer is attached to the secondary antibody. The activity of the enzyme is not affected by the attachment of immunoglobulins. The polymer HRP-antibody complex is visualized by the use of hydrogen peroxide as an enzyme substrate and an electron donor such as 3,3'-diaminobenzidine (DAB). These electron donors are known as chromogens as when oxidized they form a colored product e.g. DAB forms a brown, highly insoluble reaction product that shows the sites where the antigen of interest is located.

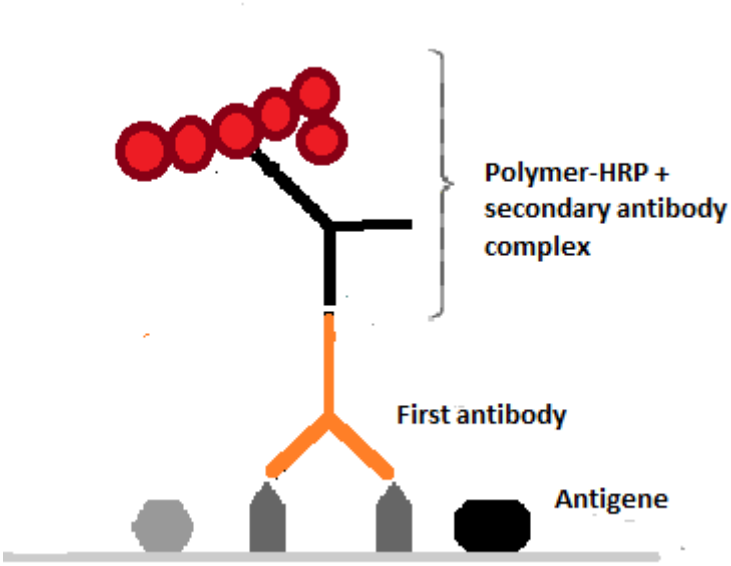

Figure 1. Polymer HRP method. The figure demonstrates the molecular interactions of the polymer HRP method.

2.7.2. qRT-PCR

Real-time PCR (RT-PCR) combined to reverse transcription has become a central tool in determining gene expression. The method allows the accumulation of amplified product to be detected and measured as the reaction progresses i.e. in real time. This is made possible by including a fluorescent molecule in the reaction that reports the increase in the amount of DNA with a proportional increase in fluorescent signal that is produced by e.g. DNA binding dyes or fluorescently labelled sequence specific primers or probes. The amplification is detected by specialized thermal cyclers equipped with fluorescence detection modules. The measured fluorescence then reflects the amount of amplified product in each cycle. Results can be either qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA). The advantages of this method are that the results can be evaluated without gel electrophoresis and closed-tube system reduces the opportunities for contamination. In quantitative RT-PCR (qRT-PCR), the data can be analyzed in two different ways. Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve as relative quantification describes the change in expression of the target gene relative to some reference group such as an untreated control.

3. AIMS OF THE STUDY

First aim of this study was to localize CA XV in mouse tissues. The second aim was to shed light on its possible compensatory role among CA isoforms in rodent physiology and why this isoform has become redundant in primates. The localization was performed by immunohistochemical studies as well as co-localization studies with other CA isoforms. Compensatory effects on protein production in knock-out tissues were also studied by immunohistochemistry. The changes in *Car15* mRNA level between wild-type and knock-out animals of other isoforms were defined by quantitative real-time PCR to detect any compensatory effects on the level of gene expression.

4. METHODS

4.1. LOCALIZATION OF CA XV IN MOUSE TISSUE

4.1.1. Western blotting

To detect CA XV in mouse tissue, a polyclonal rabbit antibody against the recombinant mouse CA XV (Hilvo et al., 2009) was raised in a rabbit by Innovagen AB (Lund, Sweden). The specificity of the rabbit anti-mouse CA XV antibody was tested by Western blotting. Recombinant mouse CA XV was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Richmond, CA). The membranes were treated for 25 minutes with Tris-buffered saline (TBS) + 0.3% Tween 20 (TBST) containing 10% cow colostral whey (Hi-Col, Oulu, Finland) and incubated for one hour with polyclonal anti-CA XV or pre-immune serum. Both the antibody and pre-immune serum were diluted 1:500 with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The membranes were washed five times for 5 minutes in TBST and incubated for one hour with HRP-linked donkey anti-rabbit IgG (Amersham Sciences, Little Chalfont, UK) diluted 1:25000 in BSA-PBS. After washing the membranes four times for 5 minutes in TBST, the antibody binding was visualized by enhanced chemiluminescence (ECL; Amersham Sciences, Little Chalfont, UK).

4.1.2. Immunohistochemistry

The deparaffinization of the sections was done using xylene and ethanol series, and then treated with methanol containing 3 % H_2O_2 for 5 minutes. After rinsing in TBS, the sections were blocked with Rodent Block M™ (Biocare Medical, Concord, CA) for 30 minutes and washed 3 times for 5 minutes with TBS. Incubation with polyclonal anti-CAs or anti-Tamm-Horsfall glycoprotein was done for 1 hour except for anti-CA IV which was incubated for 2 hours. The sections were washed 3 times for 5 minutes with TBS and incubated with a mixture of Rabbit on Rodent HRP-Polymer™ and blocking reagent XM Factor™ (20 µl XM Factor™ in 1 ml HRP-Polymer) (Biocare Medical, Concord, CA) for 30 min. After washing 3 times for 5 minutes with TBS, a 5 minute incubation with DAB (3,3'-diaminobenzidine tetrahydrochloride) solution (Zymed, Carlsbad, CA) was done. The sections were rinsed in ddH_2O, counterstained with

hematoxylin and rinsed again with ddH_2O. Finally, they were mounted in Entellan Neu™ (Merck; Darmstadt, Germany) and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss; Göttingen, Germany).

4.2. QUANTITATIVE REAL-TIME PCR

15 mouse kidneys were obtained from five *Car4*$^{-/-}$, five *Car14*$^{-/-}$, and five wild type C57BL6 mice for RNA isolation. The kidneys were homogenized (Heidolph Silent Crusher S, Colonial Scientific, VA, USA), and the RNA was isolated using an RNeasy Mini Kit™ (Qiagen Sciences, MD, USA). The concentration and purity of the isolated RNA were determined by NanoDrop™ (ThermoScientific, DE, USA).

Based on the results, one microgram of total RNA from each kidney was used as the template in reverse transcription (RT) and polymerase chain reaction (PCR). The RT reaction was performed at 37°C for 2 h followed by denaturation at 85°C for five minutes. Quantitative real time PCR was conducted using SYBR Green measured with ABI7000 (AME bioscience, Norway). Primers for murine *Car15* were the same as described in a previous study (Hilvo et al., 2005). Primers for mouse glyceraldehyde-6-phosphate dehydrogenase (mGAPDH) were used to monitor the quality and quantity of cDNA. The PCR cycling was performed at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and elongation at 58°C for one minute. Dissociation was conducted at 95°C for 15 seconds. Measurements were performed in duplicate with 0.5 µl cDNA. Transcripts for the housekeeping gene *GAPDH* were also measured, and the values of *Car15* were expressed relative to the *GAPDH* expression. The results were analyzed with χ^2 test using SPSS for Windows (version 13.0).

5. RESULTS

5.1. LOCALIZATION OF CA XV IN MOUSE TISSUE

5.1.1. Western blot

A novel rabbit antibody against the recombinant mouse CA XV produced in a baculovirus/insect cell expression system by Hilvo et al. (2009) was raised in a rabbit by Innovagen AB (Lund, Sweden). To test the specificity of this rabbit anti-mouse CA XV antibody Western blotting was performed. On the Western blot of the purified recombinant mouse CA XV, the antiserum identified a strong 34- to 36-kDa polypeptide band and a smaller 31-kDa band (Figure 2.). The result was consistent with the earlier study (Hilvo et al., 2005) where the molecular mass of glycosylated mouse CA XV protein was 34- to 36-kDa while the 31-kDa polypeptide was thought to be a non-glycosylated form of CA XV. No reaction was detected with pre-immune serum that was used as a control.

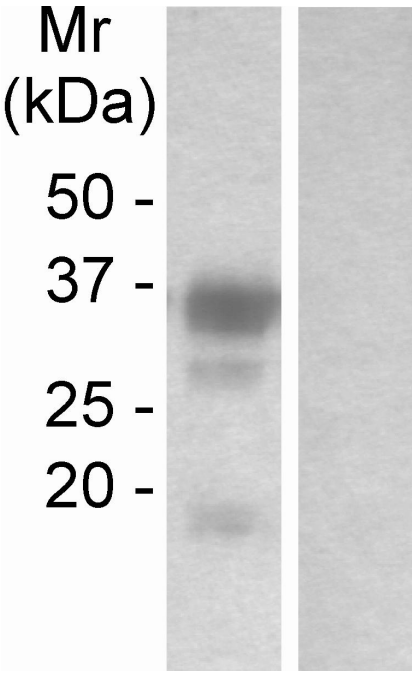

Figure 2. Western blot of the purified recombinant mouse CA XV with rabbit anti-mouse CA XV antibody. A 34- to 36-kDa polypeptide band and a smaller 31-kDa polypeptide band are identified by the antibody (left). The smaller band is suggested to be a non-glycosylated form of CA XV. Pre-immune serum showed no reaction (right). Figure adopted from Saari et al., 2010.

5.1.2. Immunohistochemistry

The localization of CA XV was studied by immunohistochemical staining in mouse tissue samples. The polyclonal rabbit antibody for CA XV was used to detect the enzyme and pre-immune serum was used as a negative control. Stainings were performed on different tissues including the kidney, testis, brain, lungs and the alimentary tract. The only tissue showing positive immunoreaction for CA XV was the kidney while the testis and brain that in a previous study by RT-PCR had given a weak band, as well as the pre-immune serum control, remained negative. In the kidney, the strongest signal was observed in the cortex region and occasional staining was also detected in the outer medulla. (Figure 3.) In the cortex region and outer medulla, CA XV seemed to be predominantly expressed in the thick ascending limb of Henle and collecting ducts. The weak staining observed deeper in the medullary region was located in the collecting ducts.

To confirm the structures of nephrons where a positive signal was detected, an antibody against Tamm-Horsfall glycoprotein was used as a marker to identify the thick ascending limbs (Kaunisto et al., 2002). The staining showed that the Tamm-Horsfall glycoprotein-positive tubules also gave a positive reaction in the CA XV immunostaining. (Figure 4.) In general, the reaction given by the marker was clearly fainter in comparison to CA XV.

Comparative immunostainings for CA XV and the other four CA isoforms present in the kidney, including CA II, IV, XII, and XIV, were performed in parallel tissue sections to receive a clear view on differences in the extent of distribution between the isoforms. The results showed that the distribution pattern of CA XV was unique among all the other isozymes. CA II is the most commonly expressed of all CA isoforms and as expected, the staining was more intense and widely spread than for CA XV involving both the proximal convoluted tubules and collecting ducts. Co-expression with CA XV was detected in the cortical collecting ducts, whereas the reactions for CA XV were much weaker in the medulla in comparison to CA II. (Figure 5.) However, CA XV-positive staining was identified in the thick ascending limbs of Henle that showed no reaction for CA II.

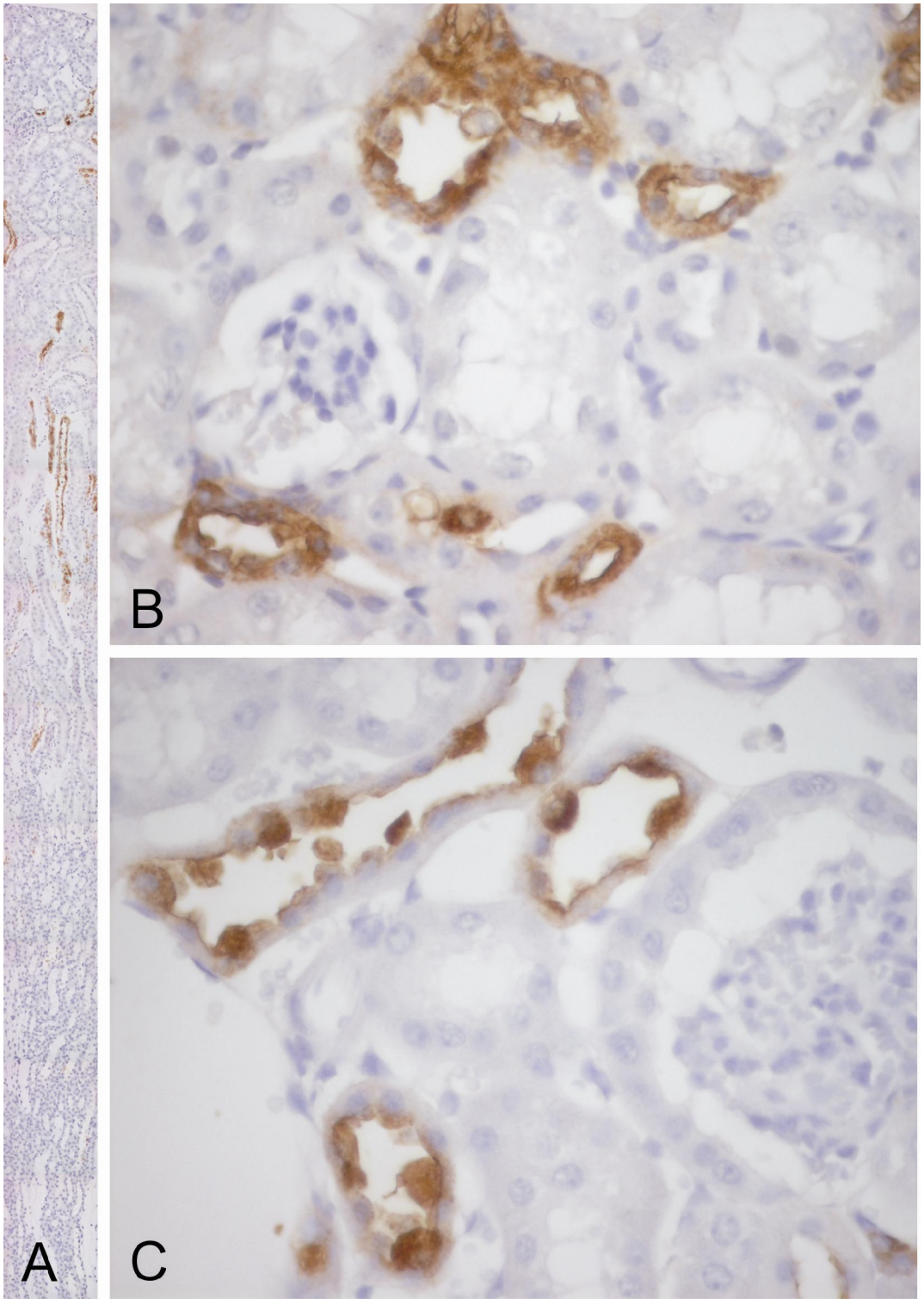

Figure 3. Immunochemical staining of CA XV in the mouse kidney. A sectional view of mouse kidney shows that CA XV is expressed in the cortex, also a weaker reaction is seen in the collecting ducts of the outer medulla (A). Intense staining is seen in the thick ascending limbs (B) and collecting ducts of the kidney cortex (C). Original magnifications x400. Figure adopted from Saari et al., 2010.

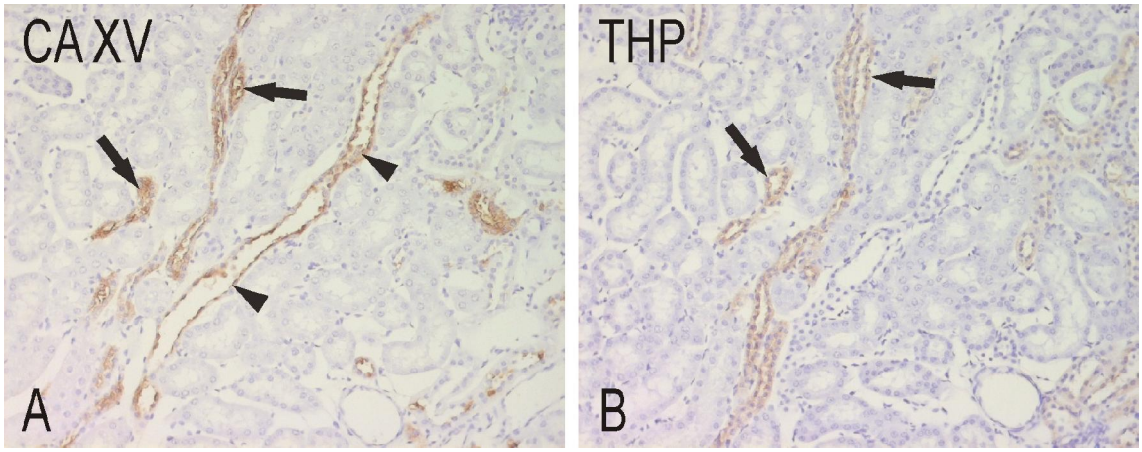

Figure 4. Confirmation of CA XV (A) immunostaining in the thick ascending limb of Henle using Tamm-Horsfall glycoprotein (THP, B) as a marker. The marker confirms that CA XV is located in the thick ascending limbs (arrows) and collecting ducts (arrowheads). Original magnifications x400. Figure adopted from Saari et al., 2010.

According to the phylogenetic studies, CA IV is the most closely related isozyme to CA XV, and CA XV has been thought to compensate the lower activity of CA IV in rodents. In the immunostainings, they both showed expression in the thick ascending limbs but no reaction was detected in the collecting ducts for CA IV (Figure 6.). A positive reaction was however shown in the proximal duct, as reported earlier by Brown et al (1990). More background signal was seen in the CA IV-staining due to a longer incubation time with the primary antibody.

CA XII has been localized to the basolateral membranes of mouse collecting ducts and to some extent to the proximal convoluted tubules (Kyllönen et al., 2003). In accordance with these findings, CA XII showed very weak reactions in the proximal tubules and a strong signal in the collecting ducts. Co-expression was observed in the collecting ducts where also CA XV showed a strong signal. (Figure 7.) Like CA XV, CA XIV is a novel member of the α-CA family and present in the rodent kidney but not in human and has also been suggested to take part in compensating the lack of CA IV activity. In immunostainings, CA XIV seemed to be predominantly expressed in the thin descending limbs of Henle and proximal convoluted tubules as found in a previous study (Kaunisto et al., 2002) and therefore, shows no co-expression in CA XV-positive structures (Figure 7.).

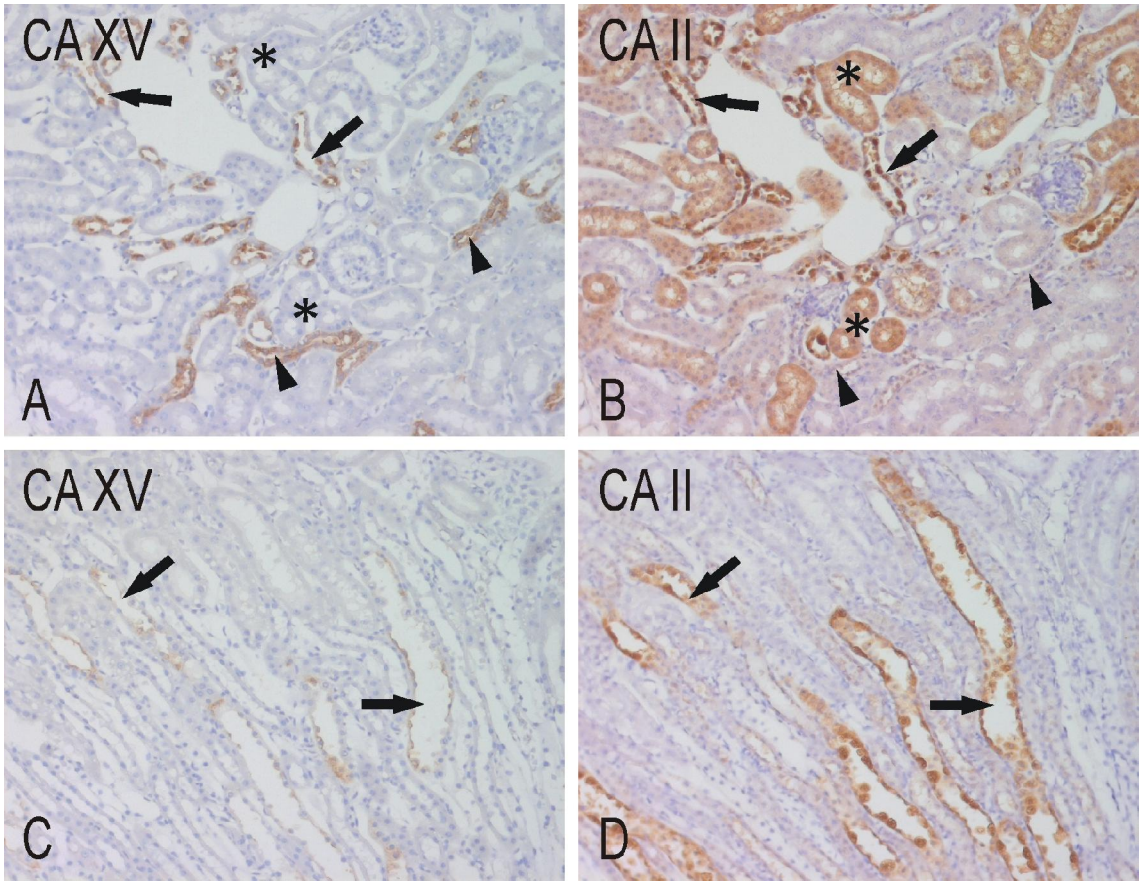

Figure 5. Immunohistochemical staining of CA XV (A, C) and CA II (B, D) in parallel sections of the mouse kidney. The reaction for CA XV in the cortex (A) is clearly more limited compared to CA II that is mainly expressed in the collecting ducts (arrows) and proximal tubules (asterisks). A positive signal is detected for CA XV in the thick ascending limbs of Henle which are shown by arrowheads. In the medulla, only a very faint reaction is seen for CA XV (C) in the collecting ducts (arrows), while the staining for CA II (D) is more intense. Original magnifications x400. Figure adopted from Saari et al., 2010.

CA XV is thought to have a compensating role in rodents due to the decreased activity of mouse CA IV in comparison to humans. Another isoform, CA XIV, is present in humans but the distribution in the kidney is different as compared to rodents. To study possible compensatory relations between these three isoforms, CA XV was also localized in the kidney specimens obtained from *Car4*$^{-/-}$ and *Car14*$^{-/-}$ mice to see if the absence of either CA IV or XIV caused any changes in the distribution or intensity of CA XV immunoreactivity. As in the wild type mice, the kidney was the only tissue showing a CA XV-positive reaction in knockout mice. One kidney specimen obtained from a *Car4*$^{-/-}$ mouse showed a more widespread immunostaining for CA XV including moderate reactivity also in the proximal convoluted tubules (Figure 8.). No significant

change in the expression levels or distribution patterns in *Car14*$^{-/-}$ or *Car4*$^{-/-}$ tissue specimens was observed.

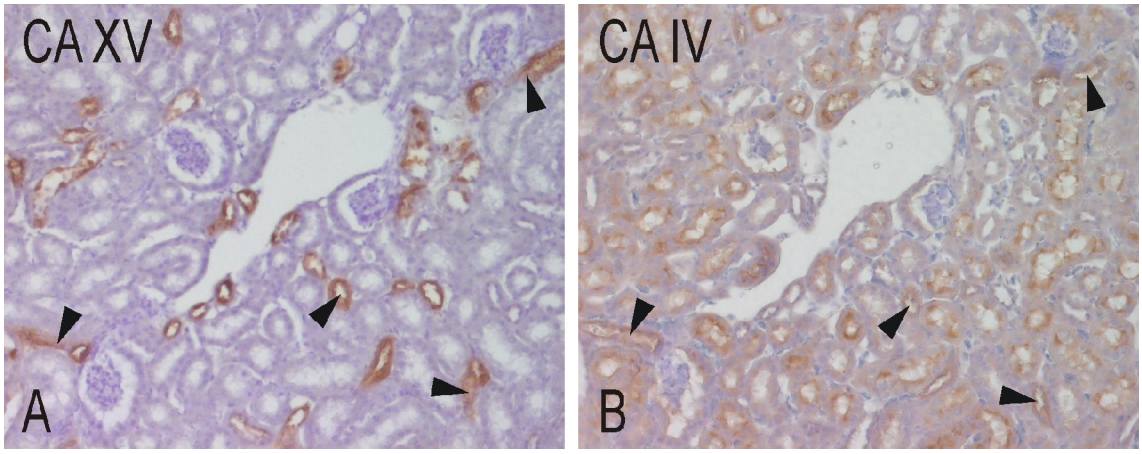

Figure 6. Immunohistochemical staining of CA XV (A) and CA IV (B) in the mouse the kidney. The staining for CA XV is more intense and restricted compared to CA IV. Similarly to CA XV, CA IV shows a reaction in the thick ascending limbs (arrowheads), and is also present in the proximal tubules, which are not stained for CA XV. Original magnifications x400. Figure adopted from Saari et al., 2010.

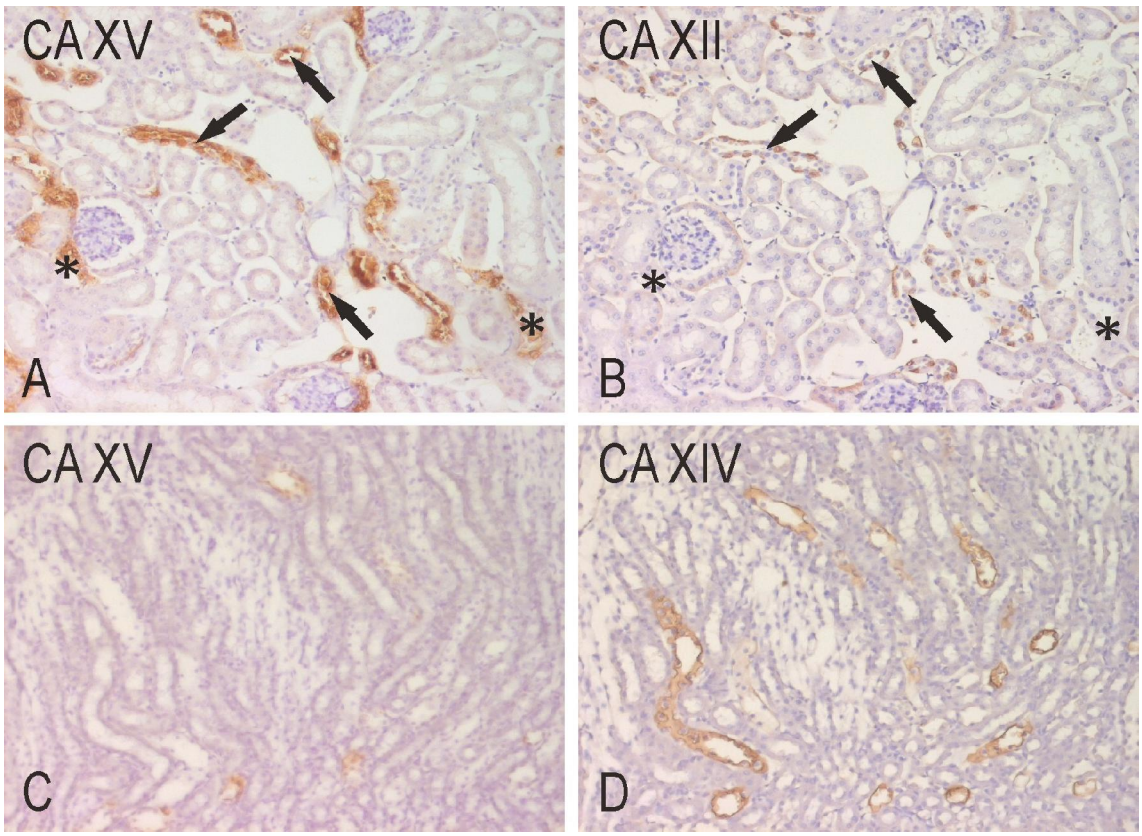

Figure 7. Immunohistochemical staining of CA XV (A, C), CA XII (B) and CA XIV (D) in the mouse kidney. CA XV and CA XII are both detected in the same cortical collecting ducts (arrows), even though the reaction for CA XV is notably more extensive and intense. CA XV is also present in the thick ascending limbs of Henle (asterisks), which are negative for CA XII. In the medulla (C,D), CA XIV is expressed in the upper portion of the thin descending limbs, as described previously by Kaunisto et al. (2002). No reaction for CA XV is detected in these segments. Original magnifications x400. Figure adopted from Saari et al., 2010.

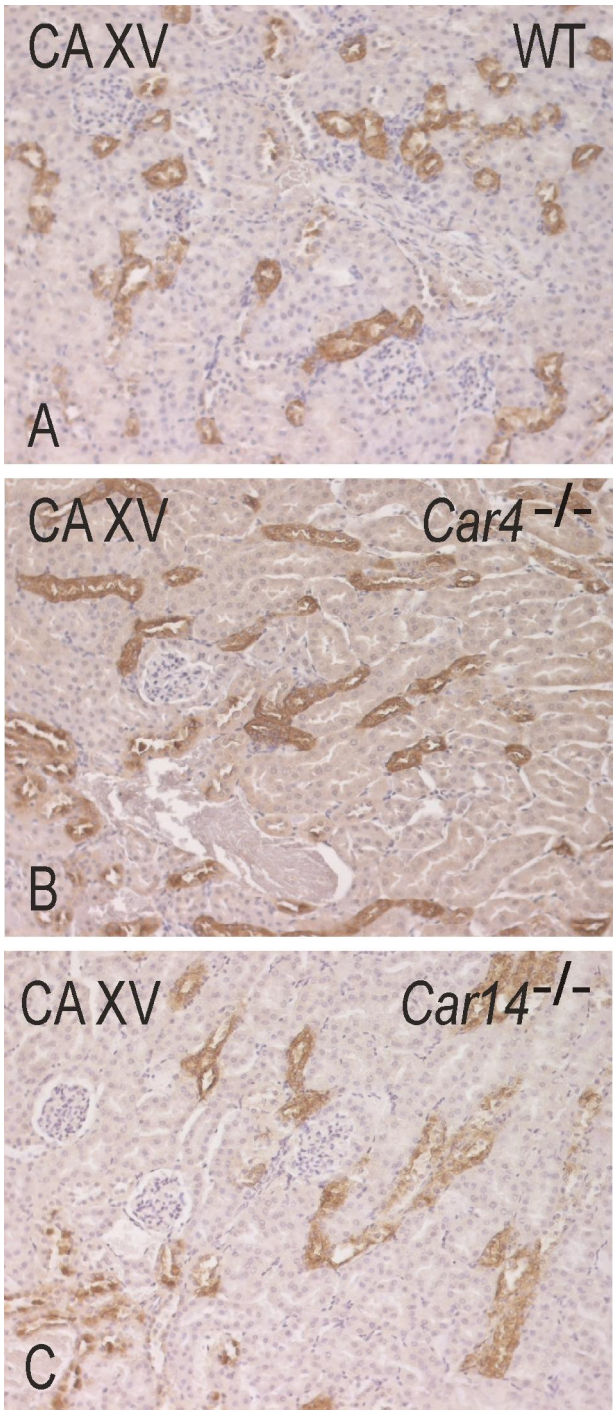

Figure 8. Immunohistochemical staining of CA XV in wild-type (A), *Car4*$^{-/-}$ (B) and *Car14*$^{-/-}$ (C) mouse kidneys. In addition to the strong immunoreactions observed in all previous sections in the collecting ducts and thick ascending limbs, weak reaction is also observed in the proximal convoluted tubules of *Car4*$^{-/-}$ kidney. Original magnifications x400. Figure adopted from Saari et al., 2010.

5.2. EXPRESSION OF CA XV IN MOUSE KIDNEY

5.2.1. qRT-PCR

The immunohistochemical stainings showed higher reactivity for CA XV in one *Car4*$^{-/-}$ kidney than in wild type or *Car14*$^{-/-}$ kidneys. To see if knocking out *Car4* causes any alterations in *Car15* gene expression, the mRNA levels of *Car15* were examined in the specimens of wild type, *Car4*$^{-/-}$ and *Car14*$^{-/-}$ mice by quantitative real time PCR. The results were expressed relative to the amounts of transcripts of the housekeeping gene *GAPDH*. No significant changes in the mRNA levels were observed. (Figure 9.) Unexpectedly, three of five kidney samples from *Car14*$^{-/-}$ mice showed increased expression of *Car15* mRNA in comparison to the wild type and *Car4*$^{-/-}$ kidneys, but the difference showed no statistical significance according to the χ^2 test.

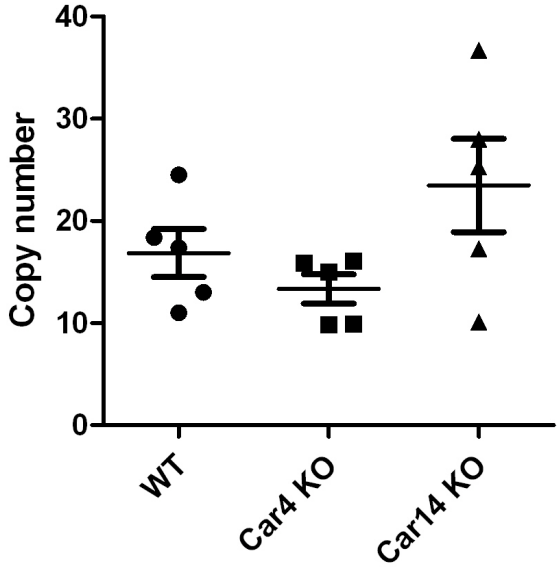

Figure 9. qRT-PCR analysis of *Car15* mRNA levels in the kidney samples from wild type (A), *Car4*$^{-/-}$ (B) and *Car14*$^{-/-}$ (C) mice. Three of five kidney samples from *Car14*$^{-/-}$ mice showed slight increase in expression of *Car15* mRNA in comparison to the wild type and *Car4*$^{-/-}$ kidneys, but the difference showed no statistical significance. Figure adopted from Saari et al., 2010.

6. DISCUSSION

6.1. LOCALIZATION OF CA XV IN MOUSE TISSUE

Kidneys function as a crucial part of acid-base homeostasis maintenance by reabsorbing and regenerating bicarbonate from the urine back to blood circulation. The protons formed in the regeneration process are secreted back to the lumen, and together with bicarbonate reabsorption, lead to urine acidification. The proximal tubule is considered as the major segment for acidification with 80 % of bicarbonate reabsorption occurring in this part of the nephron. The loop of Henle reabsorbs about 10 % of bicarbonate and the rest is absorbed by the distal tubule and collecting duct. The latter part of the nephron is considered as the major contributor in proton secretion. Among different CA isoforms, CA II is probably the main enzyme in the production of protons in different sections of the nephron, especially the proximal tubule and collecting ducts (Boron, 2006). Although CA II is responsible for 95 % of CA activity in the kidney, membrane-associated CA activity is involved in the process as well and seem to have a key role in the function of the loop of Henle (Purkerson & Schwartz, 2007). All membrane-associated activity was long attributed to CA IV but quite recently, four other membrane-associated isoforms, CA IX, CA XII, CA XIV and CA XV have been discovered to be active in the kidney (Hilvo et al., 2005, Kaunisto et al., 2002, Kyllönen et al., 2003, Brown et al., 1990, Kallio et al., 2006). CA IX is not present in the normal kidney but has been found to be active in renal carcinomas (Purkerson & Schwartz, 2007).

In rodents, CA IV is expressed on the apical membranes of the thick ascending limbs of Henle and proximal tubules, in the latter one also on the basolateral side of the epithelial cells (Brown et al., 1990). CA XII was originally identified as a tumor-associated isozyme, but has later been found also in the normal kidney where it was localized to the basolateral membranes of the proximal tubules and collecting ducts in rodents (Kyllönen et al., 2003). CA XIV is distributed on both the basolateral and apical membranes of the thin descending limbs of Henle and proximal tubules, and it has been suggested to play an even more important role in bicarbonate reabsorption than CA IV (Kaunisto et al., 2002). In a recent study, the results from RT-PCR suggested that CA XV, the most novel member of the α-CA gene family, might be expressed in the kidney

and, to lesser extent, in the brain and testis. *In situ* hybridization performed in the same study showed a positive reaction in the renal cortex (Hilvo et al., 2005). Results from the immunohistochemical stainings showed indeed a positive signal for CA XV in the kidney but no reaction was detected in any other tissue including brain and testis. Therefore, according to these findings, the distribution of CA XV seems to be more limited than in any other mammalian CA isozyme. In the kidney, most CA XV was located in the cortex, and weaker immunoreactions were seen in the medulla, which was consistent with the earlier *in situ* hybridization results by Hilvo et al. (2005). The expression was shown to be specifically restricted to the thick ascending limbs and collecting ducts of the renal cortex and outer medulla.

6.2. EXPRESSION OF CA XV IN THE MOUSE KIDNEY

CA IV is the most extensively distributed of all membrane-bound CA isoforms and, in addition to the kidney, is expressed in several different tissues including the intestine, lung, heart, gallbladder and brain (Shah et al., 2005, Zhu & Sly, 1990, Sender et al., 1998, Parkkila et al., 1996, Scheibe et al., 2006, Fleming et al., 1995). In a previous phylogenetic study by Hilvo et al. (2005), CA XV was found to be most closely related to CA IV, and these two isozymes share several similar structural properties. Both CA IV and CA XV are attached to the membrane by a GPI-anchor unlike any other membrane-bound isoform, and CA XV also contains N-linked glycosylations and stabilizing disulfide linkages, like CA IV (Hilvo et al., 2005). In addition, the immunochemical stainings in the present study showed expression partially in the same structures along the nephron. Due to these similarities and the fact that the activity of murine CA IV has been found to be only 10-20% of that of human CA IV (Tamai et al., 1996) it has been proposed that CA XV could compensate for this loss of activity in the rodent kidney. A similar role has been suggested for CA XIV which, unlike CA XV, is present in several human tissues, including the heart, liver, skeletal muscle, and brain (Parkkila et al., 2001, Parkkila et al., 2002, Wetzel et al., 2007, Scheibe et al., 2006), but in contrast to CA IV, the activity of human CA XIV is significantly lower than that of the mouse enzyme (Hilvo et al., 2007). Although CA XIV has been partially localized to the same nephron segments where CA IV is also present, mainly the proximal tubules (Kaunisto et al., 2002, Brown et al., 1990), the stainings in the present study showed no

overlapping for CA XIV with CA XV in its distribution. However, recent studies have suggested that CA XIV may have a more important role in bicarbonate reabsorption in proximal tubular cells in comparison to CA IV, as it is expressed in both S1 and S2 segments, while CA IV is mainly present in S2. In the present study, the expression of CA IV seemed to overlap that of CA XV in the thick ascending limbs and suggests that these two enzymes could share the same role in bicarbonate reabsorption within this segment. However, a reaction for CA XV was also detected in the collecting ducts of the renal cortex as well as a weaker reaction in outer medulla and in a recent study by Cheval et al. (2006) it was proposed that CA XV might participate in the control of cell proliferation during acidosis and potassium depletion in these segments of the nephron. In the study, CA XV showed different amplitude and time course of over-expression compared to other acid-base transport proteins, supporting the hypothesis that CA XV unlikely contributes to acid-base homeostasis in the collecting ducts (Cheval et al., 2006).

One of the aims of the research was to study the possible compensatory changes in the distribution and expression of CA XV when either CA IV or CA XIV was absent. In the immunohistochemical stainings, one specimen of *Car4*$^{-/-}$ kidney showed some extended reactivity for CA XV, especially in the proximal tubules, which was not evident in the wild type kidneys. However, when measured with quantitative real-time PCR, there seemed to be no significant change in the expression of *Car15* mRNA in *Car4*$^{-/-}$ kidney as compared to the wild type mice which indicates that the transcription of *Car15* gene is not increased in CA IV deficient mice. The positive reaction of the proximal tubules in the stainings may be due to a nonspecific binding of the antibody, yet this individual result is interesting as all other specimens showed a very specific reaction with basically no background staining at all. An even more interesting result was obtained when no change was detected in the distribution of CA XV in *Car14*$^{-/-}$ kidneys compared to the wild type specimens by immunohistochemical methods, but quantitative real-time PCR unexpectedly showed some mild up-regulation of *Car15* gene expression in three of five *Car14*$^{-/-}$ kidneys. The result did not reach statistical significance but may still refer to some compensatory changes in occasional cases. The compensation might be dependent on the physiological state of the animal as the three samples showing

elevated expression levels were all from female animals while the rest were received from males. This can only be considered as speculations as due to the small number of samples no conclusions can be made. However, although the compensatory properties of CA XV in absence of CA IV and CA XIV may not be as significant as presumed, they are not entirely excluded.

There is evidence of membrane-associated isozymes significantly contributing to the compensatory mechanisms in some cell types. A study by Shah et al. (2005) with knock-out mice showed that both CA IV and CA XIV contribute to buffering of the extracellular space in the brain. In double knock-out mice, the lack of both CA IV and CA XIV increased the amplitude of the alkaline transient, causing a delay in the alkaline shift. When analyzed by qRT-PCR, neither the CA IV nor the CA XIV transcript levels were up-regulated when the other was absent. However, no significant change in pH-regulation was detected in single knock-out mice compared to wild-type leading to the conclusion that the normal expression level of either CA IV or CA XIV is enough to compensate for the other isozyme. In the study by Hilvo et al. (2005), a positive signal for *Car15* mRNA in the brain was detected by both RT-PCR and *in situ* hybridization, which suggested CA XV as another candidate protein to contribute to extracellular buffering. However, no immunohistochemical signal was observed for CA XV in the normal or knock-out brain tissue (data not shown), suggesting that the protein is not produced in the brain. It is also possible that the level of expression was below the detection limit of the staining protocol used.

Over the past few years one main focus in CA research has been identification and development of different actual and potential clinical applications for CA inhibitors. These applications range from diuretics and antiglaucoma agents, to anticancer, anti-obesity and anti-epileptic drugs and finding isozyme-selective compounds is valuable to reduce side effects of the presently available drugs. However, a crucial problem in CA inhibitor design is related to the high number of isoforms, their localization in many tissues and organs, and the lack of isozyme selectivity of the presently available inhibitors. Recently, important advances have been reported in the design of compounds with high selectivity for clinically relevant isozymes, such as CA IX and XIII, over CA II inhibition which is regarded as detrimental due to the ubiquitous expression of the

isozyme. Despite the limited distribution of CA XV, the isoform possesses moderate activity and seems to be present in high amounts in rodent kidneys, and therefore, should be taken into account when investigating CA inhibitors on rodents as it might have a significant effect on the overall drug response.

7. CONCLUSIONS

The immunohistochemical staining showed that CA XV is only expressed in the kidney and therefore the distribution of the isozyme is the most limited of all CA isozymes. In the kidney, the most abundant expression of CA XV was located in the cortex region but some activity was also found in the outer medulla. Along the nephron, the isozyme is present in the thick ascending limbs of Henle and the collecting ducts, and the comparative staining showed this to be a unique distribution pattern among the isoforms present in the kidney. Although some changes in distribution were detected in *Car4*$^{-/-}$ sections in the immunohistochemical stainings, the lack of CA IV or XIV causes no compensating changes in *Car15* expression on transcriptional level. However, the intense reaction for CA XV in rodent kidney along with its moderate activity suggests a substantial role for this isozyme in renal physiology, and thus, the presence of CA XV in the kidney should be taken into account in future studies on CA inhibitors when using animal models.

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9. APPENDIX: the published article

The following article has been reproduced, with permission, from *Sina Saari, Mika Hilvo, Peiwen Pan, Gerolf Gros, Nina Hanke, Abdul Waheed, William S. Sly, and Seppo Parkkila: The most recently discovered carbonic anhydrase, CA XV, is expressed in the thick ascending limb of Henle and in the collecting ducts of mouse kidney,* PLoS One, 10:e9624, 2010.

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The Most Recently Discovered Carbonic Anhydrase, CA XV, Is Expressed in the Thick Ascending Limb of Henle and in the Collecting Ducts of Mouse Kidney

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Abstract

Background: Carbonic anhydrases (CAs) are key enzymes for physiological pH regulation, including the process of urine acidification. Previous studies have identified seven cytosolic or membrane-bound CA isozymes in the kidney. Recently, we showed by *in situ* hybridization that the mRNA for the most novel CA isozyme, CA XV, is present in the renal cortex. CA XV is a unique isozyme among mammalian CAs, because it has become a pseudogene in primates even though expressed in several other species.

Methodology/Principal Findings: In the present study, we raised a polyclonal antibody against recombinant mouse CA XV that was produced in a baculovirus/insect cell expression system, and the antibody was used for immunohistochemical analysis in different mouse tissues. Positive immunoreactions were found only in the kidney, where the enzyme showed a very limited distribution pattern. Parallel immunostaining experiments with several other anti-CA sera indicated that CA XV is mainly expressed in the thick ascending limb of Henle and collecting ducts, and the reactions were most prominent in the cortex and outer medulla.

Conclusion/Significance: Although other studies have proposed a role for CA XV in cell proliferation, its tightly limited distribution may point to a specialized function in the regulation of acid-base homeostasis.

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Introduction

Carbonic anhydrases (CAs) are zinc metalloenzymes that function as regulators of systemic acid-base homeostasis by catalyzing the interconversion of carbon dioxide and bicarbonate. Sixteen members of the α-CA gene family have been found, from which 13 possess catalytic activity [1]. CAs are distributed in different tissues to participate in a variety of physiological processes, including urine acidification. In the kidney, at least seven isozymes (CA II, IV, IX, XII, XIII, XIV, and XV) have been identified [1,2,3,4,5,6,7,8,9,10,11]. Most of these isozymes are associated with the plasma membrane, except for cytosolic CA II and XIII [2,3,4,9,11]. Nonetheless, about 95% of all CA activity in the kidney is cytosolic and probably accounts for the high activity enzyme, CA II. Most of the remaining activity has been attributed to CA IV, CA XII, and CA XIV [7,10,12,13]. Although the expression of different isozymes varies along the nephrons of different species, CA II seems to be the most widely distributed isozyme, being present in the intercalated cells of the collecting ducts as well as in the proximal tubules and the loop of Henle [14]. Both CA II and CA IV have been reported to associate with bicarbonate transporters [15]. Of the five membrane-bound CAs, CA IV is the most extensively expressed and has been found in the thick ascending limb and S2 segments of the proximal tubules of the rat kidney [5], and also in the intercalated cells of the rabbit collecting duct [16]. CA IV has been located predominantly at the luminal membranes, and some expression has also been reported at the basolateral membranes [5,17]. The luminal CA activity was long thought to be solely attributable to CA IV until the two novel CAs, CA XIV and CA XV, were isolated and characterized. CA XII was originally identified as a tumor-associated isozyme [18,19], but it was soon also demonstrated at the basolateral membranes in both S1 and S2 segments of the proximal tubules as well as in the cortical and outer medullary collecting ducts of the rat and mouse kidney [7]. In addition, it was found in the thick ascending limbs and distal convoluted tubules of the human

kidney [8]. CA XII is most closely related to the other transmembrane isozyme, CA XIV, and their CO_2 hydration activities are in the same range [20]. However, their subcellular locations are different: CA XII is confined to the basolateral membranes, whereas CA XIV is predominantly located at the luminal membranes. CA XIV is expressed in the thin descending limbs of Henle and S1 segments of the proximal tubules, and it may account for a considerable fraction of the luminal activity previously attributed to CA IV [10]. Besides CA II, CA XIII is another cytosolic isoform located in the kidney and has been found in the collecting ducts and renal corpuscle [9]. Although the low activity enzyme, CA III, may not be present in the normal kidney, it has been detected in mice and patients with proximal tubule dysfunction [21]. Transmembrane CA IX is weakly expressed in the rodent kidney [6], but its expression is highly induced in renal cell carcinoma [22,23,24].

CA XV, the most recently discovered CA isozyme, is most closely related to CA IV. Both isozymes have N-linked glycosylations and are attached to the membrane via a glycosylphosphatidylinositol (GPI)-anchor at the C-terminus of the polypeptide [1,25]. CA XV is the first member of the α-CA gene family that is expressed in several species, but its gene has become a pseudogene in primates. This finding has to be considered in context with the presence of the high activity CA IV in humans and the low activity CA IV in rodents [26]. Thus, predominantly apical CA XV and CA XIV enzymes could functionally compensate the lower activity of CA IV in rodents. The presence of the high activity CA IV enzyme could explain why CA XV is no longer needed in primates.

Since our previous study [1] showed that, among other species, CA XV is also active in rodents, we examined the distribution of *Car15* mRNA in mouse tissues with RT-PCR and *in situ* hybridization. RT-PCR of *Car15* mRNA showed a strong band for the kidney and weaker bands for the brain, testis, and 7-day and 17-day embryos. *In situ* hybridization showed the most abundant expression in the renal cortex. Recombinant mouse CA XV was expressed in a baculovirus/insect cell expression system [27], and a polyclonal antibody was raised against the purified recombinant protein. In the present study, the new antibody was used for the immunohistochemical localization of CA XV in mouse kidney. The distribution of CA XV was compared with CA II, IV, XII and XIV. Immunostainings were also performed on *Car4*$^{-/-}$ and *Car14*$^{-/-}$ kidney samples, and the reactions were compared to those observed in the wild type kidneys. We also performed quantitative real time polymerase chain reactions (qRT-PCR) to see if there were any changes in *Car15* mRNA expression between the *Car4*$^{-/-}$, *Car14*$^{-/-}$ and wild type kidneys.

Materials and Methods

Antibodies

A polyclonal rabbit antibody against the recombinant mouse CA XV [27] was raised in a rabbit by Innovagen AB (Lund, Sweden) and used to detect CA XV in tissue sections. For comparison, other anti-CA sera were also used, including rabbit anti-mouse CA II [9], rabbit anti-mouse CA IV [28], rabbit anti-mouse CA XII [7], and rabbit anti-mouse CA XIV [10,29]. A rabbit antibody against Tamm-Horsfall glycoprotein (THP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to identify the thick ascending limb of Henle in kidney sections.

Western Blotting

The rabbit anti-mouse CA XV antibody was first tested by Western blotting. Recombinant mouse CA XV produced in Sf9 insect cells was subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Richmond, CA). After transblotting, the membranes were treated for 25 minutes with Tris-buffered saline (TBS)+0.3% Tween 20 (TBST) containing 10% cow colostral whey (Hi-Col, Oulu, Finland), and then incubated for one hour with polyclonal anti-CA XV or pre-immune serum, each diluted 1:500 with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Membranes were washed five times for 5 minutes in TBST and then incubated for one hour with horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham Sciences, Little Chalfont, UK) diluted 1:25,000 in BSA-PBS. After washing the membranes four times for 5 minutes in TBST, the antibody binding was visualized by enhanced chemiluminescence (ECL; Amersham Sciences, Little Chalfont, UK).

Immunohistochemistry

Mouse tissue sections obtained from several NMRI mice (at the Animal Care Center of the University of Tampere, Finland) were cut into 3 µm-thick sections and dried onto Superfrost Plus™ microscope slides. For comparison, tissue specimens were also obtained from three *Car4*$^{-/-}$ [30], four *Car14*$^{-/-}$ [30], and four wild type C57BL6 mice. The tissues included the kidney, liver, stomach, small intestine, colon, spleen, pancreas, heart, lung, and brain. Mouse tissue collection was conducted according to the provisions of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, France). According to the national guidelines, no permission was required by authorities to collect tissue specimens from sacrificed mice.

The immunostaining method included the following steps: (1) deparaffinization of the sections using xylene and ethanol series; (2) treatment with 3% H_2O_2 in methanol for 5 min; (3) rinsing in TBS; (4) blocking with Rodent Block M™ (Biocare Medical, Concord, CA) for 30 min; (5) washing 3 times for 5 min with TBS; (6) incubation with polyclonal anti-CAs or anti-THP for 1 h (all the other antibodies except for anti-CA IV) or 2 h (anti-CA IV); (7) washing 3 times for 5 min with TBS; (8) incubation with a mixture of Rabbit on Rodent HRP-Polymer™ and blocking reagent XM Factor™ (20 µl XM Factor™ in 1 ml HRP-Polymer) (Biocare Medical, Concord, CA) for 30 min; (9) washing 3 times for 5 min with TBS; (10) incubation in DAB (3,3′-diaminobenzidine tetrahydrochloride) solution (Zymed, Carlsbad, CA) for 5 min; (11) rinsing in ddH_2O; (12) counterstaining with hematoxylin (13); and rinsing again with ddH_2O. The sections were mounted in Entellan Neu™ (Merck; Darmstadt, Germany) and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss; Göttingen, Germany).

Quantitative Real Time PCR

mRNA was isolated from 15 mouse kidneys obtained from five *Car4*$^{-/-}$, five *Car14*$^{-/-}$, and five wild type C57BL6 mice. The kidneys were homogenized (Heidolph Silent Crusher S, Colonial Scientific, VA, USA), and the RNA was isolated using an RNeasy Mini Kit™ (Qiagen Sciences, MD, USA). The RNA concentration and purity were determined by NanoDrop™ (Thermo-Scientific, DE, USA). One microgram of total RNA was used as the template in reverse transcription (RT) and polymerase chain reaction (PCR). The RT reaction was performed at 37°C for 2 h followed by denaturation at 85°C for five minutes. The PCR cycling was performed at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and elongation at 58°C for one minute. Dissociation

was conducted at 95°C for 15 seconds. Primers for mouse glyceraldehyde-6-phosphate dehydrogenase (mGAPDH) were used to monitor the quality and quantity of cDNA.

Quantitative real time PCR was conducted using SYBR Green measured with ABI7000 (AME bioscience, Norway). Primers for murine *Car15* were the same as described in a previous study [1]. Measurements were performed in duplicate with 0.5 µl cDNA. Transcripts for the housekeeping gene *GAPDH* were also measured, and the values of *Car15* were expressed relative to the *GAPDH* expression.

Results

Localization of CA XV

The novel rabbit anti-mouse CA XV serum was first tested by Western blotting. The antiserum identified a strong 34- to 36-kDa polypeptide band and a smaller 31-kDa band on the Western blot of the purified recombinant mouse CA XV (Fig. 1). The result was in line with the earlier study where the 34- to 36-kDa polypeptide represented mature CA XV, while the 31-kDa polypeptide was a non-glycosylated form of CA XV [1]. Pre-immune serum showed no reaction.

The localization of CA XV was studied by immunohistochemistry in mouse tissue samples. Kidney was the only tissue showing positive immunoreaction for CA XV, and the strongest signal was observed in the cortex and occasional staining the outer medulla (Fig. 2A). In these regions, CA XV seemed to be predominantly expressed in the thick ascending limb of Henle and collecting ducts

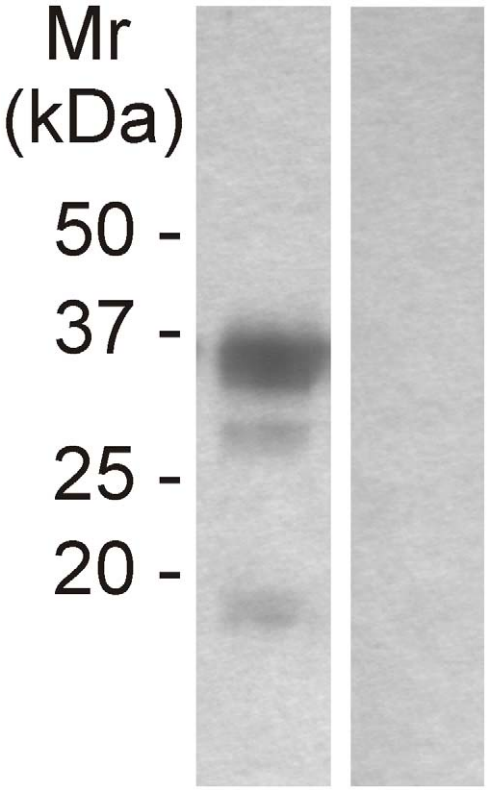

Figure 1. Western blot of recombinant mouse CA XV identified with the new anti-CA XV antibody. The antibody identifies a 34- to 36-kDa polypeptide and a smaller 31-kDa polypeptide that is suggested to be a non-glycosylated form of CA XV (left). Pre-immune serum showed no reaction (right).
doi:10.1371/journal.pone.0009624.g001

(Fig. 2B, C). The weak staining observed deeper in the medulla was located in the collecting ducts.

To confirm the location of the positive signal, an antibody against Tamm-Horsfall glycoprotein was used as a marker of thick ascending limbs [10]. Figure 3(A, B) shows that the Tamm-Horsfall glycoprotein-positive tubules were also positively identified in the CA XV immunostaining. In general, the marker gave a fainter reaction in comparison to CA XV.

We also performed immunostainings for CA XV and the other four renal CAs, including CA II, IV, XII, and XIV, in parallel tissue sections. The results showed that the distribution pattern of CA XV was unique among all the other isozymes. CA II-staining was more intense and widely spread than CA XV, as expected, involving both the proximal convoluted tubules and collecting ducts. CA XV was co-expressed in the cortical collecting ducts, whereas the reactions for CA XV were much weaker in the medulla (Fig. 4). The CA XV-positive structures identified as the thick ascending limbs of Henle remained negative for CA II.

According to the phylogenetic studies, CA IV is the most closely related isozyme to CA XV, and they both showed expression in the thick ascending limbs (Fig. 5). The proximal convoluted tubules also showed positive reaction for CA IV, as reported earlier [5]. More background signal was seen in the CA IV-staining due to a longer incubation time with the primary antibody.

CA XII has been localized to the mouse proximal convoluted tubules and more strongly to the collecting ducts [7]. In our study, CA XII showed very weak reactions in the proximal tubules and strong signal in the collecting ducts; the collecting ducts also showed strong reactions for CA XV (Fig. 6). CA XIV is predominantly expressed in the thin descending limbs of Henle and proximal convoluted tubules [10]. Therefore, CA XIV-positive structures were distinct from those of CA XV (Fig. 6).

CA XV was also localized in the kidney specimens obtained from $Car4^{-/-}$ and $Car14^{-/-}$ mice to see if the absence of either CA IV or XIV caused any changes in the amount of CA XV immunoreactivity. The kidney was the only CA XV-positive tissue in the knockout mice like in the wild type mice. One kidney specimen obtained from a $Car4^{-/-}$ mouse showed a more widespread immunostaining for CA XV including moderate reactivity in the proximal convoluted tubules (Fig. 7). In the other specimens, we observed no significant change in the expression levels or distribution patterns.

Quantitative Real-Time PCR

Because immunohistochemical staining showed higher reactivity in one $Car4^{-/-}$ kidney than in wild type or $Car14^{-/-}$ kidneys, we performed quantitative real time PCR to examine *Car15* mRNA levels in the kidney specimens of wild type, $Car4^{-/-}$ and $Car14^{-/-}$ mice. The results were expressed relative to the amounts of transcripts of the housekeeping gene *GAPDH*. There were no significant changes in the mRNA levels (Fig. 8). Unexpectedly, three of five kidney samples from $Car14^{-/-}$ mice showed increased expression of *Car15* mRNA in comparison to the wild type and $Car4^{-/-}$ kidneys, but the difference showed no statistical significance according to the chi-square (χ^2) test.

Discussion

Urine acidification is regulated by two major processes: proton secretion and bicarbonate reabsorption. Among different CA isozymes, CA II is probably the main contributor to the production of protons in different sections of the nephron and collecting ducts. There is also general agreement about the

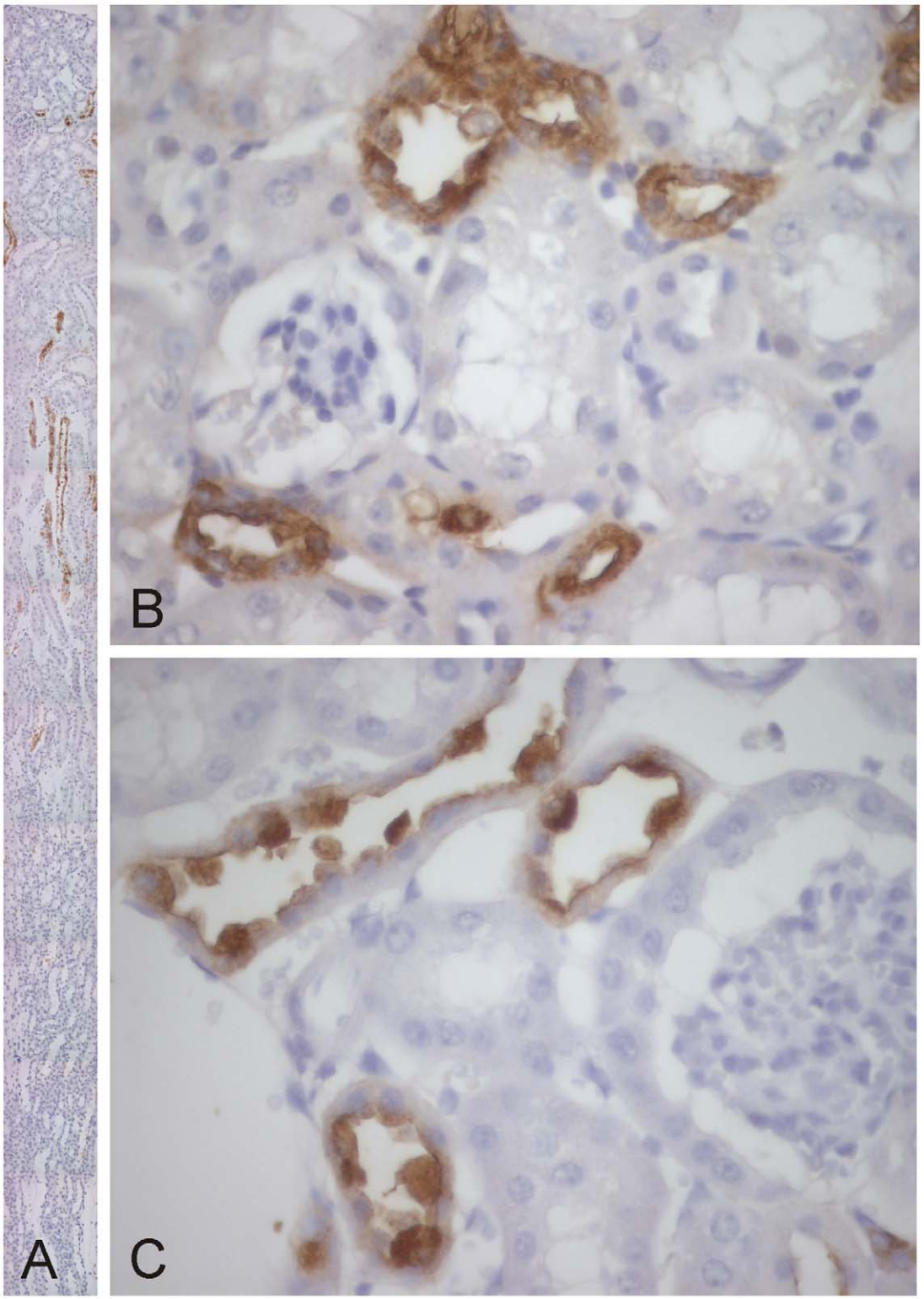

Figure 2. Immunohistochemical staining of CA XV in the mouse kidney. A sectional view of mouse kidney shows that CA XV is expressed in the cortex, and a weaker reaction is seen in the collecting ducts of the outer medulla (A). Intense staining is seen in the thick ascending limbs (B) and collecting ducts of the cortex (C). Original magnifications x100 (A), x630 (B,C).
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importance of the proximal tubule in bicarbonate reabsorption [31]. However, other downstream segments participate in this process as well [32]. In fact, the loop of Henle, under normal conditions, is able to reabsorb about 15% of the filtered bicarbonate [33]. Membrane-associated CA activity is apparently involved in this process. Even though the minority of all CA

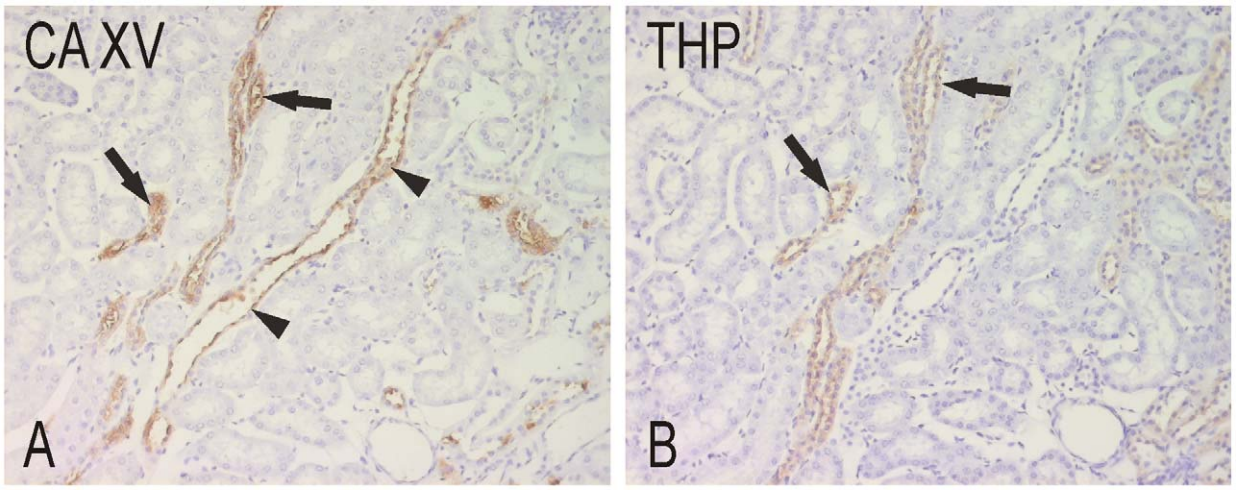

Figure 3. Confirmation of CA XV immunostaining in the thick ascending limb of Henle. Tamm-Horsfall glycoprotein (THP) antibody was used as a marker (B). CA XV (A) clearly labels the thick ascending limbs (arrows) and collecting ducts (arrowheads). Original magnifications x400.
doi:10.1371/journal.pone.0009624.g003

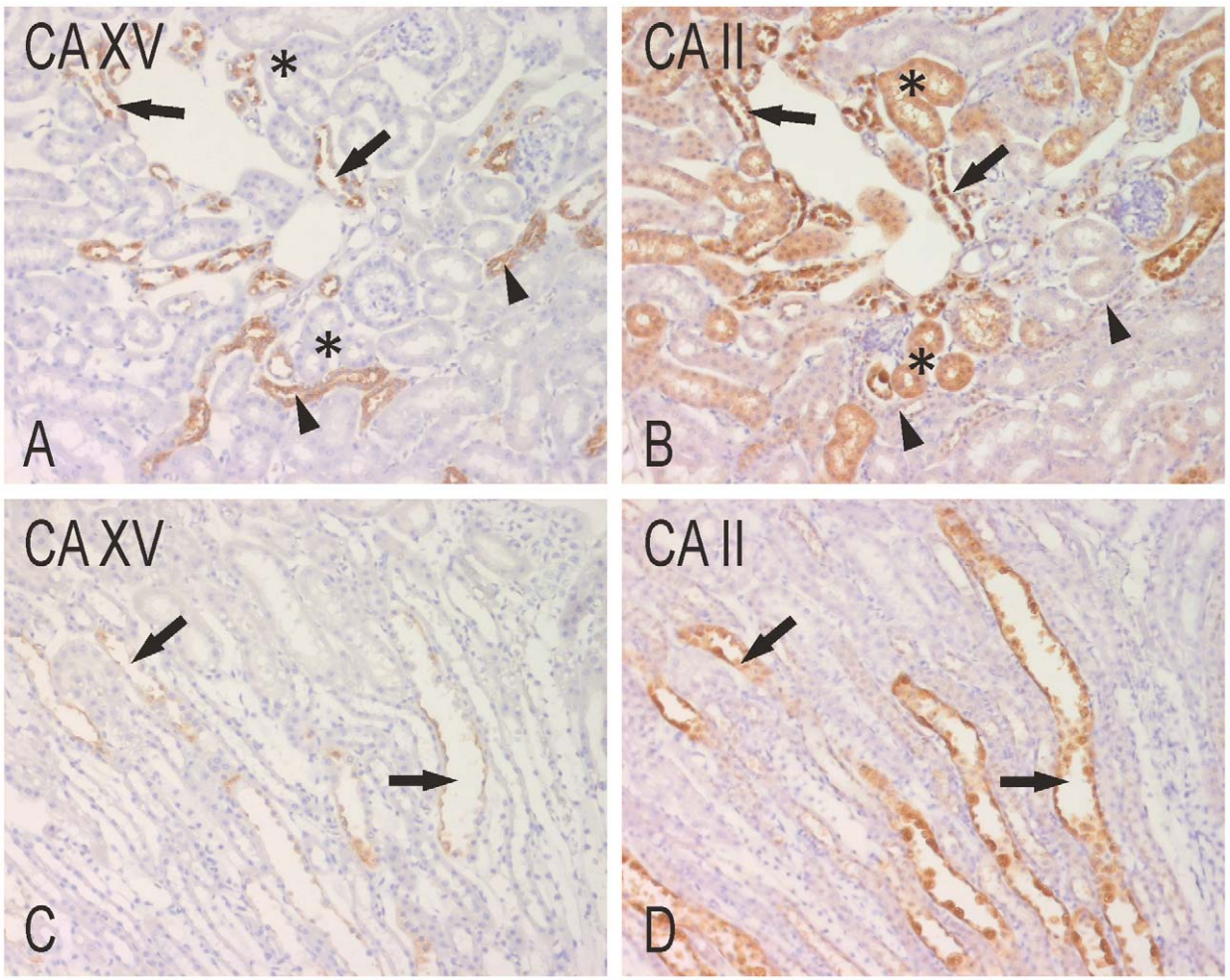

Figure 4. Immunohistochemical staining of CA XV and CA II in parallel sections of mouse kidney. In the cortex, the expression of CA XV (A) is more limited compared to CA II (B) that is mainly expressed in the collecting ducts (arrows) and proximal tubules (asterisks). Arrowheads show the thick ascending limbs of Henle, which are positively stained for CA XV. In the medulla, only a very faint reaction is seen for CA XV (C) in the collecting ducts (arrows), while the staining for CA II (D) is more intense. Original magnifications x400.
doi:10.1371/journal.pone.0009624.g004

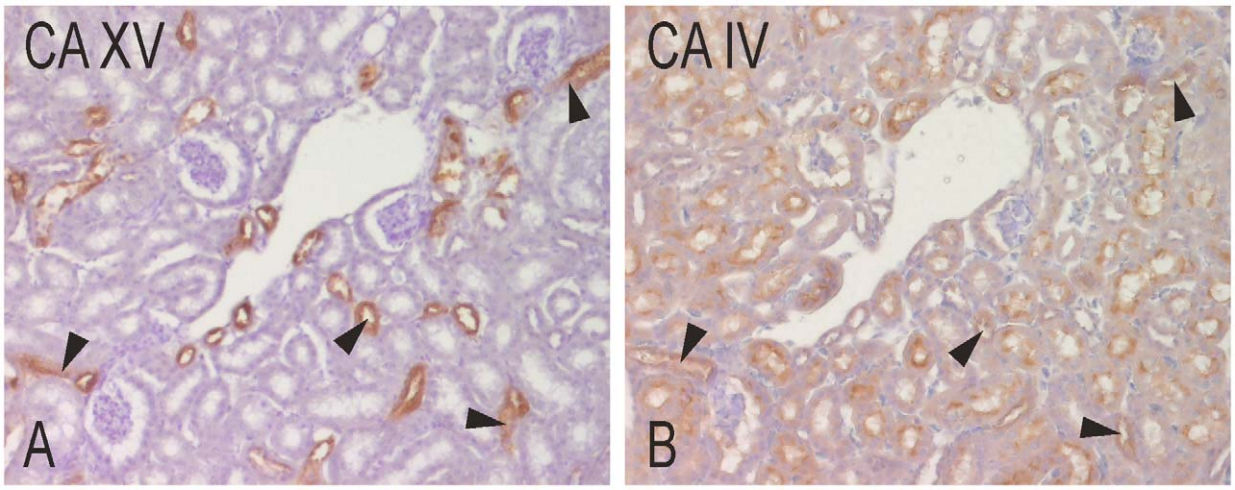

Figure 5. Immunohistochemical staining of CA XV and CA IV in mouse kidney. The staining for CA XV (A) is more intense and restricted compared to CA IV (B). CA IV shows a reaction in the thick ascending limbs (arrowheads) like CA XV, and is also present in the proximal tubules, which are not stained for CA XV. Original magnifications ×400.
doi:10.1371/journal.pone.0009624.g005

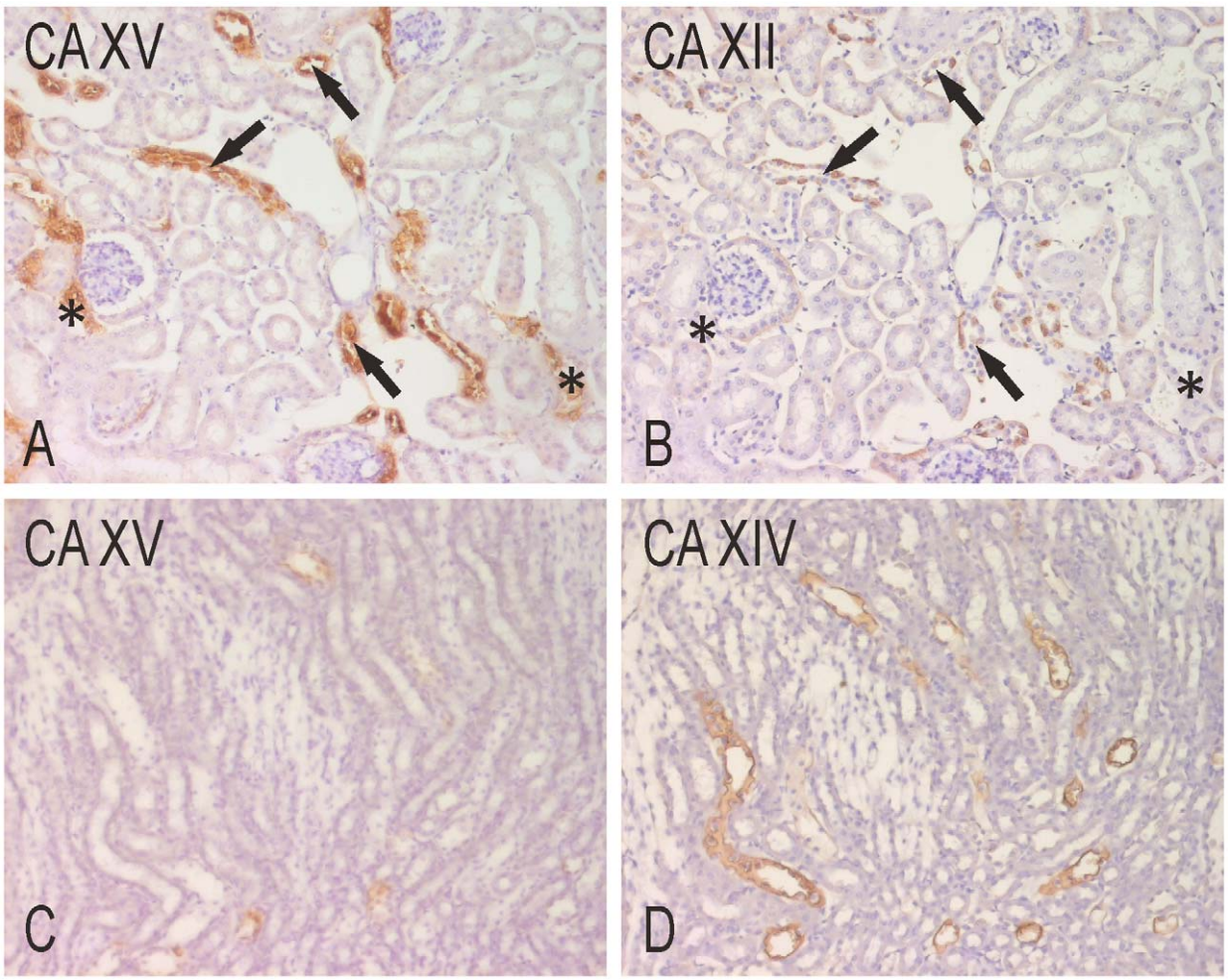

Figure 6. Immunohistochemical staining of CA XV, CA XII and CA XIV in mouse kidney. CA XV (A) and CA XII (B) label the same cortical collecting ducts (arrows), even though the reaction for CA XV is notably more extensive and intense. CA XV is also present in the thick ascending limbs of Henle (asterisks), which are negative for CA XII. In the medulla, CA XIV (D) is expressed in the upper portion of the thin descending limbs, as described previously [10]. These segments are negative for CA XV (C). Original magnifications ×400.
doi:10.1371/journal.pone.0009624.g006

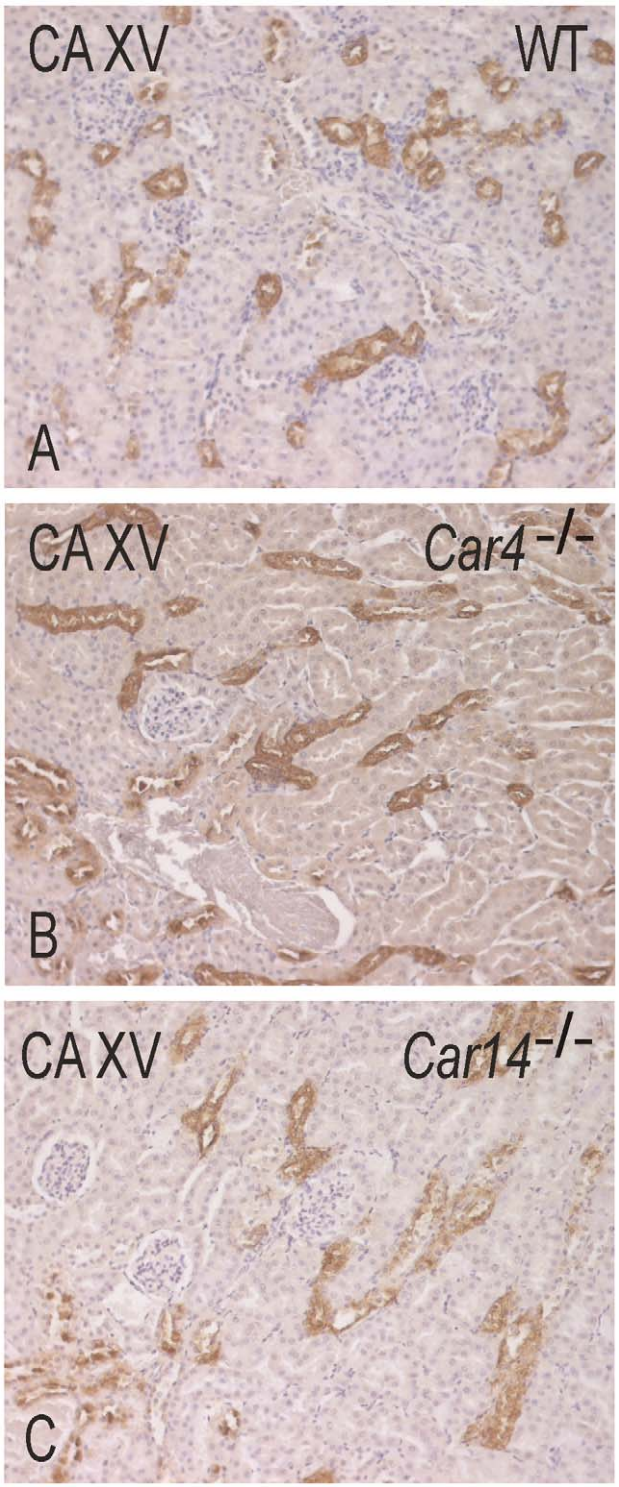

Figure 7. Immunohistochemical staining of CA XV in wild-type, *Car4*$^{-/-}$ and *Car14*$^{-/-}$ mouse kidneys. In addition to the strong immunoreactions observed in all the cases in the collecting ducts and thick ascending limbs, weak positivity is also observed in the convoluted tubules of *Car4*$^{-/-}$ kidney. Original magnifications ×400.
doi:10.1371/journal.pone.0009624.g007

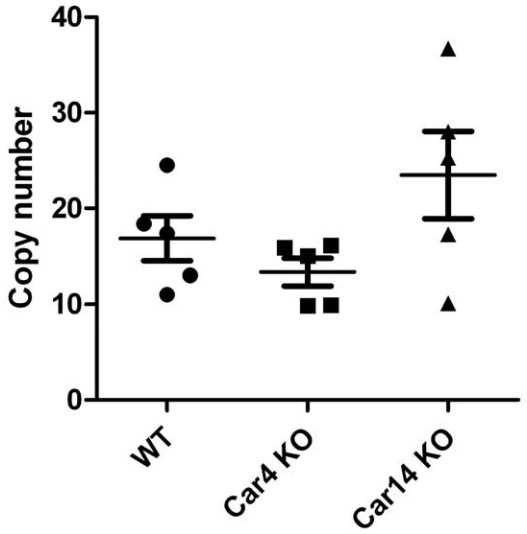

Figure 8. Quantitative real-time PCR analysis of *Car15* mRNA levels in the kidney specimens of wild type and *Car4*$^{-/-}$ and *Car14*$^{-/-}$ knockout mice. Three of five kidney samples from *Car14*$^{-/-}$ knockout (KO) mice showed slightly increased expression of *Car15* mRNA in comparison to the wild type (WT) and *Car4*$^{-/-}$ kidneys, but the difference between the groups showed no statistical significance.
doi:10.1371/journal.pone.0009624.g008

activity in the kidney is membrane-associated [12,13], five isozymes have been discovered to be responsible for it [1,5,6,7,10]. All membrane-bound CA activity was long attributed to CA IV. In rodents, it is expressed on the apical membranes of the thick ascending limbs of Henle and proximal tubules and less so on the basolateral side of the epithelial cells [5]. CA XII was originally identified as a tumor-associated isozyme, which was localized to the basolateral membranes of the proximal tubules and collecting ducts in rodents [7]. CA XIV is distributed on both the basolateral and apical membranes of the thin descending limbs of Henle and proximal tubules, and it has been suggested to play a key role in bicarbonate reabsorption along with CA IV [10]. In a recent study, the results from RT-PCR and *in situ* hybridization suggested that CA XV, the most novel member of the α-CA gene family, might be expressed in the renal cortex [1]. Our results showed positive signal for CA XV only in the kidney, and, therefore, the distribution of CA XV appears to be very limited as compared to all other mammalian CA isozymes. In the kidney, most CA XV was located in the cortex, and weaker immunoreactions were seen in the medulla, which was consistent with the earlier *in situ* hybridization results [1]. The expression was shown specifically in the thick ascending limbs and collecting ducts of the cortex and outer medulla.

CA IV is expressed in several different tissues. In addition to the kidney, it has been found, for example, in the heart, intestine, lung, and gallbladder [25,34,35,36,37]. According to our previous phylogenetic studies, CA XV is most closely related to CA IV, and these two isozymes share several similar structural properties [1]. The staining showed expression partially in the same structures along the nephron, and, unlike the other membrane-bound isozymes, both CA IV and CA XV are attached to the membrane by a GPI-anchor. CA XV also contains N-linked glycosylations and stabilizing disulfide linkages, like CA IV [1]. The activity of murine CA IV is only 10-20% of that of human CA IV [26], which has raised the question of whether the two isozymes, CA XIV and XV, could compensate for this loss of activity in the rodent kidney. Unlike CA XV, which has become a pseudogene in humans and chimpanzees, CA XIV is present in several human tissues, including the heart, liver, skeletal muscle, and brain [29,37,38,39], but the human enzyme has significantly lower

activity than the mouse enzyme [40]. Although CA XIV has been partially localized to the same nephron segments where CA IV is also produced [5,10], CA XIV showed no overlapping with CA XV in its distribution.

In rodent kidney, recent studies have suggested that CA XIV may have a more important role than CA IV in bicarbonate reabsorption in proximal tubular cells, because it is expressed in both S1 and S2 segments, while CA IV is mainly present in S2 [10]. An interesting discovery was the unexpected halo-tolerance of CA XIV, a physiological property that allows the isozyme to stay active at a lower pH than CA IV [41]. The functional significance of CA XIV may not only be to compensate for the lower activity of murine CA IV as expected but also to adapt to a more challenging environment.

In addition to proximal tubules, CA IV is expressed in the thick ascending limbs of Henle [5] together with CA XV. Based on the similar localization, one could argue that these two enzymes can share the same role in bicarbonate reabsorption within this segment. However, it was recently proposed that CA XV might participate in the control of cell proliferation during acidosis and potassium depletion in the collecting ducts [42]. CA XV showed a different amplitude and time course of over-expression compared to other acid-base transport proteins, supporting the hypothesis that CA XV unlikely contributes to acid-base homeostasis in the collecting ducts.

In the present study, we also evaluated the possible compensatory changes in *Car15* gene expression when either CA IV or CA XIV was absent. In one specimen of $Car4^{-/-}$ kidney, we observed some reactivity for the CA XV antibody, especially in proximal tubules, that was not evident in the wild type kidneys. However, when measured with quantitative real time PCR, there seemed to be no significant change in the expression of *Car15* mRNA in $Car4^{-/-}$ kidney as compared to the wild type mice. This result indicated that the transcription of *Car15* gene is not increased in CA IV deficient mice, and the positive reaction of the proximal tubules may be due to an artifact caused by nonspecific binding of the antibody. By immunohistochemistry, no change was detected in the distribution of CA XV in $Car14^{-/-}$ kidneys compared to the wild type specimens, but quantitative real time PCR unexpectedly showed some mild up-regulation of *Car15* gene expression in three of five $Car14^{-/-}$ kidneys. Even though the difference did not reach statistical significance, this slight tendency may point to some compensatory changes in occasional cases, depending on the physiological needs of the mice. Though the role of CA XV as a compensating isozyme for CA IV and CA XIV may not be as significant as presumed, it is not entirely excluded.

A study with knock-out mice showed that both CA IV and CA XIV contribute to buffering of the extracellular space in the brain [30]. The lack of both CA IV and CA XIV in double knock-out mice increased the amplitude of the alkaline transient, causing a delay in the alkaline shift. Neither the CA IV nor the CA XIV transcript levels were up-regulated when the other was absent, yet there was no significant change in pH-regulation in single knock-out mice compared to wild-type. These results suggested that the normal expression level of either CA IV or CA XIV is enough to compensate for the other isozyme. Both RT-PCR and *in situ* hybridization showed a positive signal for *Car15* mRNA in the brain [1], and, therefore, CA XV emerged as another candidate protein to contribute to extracellular buffering. However, we observed no immunohistochemical signal for CA XV in the normal or knock-out brain tissue (data not shown), suggesting that the protein is not produced in the brain or that the level of expression was below the detection limit of our staining protocol. Nevertheless, the membrane-associated isozymes may significantly contribute to the compensatory mechanisms in some cell types.

Author Contributions

Conceived and designed the experiments: SS MH PP GG NH AW WS SP. Performed the experiments: SS MH PP GG NH. Analyzed the data: SS SP. Contributed reagents/materials/analysis tools: SS MH PP GG NH AW WS SP. Wrote the paper: SS MH PP GG NH AW WS SP.

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