Expression studies on carbonic anhydrase IX

Master's Thesis

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Hiilihappoanhydraasi IX (CA IX) on ominaisuuksiltaan ainutlaatuinen hiilihappoanhydraasi-entsyymiperheen jäsen. Hiilihappoanhydraasien pääasiallinen tehtävä on säädellä elimistön happoemästasapainoa. Toisin kuin muut hiilihappoanhydraasit, CA IX osallistuu todennäköisesti myös solujen jakautumisen säätelyyn, kiinnittymiseen ja pahanlaatuisten kasvainten muodostumiseen. Tässä tutkimuksessa selvitettiin CA IX:n ilmentymistä hiiren ja ihmisen elimistössä. Tutkimuksella oli kaksi tavoitetta: ensimmäinen tavoite oli tutkia CA IX:n ilmentymistä hiiren kudoksissa, ja toinen tavoite oli kehittää immunomääritysmenetelmä, jolla voitaisiin mitata CA IX:n pitoisuutta ihmisen seerumissa.

Tutkimusmenetelmät: Lähetti-RNA:n transkriptiota tutkittiin hiiren kudoksissa käänteiskopiointi-PCR:n (RT-PCR) avulla. Hiiren CA IX –proteiinia tutkittiin Western blotilla ja immunohistokemian avulla. Immunomääritysmenetelmän periaatteena oli tunnistaa ihmisen CA IX –proteiini monoklonaalisten vasta-aineiden sekä kemiluminesenssiin perustuvan reaktion avulla. Immunomääritysmenetelmää sovellettiin kontrolliseeruminäytteisiin sekä munuais- ja rintasyöpäpotilaiden seerumeihin.

Tutkimustulokset: Hiiren kudoksissa voimakkain CA IX:n ilmentyminen havaittiin mahalaukun seinämässä. CA IX:ää löytyi paljon myös paksunsuolen enterosyyteistä ja haiman asinuksista. RT-PCR yllättäen näytti voimakkaan signaalin CA IX:n lähetti-RNA:lle munuaisessa ja luustolihaksessa, kun taas vastaavaa proteiinia ei havaittu immunoentsymaattisilla menetelmillä. Immunomääritysmenetelmällä ei saatu selville CA IX:n tarkkaa konsentraatiota seerumissa, vaikka menetelmä tunnisti CA IX –proteiinin. Joillakin munuaissyöpäpotilailla havaittiin korkeampia CA IX-pitoisuuksia seerumissa verrattuna kontrollihenkilöihin ja rintasyöpäpotilaisiin.

Johtopäätökset: Lähetti-RNA:n ja proteiinin välinen ero munuaisessa ja lihaksessa viittaa näille kudoksille ominaiseen transkription jälkeiseen säätelyyn, joka saattaa liittyä kudosten fysiologisiin ominaisuuksiin. Immunomääritysmenetelmän tulokset vahvistivat, että CA IX:n soveltuvuutta syövän merkkiaineeksi kannattaa selvittää jatkotutkimuksissa.

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Abstract

Background and aims: Carbonic anhydrase IX (CA IX) is a unique member of the CA family. It participates in the regulation of acid-base balance, cell proliferation, adhesion, and malignant processes. This study consisted of two goals: the first was to study the expression of CA IX in mouse tissues, and the second was to develop an immunoassay to detect CA IX levels in human serum.

Methods: mRNA transcription was studied by reverse transcriptase PCR (RT-PCR). CA IX protein was studied by Western blot and immunohistochemistry. The principle behind the immunoassay was to detect CA IX with monoclonal antibodies and chemiluminescent reaction: it was applied to serum samples of controls, and to renal and breast cancer patients.

Results: In mouse tissues, strong expression was observed in the gastric mucosa. Moderate reactions were seen in the colonic enterocytes and pancreatic acini. RT-PCR surprisingly revealed strong signal for CA IX mRNA in the kidney and skeletal muscle, while signal for the protein could not be observed. The exact concentration of CA IX in serum could not be revealed with the immunoassay, although it seemed to detect CA IX protein. Higher values were observed in some serum samples obtained from renal cancer patients compared to the other groups.

Conclusions: The discrepancy between mRNA and protein in the kidney and muscle suggests a tissue-specific post-transcriptional control mechanism for CA IX, possibly related to physiological demands. The immunoassay results confirm that further studies are reasonable to evaluate the value of CA IX as a tumor marker.

Contents

Abbreviations

aa	amino acid
AE	anion exchanger
bp	base pair
BSA	bovine serum albumin
CA	carbonic anhydrase
CA IX	carbonic anhydrase IX
CA9	*carbonic anhydrase 9* (refers particularly to the human gene)
Car9	*carbonic anhydrase 9* (refers particularly to the mouse gene)
CA-RP	carbonic anhydrase related protein
CAI	carbonic anhydrase inhibitor
CAM	cell adhesion molecule
ccRCC	clear cell renal carcinoma
cDNA	complementary deoxyribonucleic acid
DAB	3,3'-diaminobenzidine tetrahydrochloride
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
GST-PGCA	fusion protein consisting of glutathione S-transferase as well as CA IX catalytic and proteoglycan domains
HIF	hypoxia inducible factor
HRP	horseradish peroxidase
IHC	immunohistochemistry
kDa	kilodalton
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase-antiperoxidase
pVHL	von Hippel-Lindau protein
PBS	phosphate-buffered saline
PHD	prolyl-4-hydroxylase
PCR	polymerase chain reaction
RCC	renal cell carcinoma
RLU	relative light unit
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
sCA IX	soluble form of CA IX
SDS	sodium dodecyl sulfate
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau (gene or disease)
WB	Western blot

1. Introduction

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes whose main function is to participate in the regulation of acid-base balance. Mammals have at least 12 active isozymes that belong to the α-CA family. The CA isozymes differ in their subcellular localization, kinetic properties, and inhibition profiles. In addition, each CA isozyme has a unique distribution in tissues (Lehtonen et al., 2004). The expression pattern for each isozyme is important to know, since it reflects the physiological function of the CA enzyme in question.

Carbonic anhydrase IX (CA IX) is a transmembrane protein. Its expression has been studied intensively in humans as well as in rat alimentary tract. CA IX has a limited distribution in normal tissues: it is expressed mainly in the gastrointestinal tract (Pastorekova et al., 1997). A knock-out mouse model generated for CA IX revealed that it has an important role in gastric morphogenesis (Ortova Gut et al., 2002). However, no thorough study has been carried out to reveal the distribution of CA IX in mouse tissues. The expression of CA IX in mouse tissues formed the first specific goal of this thesis.

The unique structural feature of CA IX is its proteoglycan domain. In addition to pH regulation, CA IX participates in cell-cell adhesion with its proteoglycan domain. The dual function of CA IX has importance especially in several tumors. Although CA IX has a limited distribution in normal human tissues, a number of malignancies express high levels of CA IX. It is overexpressed especially under hypoxic conditions, and the expression of CA IX is regulated mainly by the VHL/HIF pathway (von Hippel-Lindau / Hypoxia Inducible Factor). This regulatory pathway explains why for example every clear cell renal carcinoma (ccRCC) expresses CA IX, although normal human kidney is negative for CA IX. At the moment a few studies are being carried out in order to reveal if CA IX could be used as a target for cancer therapy (Pastorekova & Zavada, 2004).

Overexpression of CA IX has also raised a question if this enzyme could be used clinically as a tumor marker. It has been shown that RNA extraction and RT-PCR from renal cancer patients' blood reveals circulating cancer cells (McKiernan et al., 1997). CA IX protein is also known to be shed from the cell membrane of renal cancer cells to blood (Zavada et al., 2003). Another diagnostic approach would be to study if CA IX protein in the serum could be used as a tumor marker. The second specific goal of this thesis was to develop an immunoassay method that could be utilized to monitor the level of CA IX in human serum samples.

2. Review of the literature

2.1. Acid-base balance

Enzymes are proteins that catalyze biochemical reactions, and thus they form the basis of life. Each enzyme has a characteristic pH value where its catalytic activity is optimum, and on either side of the optimum pH the activity often declines sharply. Because even small changes of pH can cause large changes in some crucial reactions, the control of the acid-base balance of cells and body fluids is important to physiological functions (Nelson & Cox, 2000).

Each part of the human body has its own peculiar pH. The normal pH of arterial blood and interstitial fluid is about 7.4. Intracellular pH is slightly lower than plasma pH because the metabolism produces acid, especially H_2CO_3. Depending on the type of cells, the pH of intracellular fluid has been estimated to range from 6.0 to 7.4. The pH of urine can range from 4.5 to 8.0, depending on the acid-base status of the extracellular fluid. An extreme example of acidic body fluid can be found in the stomach, where the pH can be as low as 0.8. There are three primary systems that regulate the hydrogen ion concentration in the body fluids to prevent acidosis or alkalosis: 1) the chemical acid-base buffer system that reacts within a fraction of a second 2) the respiratory system, which acts within a few minutes, and 3) the kidneys that regulate long-term acid-base balance (Guyton & Hall, 2000).

Buffers are aqueous systems that resist changes in pH when small amounts of acid or base are added. A buffer system consists of a weak acid and its conjugate base. The organisms have in general four main pH buffering mechanisms. 1) The cytoplasm of most cells contains high concentrations of proteins, which contain many amino acids with functional groups that are weak acids or weak bases. One such example is the side chain of histidine that has a pK_a of 6.0 (Nelson & Cox, 2000). In blood, one buffering agent is hemoglobin. It is normally slightly dissociated into hydrogen ions (protons) and hemoglobin anions: $HHb \Leftrightarrow H^+ + Hb^-$ (Nienstedt et al., 2002). 2) Some highly specialized organelles and extracellular compartments have high concentrations of compounds that contribute to buffering capacity, for example the ammonia buffers urine. 3) Phosphate buffer system, which mainly acts in the cytoplasm of cells, consists of $H_2PO_4^-$ as proton donor and HPO_4^{2-} as proton acceptor: $H_2PO_4^- \Leftrightarrow H^+ + HPO_4^{2-}$

(Nelson & Cox, 2000). 4) The bicarbonate buffering system buffers pH according to the following reactions: $CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$. This system is important because breathing eliminates excessive carbon dioxide very rapidly (Guyton & Hall, 2000). For example, if the pH of blood is lowered (the concentration of H^+ is raised), the equilibriums of both reactions are readjusted: bicarbonate and proton are fused and produce more H_2CO_3, which in turn dissolves into water and carbon dioxide, and finally CO_2 is exhaled. On the other hand, when the pH of blood is raised, the H^+ concentration is lowered, which causes H_2CO_3 to dissolve into bicarbonate and proton. This, in turn results in reduced exhalation of CO_2 in lungs (Nelson & Cox, 2000).

The reaction $CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$ is catalyzed by a family of enzymes called carbonic anhydrases (Sly & Hu, 1995).

2.2. Carbonic anhydrases (CAs)

2.2.1. General aspects

Evolution has produced three unrelated carbonic anhydrase families named α-CA, β-CA and γ-CA. The α-genes are present in vertebrates and also in many algae, plants and some eubacteria. The β-genes are present in vascular plants, eubacteria, archaebacteria and certain algae. The γ-genes can be found mainly at archea and some eubacteria (Chegwidden & Carter, 2000; Hewett-Emmett, 2000; Supuran, 2004). The focus of this thesis will be on mammals and therefore on α-CAs.

At the moment twelve active α-CAs have been characterized: CAs I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, and XIV (Lehtonen et al., 2004). Recent results have shown that a thirteenth member, named CA XV, also belongs to the family (Hilvo et al., unpublished). The main function of these isoenzymes is to catalyze the reversible hydration of carbon dioxide: $CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$. CAs belong to metalloenzymes since they contain a zinc-atom in their active site. It has been proposed that the central catalytic step in CAs is a reaction between CO_2 and a zinc-bound OH^- ion yielding a coordinated HCO_3^- ion, which is displaced from the metal ion by water molecule. The mechanism is illustrated schematically in Equation 1. The regeneration of OH^- involves the transfer of H^+ from the zinc-bound water molecule to the solution which is shown in Equation 2 (Lindskog & Silverman, 2000; Supuran, 2004).

$$(1)\quad EZn^{2+} - OH^- + CO_2 \Leftrightarrow EZn^{2+} - HCO_3^- \overset{H_2O}{\Leftrightarrow} EZn^{2+} - OH_2 + HCO_3^-$$

$$(2)\quad EZn^{2+} - OH_2 \Leftrightarrow EZn^{2+} - OH^- + H^+$$

The zinc-atom is coordinated by three histidine residues that are crucial for the CA activity of these enzymes (Stams & Christianson, 2000). CA-related proteins, designated CA-RPs, lack one or more of these critical histidine residues and thus they do not have the CA catalytic activity. Three CA-RPs, named CA-RPs VIII, X, and XI, have been characterized. The functional significance of these proteins is still unknown, but for example CA-RP VIII has been reported to be overexpressed in some carcinomas. Homologous CA-like domains without the critical histidine residues have also been found in extracellular parts of the receptor-type protein tyrosine phosphatases, RPTPβ and RPTPγ (Nishimori, 2004).

Two main classes of carbonic anhydrase inhibitors (CAIs) are the metal-complexing anions and the unsubstituted sulfonamides, which bind to the Zn(II) ion of the enzyme either by substitution (Equation 3) or addition to the metal coordination sphere (Equation 4). Sulfonamides are the most important CAIs: they bind in a tetrahedral geometry of the Zn(II) ion. The inhibitor forms an extended network of hydrogen bonds involving several amino acid residues as well as the metal ion. The aromatic or heterocyclic parts of the inhibitor interact with the hydrophilic and hydrophobic residues of the cavity (Supuran, 2004).

$$(3)\quad EZn^{2+} - OH_2 + I \Leftrightarrow EZn^{2+} - I + H_2O \quad \text{(substitution)}$$

$$(4)\quad EZn^{2+} - OH_2 + I \Leftrightarrow EZn^{2+} - OH_2(I) \quad \text{(addition)}$$

The active α-carbonic anhydrases differ in their subcellular localization, distribution in tissues, kinetic properties, and inhibition profiles. The subcellular localizations as well as affinity for sulfonamides (CA inhibitors) are listed in Table 2.1. Table 2.2 compares the catalytic activity of different CA isozymes. In the next sections, CA isozymes are described in the order of their subcellular localizations.

Table 2.1. Subcellular localizations and affinities for the sulfonamides of the CA isozymes. The data on subcellular localization has been extracted from (Lehtonen et al., 2004), except for CA XV (Hilvo et al., unpublished results). The information on the affinity for sulfonamides has been obtained from (Supuran, 2004).

CA isozyme	Subcellular localization	Affinity for sulfonamides
CA I	cytosol	medium
CA II	cytosol	very high
CA III	cytosol	very low
CA IV	membrane-bound	high
CA VA and VB	mitochondria	high
CA VI	secreted	medium-low
CA VII	cytosol	very high
CA IX	transmembrane	high
CA XII	transmembrane	high
CA XIII	cytosol	high
CA XIV	transmembrane	high
CA XV	membrane-bound	-

Table 2.2. The catalytic activity of the CA isozymes. CA XV is excluded from the table, since no activity data is available. The scale of CO_2 hydration activity is somewhat arbitrary.

CA isozyme	k_{cat} [a] (s^{-1})	k_{cat} / K_M [b] ($M^{-1} \times s^{-1}$)	Conditions (T, pH)	CO_2 hydration activity	Reference
human CA I	2.0×10^5	5.0×10^7	25 °C, 7.5	moderate	(Lehtonen et al., 2004)
human CA II	1.4×10^6	1.5×10^8	25 °C, 7.5	high	(Lehtonen et al., 2004)
human CA III	1.0×10^4	3.0×10^5	25 °C, 7.5	low	(Lehtonen et al., 2004)
human CA IV	1.0×10^6	5.0×10^7	25 °C, 7.5	high	(Lehtonen et al., 2004)
mouse CA V	7.0×10^4	3.0×10^7	25 °C, 7.5	moderate[c]	(Lehtonen et al., 2004)
CA VI	-	-	-	moderate	(Supuran, 2004)
human CA VII	9.5×10^5	8.3×10^7	20 °C, 7.5	high	(Vullo et al., 2005b)
human CA IX	3.8×10^5	5.5×10^7	25 °C, 7.5	high	(Lehtonen et al., 2004)
human CA XII	4.2×10^5	3.5×10^7	20 °C, 7.5	moderate	(Vullo et al., 2005a)
mouse CA XIII	8.3×10^4	4.3×10^7	25 °C, 7.5	moderate	(Lehtonen et al., 2004)
mouse CA XIV	-	-	-	high	(Whittington et al., 2004)

[a] k_{cat} is equivalent to the number of substrate molecules converted to product in a given enzyme molecule when the enzyme is saturated with substrate (Nelson & Cox, 2000)
[b] $kcat/k_M$ is the best way to compare the catalytic efficiencies of different enzymes; this parameter is a rate constant for the conversion of enzyme and substrate to enzyme and product (Nelson & Cox, 2000)
[c] The activity is high at pH 8.2 or higher pH (Supuran, 2004)

2.2.2. Cytosolic CAs

CA II is the most widely distributed isozyme: it is present in some cells of virtually all tissue types. CA II is also one of the fastest enzymes known and this fact also

emphasizes its importance in many physiological functions. In erythrocytes, CA II catalyzes the hydration of CO_2 to HCO_3^- while in renal tubules and collecting ducts it is important for the acidification of urine (Chegwidden & Carter, 2000). CA II also contributes to H^+ secretion by gastric parietal cells and it helps in the provision of H^+ ions in osteoclasts for bone resorption. CA II is involved in the production of numerous biological fluids. It promotes HCO_3^- secretion by pancreatic duct cells to pancreatic juice, provides HCO_3^- for bile in the liver epithelial duct cells and also catalyzes the production of HCO_3^- to saliva. CA II also regulates pH of the cerebrospinal fluid produced in the choroid plexus, and is involved in the production of aqueous humor in the eye. In distal colonic epithelium H^+ and HCO_3^- secretion catalyzed by CA II are coupled to Cl^- and Na^+ reabsorption and contribute to electrolyte and water balance. CA II has also been suggested to participate in fatty acid and amino acid synthesis (Sly & Hu, 1995; Chegwidden & Carter, 2000). CA II functionally and physically interacts with members of the anion exchange (AE) family of bicarbonate/chloride transporters. Also Na^+/H^+ exchanger 1 (NHE1) and NBC3 Na^+/HCO_3^- contransporter seem to interact with this isozyme (Loiselle et al., 2004).

CA II deficiency syndrome in humans is associated with osteopetrosis (excessive formation of dense bones), renal tubular acidosis and cerebral calcification. Over 90% of these patients are mentally retarded and they have brain calcification, indicating that CA II has an important function in normal brain development. CA II deficiency is autosomal, recessive disorder. The loss of CA II catalytic activity may result from several different mutations in the *CA2* encoding gene. It has been proposed that even slight remanence of CA II catalytic activity may save the patient from mental retardation. However, this has become recently debatable, since one patient having homozygous acatalytic CA II was shown to have near normal mental development (Sly & Hu, 1995; Shah et al., 2004).

CA inhibitors (CAIs) have major applications in ophthalmology. CAIs, such as acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide are widely used as systemic antiglaucoma drugs. They inhibit CA II and CA IV, which are present in the ciliary processes of eye, and thus reduce the secretion of bicarbonate and aqueous humor, and lower the intraocular pressure characteristic of this disease. Because CA II and CA IV are present also in many other tissues, inhibitors given systematically cause

a number of side effects. To avoid these undesired side effects, topically effective inhibitors, dorzolamide and brinzolamide, have been developed (Mincione et al., 2004). CAIs have also applications in neurology. For several decades, acetazolamide was used to treat epilepsy. It was primarily used in combination with other antiepileptic medications in both children and adults. More recently, a widely used antiepileptic drug, topiramate, has been shown to be an efficient inhibitor of CA activity. CA II promotes cerebrospinal fluid production, and therefore acetazolamide inhibits this process. It is also known that acetazolamide dilates intracranial vessels. Because of these properties, acetazolamide has been used to treat patients with increased intracranial pressure (Parkkila et al., 2004).

CA I possesses moderate catalytic activity that is approximately 15% of that of CA II. CA I is five to six times as abundant as CA II in human erythrocytes but because of its low activity it contributes to approximately 50% of the total CA activity in these cells (Dodgson et al., 1988). The presence of CA I may explain why CA II deficient people have no defects in their erythrocytes. In addition to erythrocytes, CA I is expressed at lower levels in the epithelium of the large intestine, corneal epithelium, the lens of the eye, the A cells of Langerhans islets, and the placenta and foetal membranes (Muhlhauser et al., 1994; Parkkila et al., 1994; Sly & Hu, 1995). The role of this isozyme is still somewhat enigmatic (Chegwidden & Carter, 2000). Recent results have shown CA I to be overexpressed in chronic myeloproliferative disorders (Bonapace et al., 2004a).

CA III is a very low-activity isozyme, its activity being only about 3% of that of CA II. CA III is very abundant in skeletal muscle and adipocytes, constituting up to 8% and 25% of the soluble protein fraction of these tissues. This gene is expressed abundantly also in the rodent liver (Kim et al., 2004a). Since the CA catalytic activity of this isozyme is so low, it is thought that CO_2 hydration is not the main function of this enzyme. Although CA III has been studied quite much, still the main functional role of this enzyme is unknown. CA III was believed to have a phosphatase activity but later results have proven this assumption to be wrong (Kim et al., 2000). The production of CA III is lower in the fat tissue and liver of obese rats than in normal rats (Lynch et al., 1993). CA III has two sulfhydryl groups that can conjugate to glutathione through a disulfide link in a process called S-glutathionylation. CA III is rapidly glutathionylated

in vivo and *in vitro*, when the cells are exposed to oxidative stress, and thus, it has been proposed to have a role in cellular response to oxidative stress. Recently, a knock-out mouse model was generated to study the function of CA III: the mice showed no morphological or physiological abnormalities (Kim et al., 2004a). Another study of CA III deficient mice suggested a role for anti-oxidative response in skeletal muscle (Zimmerman et al., 2004). It is possible that CA III has an anti-oxidative rather than CA catalytic function. But for instance the lack of phenotype in CA III knock-out mice demonstrates that still more functional studies are needed to understand the role of this enzyme.

CA III and myoglobin are released from skeletal muscle during tissue injury. Cardiac muscle contains myoglobin but no CA III. Myocardial infection releases myoglobin that has been a marker of this injury. Myoglobin, however, is not specific to myocardial infection. To increase the specificity of this marker, also levels of CA III have been measured. Myocardial infection patients show significantly elevated ratio of myoglobin / CA III (Vaananen et al., 1990; Beuerle et al., 2000). The same ratio has also been used as a marker of reperfusion after myocardial infection (Vuotikka et al., 2003).

CA VII has a high CA catalytic activity. In terms of evolution, it is the most highly conserved of the active CA isozymes, which may imply a significant function for this isozyme (Sly & Hu, 1995). The function has remained obscure for long, since very few studies have been carried out to study this enzyme. Recent results, however, suggest a peculiar function for CA VII in a developmental process that enables synchronous firing of CA1 pyramidal neurons (Ruusuvuori et al., 2004).

CA XIII was characterized recently. Its expression was studied in human and mouse tissues that showed some interspecies differences. In humans CA XIII was expressed in a number of tissues of the alimentary tract. In addition, human reproductive tissues seemed to express CA XIII: it was shown to be expressed at all stages of developing sperm cells as well as in the uterine cervix and some endometrial glands (Lehtonen et al., 2004). The detailed physiological function of this isozyme remains a target for future studies.

2.2.3. Membrane-bound CAs

CA IV is a high-activity enzyme and a unique member of the CA family because it is attached to the cell membrane by glycosylphosphatidylinositol (GPI) anchor. This extracellular protein is produced in a number of tissues. In kidney, it is present mainly on the brush border membrane of the proximal tubular cells and on the cells of the thick ascending limbs of Henle, where its physiological role is to facilitate bicarbonate reabsorption (Brown et al., 1990; Zhu & Sly, 1990). In lung, CA IV is localized on the luminal surface of pulmonary endothelial cells where it catalyzes the dehydration of bicarbonate in the serum to yield CO_2 (Zhu & Sly, 1990; Fleming et al., 1993). CA IV is localized in the capillary endothelium of skeletal and heart muscle, and in the latter it can be also found in special sarcolemmal structures and sarcoplasmic reticulum (Sender et al., 1994; Sender et al., 1998). In distal small and large intestine CA IV participates in ion and fluid transport (Fleming et al., 1995). CA IV participates in acidification of epididymal fluid and is also expressed in the brain capillary endothelial cells (Ghandour et al., 1992). In addition, CA IV has been reported to be expressed in human pancreas, salivary glands (Fujikawa-Adachi et al., 1999a), gallbladder epithelium (Parkkila et al., 1996), choriocapillaris of the eye (Hageman et al., 1991) and erythrocytes (Wistrand et al., 1999). CA IV has been shown to form physical complexes with chloride/bicarbonate exchange proteins, and therefore, it facilitates the rate of bicarbonate transportation (Sterling et al., 2002). CA IV is also crucial for the function of the NBC1 sodium/bicarbonate co-transporter (Alvarez et al., 2003). Recently, an apoptosis-inducing mutation has been identified in the signal sequence of *CA4* gene which causes the RP17 form of retinitis pigmentosa (Bonapace et al., 2004b; Rebello et al., 2004).

Transmembrane protein CA IX is reviewed in detail in section 2.3. CA XII is also a transmembrane protein. Like CA IX, it is a tumor-related protein that is induced by hypoxia (Watson et al., 2003). The activity of CA XII is moderate being approximately same as the activity of CA I (Ulmasov et al., 2000). In the native state, CA XII has been shown to appear as dimers (Whittington et al., 2001). CA XII is produced in normal human endometrial epithelium, where its function is unclear (Karhumaa et al., 2000). CA XII localizes to the basolateral membranes in renal tubules, where it may promote the acidification of urine together with CA IV and CA XIV (Kyllonen et al., 2003). 10% of patients with renal cell carcinoma (RCC) overexpress this gene in kidney (Tureci et

al., 1998). Normal gastric mucosa produces little CA XII and the expression is slightly higher in gastric tumors (Leppilampi et al., 2003). CA XII has been reported to be a good prognostic marker in patients with invasive breast carcinoma (Watson et al., 2003).

Transmembrane CA XIV was characterized in 1999. Recent results suggest that the catalytic activity of CA XIV may be even higher than that of CA II (Whittington et al., 2004). CA XIV is expressed widely in different tissues. It has been shown to be abundant on neuronal membranes and axons in brain where it may have a role in the extracellular alkaline shift after excitatory synaptic transmission (Parkkila et al., 2001). As it was pointed out earlier, CA XIV has probably a major role in bicarbonate reabsorption in kidney (Kaunisto et al., 2002). The expression of CA XIV was also shown in the murine hepatocytes (Parkkila et al., 2002). There are few studies considering CA XIV, and the role of this high-activity enzyme will be probably understood better in the near future.

Unpublished results show that most mammals have still another membrane-bound CA isozyme, CA XV. It has become a pseudogene in humans and chimpanzees but seems to be expressed in many species like mouse, rat, dog, chicken, and many fish species. It is attached to the cell membrane with a GPI anchor like CA IV, and these enzymes seem to have also many other common biochemical properties (Hilvo et al., unpublished).

2.2.4. Mitochondrial and secreted CAs

Mitochondrial CA V was identified in 1990 (Carter et al., 1990). In year 1999 it was revealed that the human genome actually includes two nuclear genes encoding CA isoforms that are located within the mitochondria (Fujikawa-Adachi et al., 1999b). After this finding, CA V was designated as CA VA, and the novel enzyme was designated CA VB since these proteins are very closely related to each other. The distribution of these two enzymes can be generalized in a way that CA VA is expressed mainly in the liver and also to some extent in skeletal muscle and kidney. CA VB has expression in many tissues, but not in the liver (Shah et al., 2000). The catalytic activity of CA V is moderate for example at pH 7.4, but high at pH 8.2 or higher pH (Supuran, 2004). Mitochondrial CAs have been proposed to take part in two metabolic pathways: one is

gluconeogenesis where CA activity may supply HCO_3^- to pyruvate carboxylase. The second is ureagenesis, where mitochondrial CAs may be needed to supply HCO_3^- to carbamyl phosphate synthetase (Sly & Hu, 1995). The role of CA V in pyruvate carboxylation has been considered also to be have importance in lipogenesis (Lynch et al., 1995; Hazen et al., 1996). Mitochondrial CAs are also produced in the pancreatic insulin-producing beta-cells, brain tissue as well as in the gastrointestinal tract, but their role in these tissues is still not completely clear (Parkkila et al., 1998; Saarnio et al., 1999; Sato et al., 2002).

CA VI is the only known secretory isoform of the CA family. CA VI is produced in the serous acinar and ductal cells of the parotid and submandibular glands and secreted into saliva (Parkkila et al., 1994). A competitive time-resolved immunofluorometric assay was developed to measure the concentration of CA VI (Parkkila et al., 1993). Application of this assay showed that CA VI secretion into saliva follows circadian period: it is low during the night and rises rapidly to daytime levels after awakening (Parkkila et al., 1995; Kivela et al., 1997). CA VI has been demonstrated to locate in the enamel pellicle, which is a thin layer of proteins between the enamel of the tooth and the bacterial plaque. Therefore, it is located in the optimal site on dental surfaces for catalyzing the conversion of salivary bicarbonate and microbe-delivered hydrogen ions to carbon dioxide and water (Leinonen et al., 1999; Parkkila, 2000). Indeed, low salivary CA VI concentrations are associated with increased caries prevalence, particularly in subjects with neglected oral hygiene (Kivela et al., 1999). In addition, saliva containing CA VI seems to offer mucosal protection in the upper alimentary tract (Parkkila et al., 1997). Some acinar cells of the lacrimal gland produce a small amount of CA VI (Ogawa et al., 1995; Ogawa et al., 2002). CA VI has also been found in human and rodent milk; especially colostrum has a high concentration of this enzyme. It was shown that salivary factor named gustin, that has a role in taste bud growth, is actually CA VI. Combining these two facts it can be predicted that CA VI is, indeed, involved in normal growth and development of the infant alimentary tract (Thatcher et al., 1998; Karhumaa et al., 2001b). Recently, CA VI was demonstrated in the mouse nasal gland (Kimoto et al., 2004) and was suggested to have a mucosa-protective function also in the respiratory tract (Leinonen et al., 2004).

2.3. Carbonic anhydrase IX (CA IX)

2.3.1. General aspects

In the literature CA IX is known by several names because of historical reasons. In 1992, a new membrane-bound, tumor-associated protein was discovered, and it was given the name MN (Pastorekova et al., 1992; Pastorek et al., 1994). It was soon realized that this protein was a new member of the CA family, and therefore, it was designated as MN/CA9 or MN/CA IX (Opavsky et al., 1996). In the year 1986, however, another group had reported that their monoclonal antibody G250 recognized some antigen in renal cell carcinoma (RCC) cells (Oosterwijk et al., 1986). It was not until the year 2000, when G250 protein was confirmed to be CA IX (Grabmaier et al., 2000). The researchers in the Netherlands studying CA IX have been focusing on the clinical use of the antibody G250. The Slovak and Czech researches developed antibody named M75 that recognizes both denatured and native forms of CA IX. Therefore, this antibody has allowed extensive studies focusing on the characterization and molecular biology of this exciting protein (Pastorekova & Zavada, 2004). Nowadays, CA IX has raised also the interest of many other research groups. In the literature, all of the mentioned names for CA IX are still used and in various combinations.

CA IX is a high-activity enzyme, which is composed of 459 amino acids (aa). It is a transmembrane protein whose N-terminal extracellular part is composed of 37 aa signal peptide, 59 aa region with similarity to keratan sulfate-binding domain of a large proteoglycan aggregan and a 257 aa CA catalytic domain. The transmembrane region of CA IX is 20 aa and a 25 aa C-terminal intracellular tail resides in the cytosol. CA IX usually assembles into trimers. CA IX is the only member of the CA family that contains a proteoglycan domain (Pastorekova & Zavada, 2004).

2.3.2. CA IX in normal tissues

The expression of CA IX has been studied thoroughly in human and rat alimentary tracts by immunohistochemical methods (Pastorekova et al., 1997; Saarnio et al., 1998b). The results are summarized in Table 2.3. No positive reaction was found in oral or esophageal epithelium. CA IX is expressed abundantly in the stomach. CA IX is present throughout the gastric mucosa from the gastric pits to the deep gastric glands and confined to the basolateral surface of the epithelial cells. All major cell types of the gastric epithelium express CA IX. In the rat intestine, epithelial cells of the duodenum and colon produce CA IX, while jejunum and ileum are negative tissues. In the intestine CA IX is also confined to the basolateral membrane. The basolateral membranes of the epithelial cells were found to be positive throughout the colon, but in the distal segments the production is much lower. In the human intestine, duodenum, jejunum, ileum and proximal colon are positive tissues and again the expression decreases toward the distal segments. The expression in the ileum and proximal colon is somewhat focal. The distribution of CA IX in the mucosal layer is different in humans and rats. In the rat colon, CA IX is most intensively produced in the surface epithelium, while in the human colon the expression is highest in the base of the crypts of Lieberkühn (Pastorekova et al., 1997; Saarnio et al., 1998b).

Submandibular and parotid glands do not produce CA IX. A faint signal has been observed in the basolateral membrane of epithelial cells in the human pancreatic ducts, while the rat pancreatic ducts are negative. Both human and rat bile ducts show a positive signal at the basolateral surface of the epithelial cells. The human gallbladder epithelium seems to be an abundant site of CA IX expression (Pastorekova et al., 1997).

Weak expression of CA IX has been shown in male excurrent ducts. CA IX has been observed in the basolateral membrane of the efferent duct epithelium but the epididymal duct epithelium has remained negative (Karhumaa et al., 2001a). Some expression of CA IX has also been observed in the lining cells of the body cavity, rete testis, rete ovarii and surface coelomic epithelium, in ventricular linings of the central nervous system and choroid plexus. However, many studied tissues in humans are CA IX-negative (Ivanov et al., 2001). In conclusion, it can be stated that CA IX expression is mainly limited to the gastrointestinal tract.

Table 2.3. Distribution of CA IX in human and rat alimentary tract. Data adopted from (Pastorekova et al., 1997; Saarnio et al., 1998b).

Organ	Histological site	Rat	Human
oral mucosa	surface epithelial cells	ND	-
parotid gland	*several*	-	-
submandibular gland	*several*	-	-
esophagus	*several*	ND	-
stomach	surface epithelial cells	+++	+++
	parietal cells	+++	+++
	chief cells	+++	+++
duodenum	enterocytes	++	+++
	Brunner's gland	-	-
jejunum	enterocytes	-	+++
ileum	enterocytes	-	++
colon (proximal)	enterocytes	+++	++
colon (middle)	enterocytes	++	+
colon (distal)	enterocytes	+	+
liver	hepatocytes	-	-
	duct cells	+	++
gallbladder	luminal epithelial cells	ND	+++
pancreas	acinar cells	-	-
	islets of Langerhans	-	-
	duct cells	-	+

Scores in immunohistochemistry: -, no staining; +, weak staining; ++, moderate staining; +++, intense staining; ND, not done.

A knock-out mouse model was constructed to study the function of CA IX. In this study, the expression pattern of *Car9* mRNA was studied briefly in normal mouse tissues: the highest level of mRNA was observed in the stomach, medium level was found in the small intestine and colon, whereas kidney and brain showed very weak expression. Liver and spleen were negative. Signal was also present in the mouse embryo at the age of embryonic day E18.5, whereas it was absent in embryonic stem cells and E10.5 embryo (Ortova Gut et al., 2002).

CA IX functionally participates in both pH regulation and cell-to-cell adhesion. The expression pattern of CA IX in normal tissues suggests that it participates in the regulation of acid-base balance on the basolateral surfaces of the gastrointestinal tract epithelia. The knock-out mouse model confirmed the previous results that CA IX functions also as a cell adhesion molecule (CAM). The mice homozygous for the disrupted *Car9* allele developed gastric hyperplasia of the glandular epithelium with numerous cysts. The first changes were observed in the newborn animals, and the hyperplasia became prominent at the end of gastric morphogenesis in 4-week-old mice. In adult knock-out mice the hyperplastic changes affected only the glandular stomach epithelium, whereas the squamous epithelium of the non-glandular fore stomach

remained normal. The most pronounced hyperplasia was observed in the corpus region. The mucosa of CA IX-deficient mice contained approximately 30% more cells than the control epithelium (Ortova Gut et al., 2002). The functional role of CA IX has raised particularly interest in tumors, and this will be the topic of the next section.

2.3.3. Function of CA IX in tumors

CA IX has been proposed to play a role in malignant processes since many tumors overexpress this enzyme. Because of their rapid growth, tumors commonly experience hypoxia (limited oxygen supply) since they initially have no extensive capillary network to supply the tumor cells with oxygen. As a result, cancer cells more than 100 to 200 µm from the nearest capillaries depend on anaerobic glycolysis for much of their energy production (Nelson & Cox, 2000). The anaerobic tumor metabolism generates excess of acidic products, such as lactic acid and H^+ that have to be exported from the cell interior in order to maintain the neutral intracellular pH. This results in low extracellular pH that is a common feature for solid tumors. In addition to lactic acid, CO_2 is a significant source of acidity in tumors. A role for CA IX in this process appears to involve

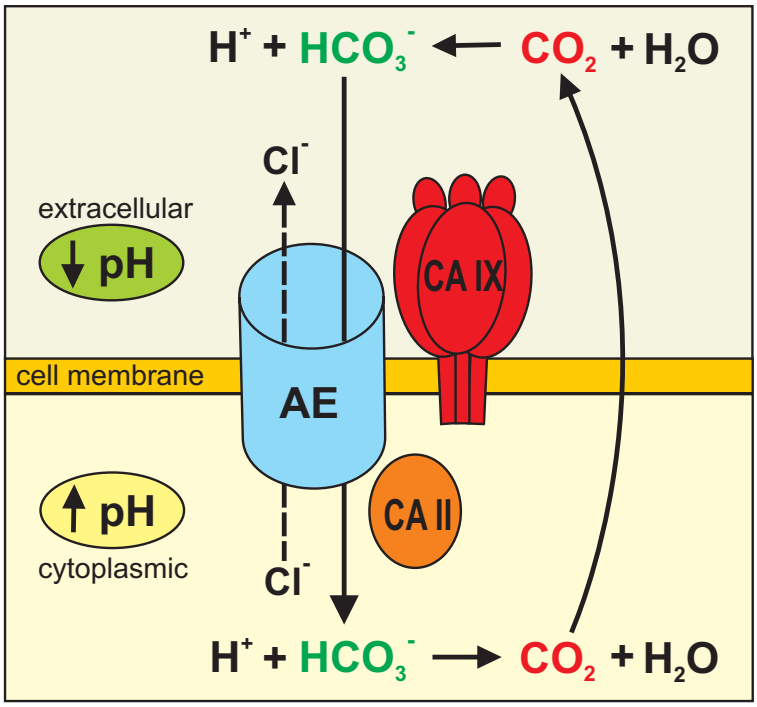


Figure 2.1. Proposed hypothesis of the role of CA IX in the pH regulation of tumors. This model is based on the formation of a transport metabolon composed of anion exchanger (AE) and CAs (in analogy to CA IV-AE-CA II metabolon described by (Sterling et al., 2002)). CA IX as an extracellular component of the metabolon hydrates CO_2 and provides bicarbonate anions to AE, which transports them to the cytoplasm in exchange for Cl^- ions. At the intracellular side, CA II converts HCO_3^- to CO_2, which diffuses out through the plasma membrane. Intracellular activity consumes H^+ neutralizing pH, whereas extracellular pH becomes lower because of excess H^+. The figure is adapted from (Pastorekova & Zavada, 2004).

catalytic conversion of CO_2 to bicarbonate and proton at the extracellular side of the plasma membrane and facilitation of the bicarbonate transport to the cell cytosol. The protons produced by CA IX from hydration of CO_2 may remain outside and facilitate the acidosis of the microenvironment. In the cytosol HCO_3^- is dehydrated back to CO_2, which then leaves the cells by diffusion. This dehydration consumes H^+ and thereby helps to neutralize the intracellular pH. It is proposed, that CA IX may interact with anion exchangers in this process similar to CA IV. In summary, the net result is the increase in intracellular pH and decrease in extracellular pH. The proposed mechanism of acidification of the extracellular environment is illustrated in Figure 2.1. Recent results confirmed that hypoxia activates the capacity of CA IX to acidify extracellular pH (Svastova et al., 2004). It is known that the acidic extracellular environment induces production of growth factors, increases genomic instability, perturbs cell-to-cell adhesion, and facilitates tumor spread and metastasis (Helmlinger et al., 2002; Pastorekova & Pastorek, 2004; Pastorekova & Zavada, 2004). Hypoxia predicts poor outcome in many cancers, because it is also associated with resistance to chemotherapy and radiotherapy (Wouters et al., 2002).

In addition to pH regulation, CA IX also has an important role in the cell-to-cell adhesion in tumors. For example, CA IX can facilitate the attachment of cells to non-adhesive solid support with its N-terminal proteoglycan domain (Zavada et al., 2000). The cell-adhesion property of CA IX was studied by transfecting polarized epithelial MDCK cells with human *CA9* cDNA. The results of this experiment showed that CA IX reduces E-cadherin-mediated cell-adhesion by interacting with β-catenin. E-cadherin is a key adhesion molecule whose loss or destabilization is linked to tumor invasion. β-catenin plays a role in the formation of adherent junctions between epithelial cells by connecting E-cadherin to α-catenin and thereby to the cytoskeleton. The formation of complexes between E-cadherin and β-catenin is essential for cell-adhesive function. The results supported a functional relationship between E-cadherin and CA IX, since overexpression of CA IX in MDCK cells reduced binding of E-cadherin to β-catenin and by this mechanism destabilized intercellular adhesion. A similar mode of action has been observed earlier by important regulatory molecules such as EGFR, ErbB2 (Her2), MUC1, and IQGAP. Destabilization of intercellular contacts plays an important role in tumor progression, because it allows for detachment of cells from the tumor mass, thus facilitating invasion and metastasis. It has been proposed that tumor hypoxia is an

initiating factor that causes a tumor to metastasize. It is also known that increased invasiveness and loss of E-cadherin function are related processes. Since CA IX is upregulated by hypoxia, the study suggested that it may be one of the key molecules in tumor invasiveness and metastasis (Svastova et al., 2003).

A nuclear protein called Ki-67 is known to be a reliable marker of cell proliferation in the gastric mucosa. Comparison of CA IX and Ki-67 expression pattern in colorectal tumors showed that CA IX is expressed in areas with high proliferative capacity. Therefore CA IX is likely to have a role in increased cell proliferation in tumors (Saarnio et al., 1998a). As it was pointed out in section 2.2.1, some receptor-type protein tyrosine phosphatases have domains that are homologous to CA proteins. Thus it may be possible that CA IX could also mediate the communication between cells and transmit signals, but there is no conclusive evidence for this so far (Pastorekova & Zavada, 2004).

2.3.4. Several tumors overexpress CA IX

The most intense overexpression of CA IX has been observed in renal cell carcinomas (RCCs), especially of the clear cell type (Oosterwijk et al., 1986; McKiernan et al., 1997). Majority of RCCs and all clear cell RCCs (ccRCCs) overexpress CA IX. The reason why ccRCCs overexpress CA IX is explained in the next section. Recent results have suggested that low CA IX expression may indicate poor survival for an RCC patient (Bui et al., 2004). CA IX has been detected in the human cervical carcinoma cell line HeLa and also in carcinomas of ovary, endometrium and uterine cervix, but not in normal tissues from corresponding organs or from placenta (Zavada et al., 1993). More than 90% of dysplastic or malignant cervical tissues show immunoreactivity to CA IX, while in normal uterine cervix CA IX is almost absent (Liao et al., 1994). 72% of early-stage non-small cell lung cancers were CA IX-positive and CA IX was associated with poor disease-free survival (Kim et al., 2004b). Other studies confirmed that approximately 80% of non-small cell lung cancers express CA IX, and the expression corresponds to poor prognosis. Morphologically normal epithelium is CA IX-negative. Cells expressing CA IX can survive farther from blood vessels than CA IX-negative tumor cells (Vermylen et al., 1999; Swinson et al., 2003).

A study performed in the year 1997 showed that all esophageal squamous cell carcinomas express CA IX although in normal tissues there was only weak expression in the basal cells of normal squamous epithelium. 80% of esophageal adenocarcinomas were positive for CA IX (Turner et al., 1997). Expression of CA IX was also studied in head and neck squamous cell carcinoma, and the results indicated, that CA IX expression correlated with tumor necrosis, higher microvessel density, and advanced stage (Beasley et al., 2001). As it was explained in the section 2.3.3, CA IX shows abnormal expression in colorectal neoplasms, suggesting its involvement in their pathogenesis (Saarnio et al., 1998a). In pancreas, CA IX shows occasional staining in some areas of acinar and ductal epithelia. Of 29 studied malignant tumors of pancreas, 10 showed increased expression of CA IX (Kivela et al., 2000). 66% of studied soft tissue sarcomas were positive for CA IX and again patients with CA IX-positive tumors had significantly lower disease-specific and overall survival rates than patients with CA IX negative tumors (Maseide et al., 2004). CA IX is absent from normal breast tissues, but approximately 50% of breast cancers express this enzyme. CA IX is associated with high-grade, steroid receptor-negative cancer tissues as well as tumor necrosis. CA IX expression has relationship to the expression of ErbB2. For the patient, expression of CA IX predicts a higher relapse rate as well as poorer overall survival (Chia et al., 2001; Wykoff et al., 2001; Bartosova et al., 2002; Span et al., 2003). Ovarian tissues are CA IX-negative, but ovarian tumors express CA IX (Hynninen et al., unpublished). CA IX is expressed also in bladder carcinomas, especially at the luminal surface (Turner et al., 2002).

Ivanov et al. screened a number of cell lines and human normal as well as cancerous tissues to reveal the expression of CA IX. Below are listed only those tissues that have not been mentioned earlier. Mesothelial cells, the lining cells of the body cavities, as well as mesotheliomas were CA IX-positive. Normal human neurons were negative and the choroid plexus was positive. The corresponding tumors were positive for CA IX to variable degree. Testis and germ cell tumors showed some positive staining. In skin, basal cells of hair follicle were positive. Some squamous and basal cell carcinomas were also CA IX-positive. Prostate gland and prostate tumors were negative (Ivanov et al., 2001).

Interestingly, tumors originating from CA IX-positive tissues tend to have lowered expression of the enzyme. CA IX is highly expressed in the normal gastric mucosa. In gastric adenomas, CA IX expression decreases towards the high grade dysplasia. In well differentiated adenocarcinomas, CA IX expression is as high as in the normal mucosa. However, in less differentiated carcinomas the expression declines (Leppilampi et al., 2003). Similarly, hepatobiliary epithelial tumors show decreasing levels of CA IX with increasing grades of dysplasia and carcinoma (Saarnio et al., 2001). The expression of CA IX in normal and malignant tissues is summarized in Table 2.4.

Table 2.4. *Expression of CA IX in normal tissues and tumors. The data is achieved from the text as well as from (Pastorekova & Pastorek, 2004). The scale is simplified and therefore somewhat arbitrary. CNS = central nervous system.*

Normal tissues	Level	Tumor tissues
CNS – neurons		glioma / ependymoma
CNS – choroid plexus		choroid plexus tumor
body cavity linings		mesothelioma
esophagus		esophageal / head / neck carcinoma
respiratory tract		lung carcinoma
stomach / duodenum		stomach carcinoma
colon		colon carcinoma
gallbladder / bile ducts		biliary carcinoma
pancreas		pancreatic carcinoma
kidney		renal cell carcinoma
prostate		prostate carcinoma
testis		germ cell tumor
ovary		ovarian cancer
uterine cervix		carcinoma of cervix uteri
endometrium		endometrial carcinoma
breast		breast carcinoma
skin		squamous / basal cell carcinoma

Scale: negative tissue weak expression strong expression

2.3.5. Regulation of expression

Analysis of *CA9* promoter region showed that it contains five regulatory regions containing several *cis*-acting elements (Kaluz et al., 1999). Further studies revealed that two regions adjacent to the transcription site bind AP-1 (Activator Protein 1) and SP (Specificity Protein) transcription factors. Their synergistic co-operation is necessary for *CA9* transcriptional activity (Kaluzova et al., 2001). However, the most important

region regulating expression is hypoxia response element (HRE) which is located on the antisense strand at position -10/-3 relative to transcription start site. The HRE element consists of the following nucleotide sequence, 5'-TACGTGCA-3', and it is recognized by HIF-1 (hypoxia-inducible factor-1) (Wykoff et al., 2000).

The mechanism for the regulation of CA IX in hypoxia and normoxia is illustrated in Figure 2.2. At normal oxygen levels (normoxia), prolyl-4-hydroxylases (PHDs) hydroxylate two conserved proline residues of HIF-1α. The von Hippel-Lindau tumor suppressor protein (pVHL) binds hydroxylated HIF-1α and targets it for degradation by the ubiquitin-proteasome system. Under hypoxia, HIF-1α is not hydroxylated because PHDs are inactive in absence of dioxygen. Non-hydroxylated HIF-1α is not recognized by pVHL, it is stabilized and thus accumulates. After translocation to nucleus, HIF-1α dimerizes with HIF-1β, which is a constitutively expressed subunit. This complex forms an active transcription factor HIF-1 that binds to the HRE element of target genes, and activates their transcription. Target genes include glucose transporters (GLUT-1 and GLUT-3) that participate in glucose metabolism; vascular endothelial growth factor (VEGF), which triggers neoangiogenesis; erythropoietin (EPO-1) that is involved in erythropoiesis; CA IX that contributes to pH regulation; and additional genes with functions in cell survival, proliferation, metabolism and other processes (Pastorekova & Pastorek, 2004). This model explains why CA IX can be used as a marker of tumor hypoxia.

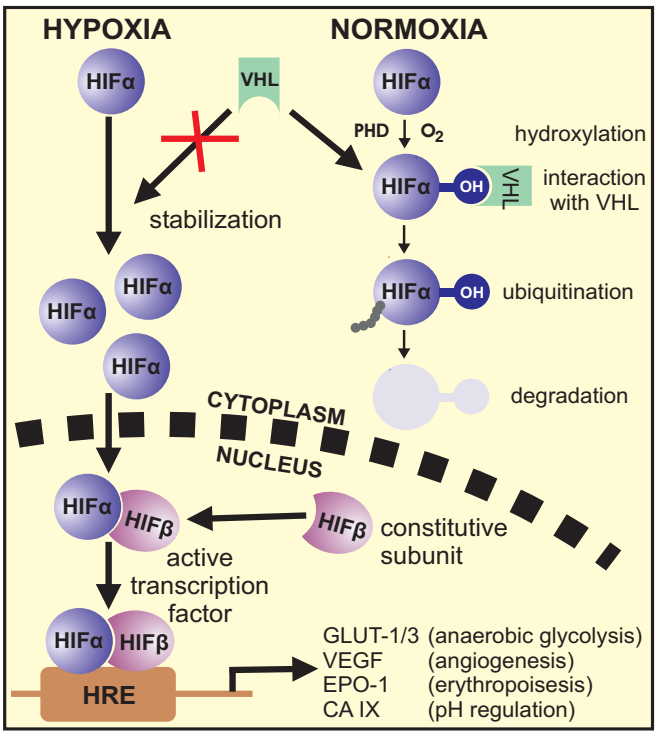


Figure 2.2. The VHL/HIF pathway. The mechanism illustrated in this figure is explained in the text. The figure is adapted from (Pastorekova & Pastorek, 2004).

Germline mutations of the *VHL* gene in humans cause a hereditary cancer syndrome, which is called the von Hippel-Lindau disease. One frequently occurring cancer among these patients is ccRCC (Kondo & Kaelin, 2001). In one study, 57% of ccRCCs had mutations in *VHL* tumor suppressor gene, and loss of heterozygosity was observed in 98% of those samples (Gnarra et al., 1994). The frequent absence of functional *VHL* gene in ccRCCs explain, why CA IX is so frequently overexpressed in these tumors: the loss of functional pVHL releases the transcription of CA IX (Ivanov et al., 1998).

Hypomethylation of CA IX promoter was also shown to induce the expression of CA IX in renal cell carcinoma cell lines (Cho et al., 2000). It was also revealed that in RCCs with *VHL* mutations, the expression of CA IX does not occur without hypomethylation of the promoter, particularly at CpG sites -74 and -6 (Ashida et al., 2002). In addition to hypoxia, increased cell density also induces the expression of CA IX. This phenomenon seems to be related to lowered oxygen tension as well as increased phosphatidylinositol 3'-kinase (PI3K) activity, and subhypoxic levels of HIF-1α.

2.3.6. CA IX as a potential diagnostic tool

The widest overexpression of CA IX has been observed in RCC. It is the most common malignant lesion of the kidney, accounting for approximately 85% of all renal cancers. On the other hand, renal cancer accounts for approximately 3% of all cancers. Of renal cell carcinomas, 80% are ccRCCs. Most commonly, renal tumors are discovered incidentally during the course of various diagnostic studies. Usually only patients with advanced disease have symptoms. The most important treatment for RCC is surgical removal because they are usually resistant to radiotherapy and chemotherapy. Immunotherapy is considered as one option of future treatments. Five-year survival rates after surgical removal are approximately 94% for stage I and 79% for stage II cancers. Patients with renal vein or inferior vena caval involvement have a survival rate of 25% to 50%, and patients with regional lymph node involvement or extracapsular extension have a survival rate of 12% to 25% (Holland & Frei, 2003). Because no good adjuvant therapy is available for renal carcinomas, early diagnosis of the disease is critical for the survival of the patient. Unfortunately, there are not available any good biomarkers for the laboratory detection of RCC. Some studies have suggested CA IX as

a biomarker for renal cancer, and this is the topic of this section. CA IX and therapeutical approaches are considered in the next section.

A few studies have shown that examining the presence of *CA9* mRNA in the human blood could have diagnostic value. An enhanced RT-PCR assay was used to find renal carcinoma cells in the peripheral blood. The results showed that 1.8% of control patients, 46% and 56% of patients with a localized RCC or metastatic disease, gave positive results, respectively. All blood test results for patients with ccRCC were noted to be positive (McKiernan et al., 1999). Another project used both *CA9* and prostate-specific membrane antigen (PSMA) to detect specific mRNAs in renal cancer patients' blood. *CA9* was detected in 19% of samples, PSMA was detected in 20% of samples, and one or both of these tumor markers were observed in 36% of samples. Control samples gave negative results. Thus it was proposed that *CA9* together with PSMA could be used as a tumor marker (de la Taille et al., 2000). The problem that restricts the use of RT-PCR clinically is that it requires many steps of sample preparation: RNA has to be extracted from the blood, and then reverse transcribed to cDNA. This cDNA is then used as a template in PCR. One option to make a quicker test would be to use column extraction of total RNA combined with one-step RT-PCR as described recently (Li et al., 2003).

Another strategy could be to study the concentration of CA IX protein in human serum. It has been shown that human CA IX has two major forms. One is the normal cell-associated form that gives a twin band of 54/58 kDa in Western blots. The other is a soluble sCA IX of 50/54 kDa which is released into the body fluids, probably by cleavage of the extracellular part from transmembrane and intracellular sequences. However, the concentration of sCA IX in body fluids is very low: 20 pg - 3.6 ng/ml in the sera of RCC patients, and 5-25 pg/ml in control sera. The concentrations in urine were 20 pg - 3 ng/ml, and 0-2 pg/ml respectively (Zavada et al., 2003). The sCA IX seems to be rapidly cleared from the blood, but until now it is not known whether this is due to absorption in unknown deposits, degradation or excretion into urine (Pastorekova & Zavada, 2004). The previously described method to determine the level of CA IX in body fluids was time-consuming and the quantification appeared to be more or less inaccurate. Even though these facts decreased the utility of the described CA IX detection as a routine laboratory method, there is still a great need for clinical chemistry

tools for the detection of RCC especially in patients with clinically presymptomatic disease. Therefore, it would be attractive to develop a new sensitive method that could accurately measure the concentration of sCA IX protein directly from the serum samples.

2.3.7. CA IX as a potential target for cancer therapy

The wide distribution of CA IX in several tumors has raised interest to develop cancer treatment based on this molecule. One potential drug is indisulam (E7070), a sulfonamide that has shown several antitumor properties: it has shown to restrict G1/ S and G2/M transitions of the cell cycle, inhibit retinoblastoma protein (pRb) phosphorylation, reduce the expression of cyclin A, cyclin B1, cyclin-dependent kinase 2 (CDK2) and cell division cycle kinase 2 (CDC2) and to suppress CDK2 catalytic activity with the induction of p53 and p21 proteins. Indisulam is a cell-cycle inhibitor that affects multiple cell-cycle checkpoints (Supuran, 2003). Indisulam also shows differential expression of many additional genes known to participate in metabolism, the immune response, signaling, and cell adhesion (Pastorekova & Pastorek, 2004). In addition, indisulam also seems to be a potent inhibitor of CA I, II, and IX (Abbate et al., 2004). Indisulam has progressed to phase I and phase II clinical trials for the treatment of solid tumors (Supuran, 2003).

The Wilex company (http://www.wilex.com) has announced that it is developing chimeric monoclonal antibody WX-G250 (Rencarex®) (anti-human CA IX) to treat renal cancer. This antibody consists of 75% of human sequence and 25% of murine sequence. It has passed to phase III trials in non-metastatic RCC (monotherapy), and phase II in metastatic RCC (combination therapy with cytokines IL-2 (Interleukin-2) and IFN (Interferon)). The major mechanism of action of this novel drug is antibody-dependent cellular cytotoxicity (ADCC).

Several other therapeutic approaches exploiting CA IX have been developed. A number of clinical trials have concentrated on radioimmunotherapy of RCC with monoclonal antibodies labeled with ^{131}I. These approaches have the advantage that they can be used both in diagnostic and therapeutic formats. The chimeric ^{131}I-G250 antibody proved to be very suitable for scintigraphic imaging of RCC in the patients with a very high

specificity and extremely low background, enabling detection of very small tumors (less than 1 cm). However, intratumoral heterogenous distribution makes therapeutic use difficult. Some anti-cancer vaccines have been promising in mice. Antifection is a technique in which therapeutic genes are chemically conjugated with monoclonal antibody, and internalized into tumor cells. Another strategy of the gene therapy is unlimited supply of cytotoxic lymphocytes (CTL) specifically destroying the tumor without damaging normal tissues. In mice, temporary growth arrest of tumors was achieved by immunomagnetic hyperthermia: in this technique magnetic particles were enclosed in liposomes with covalently linked Fab fragment of the G250 antibody, and then the mice were exposed to oscillating magnetic field, which induced vibrations of the magnetic particles. As a consequence, the temperature of the tumors increased to 43 ºC. Finally, targeted oncolytic viruses have been tested for the infection of tumor cells (Pastorekova & Zavada, 2004).

2.4. Theory behind the methods

This study uses several techniques that are widely used in the field of biotechnology: the theory behind these methods is not reviewed. However, the theory of peroxidase-antiperoxidase staining and chemiluminescence are not necessarily widely known, and therefore these are explained briefly.

2.4.1. Peroxidase-antiperoxidase method

Peroxidase-antiperoxidase (PAP) method is an indirect immunohistochemical staining method. Figure 2.3 illustrates the PAP method. (1) First the sample is incubated with primary antibody that recognizes the studied antigen in the sample. Then excess antibody is washed out and (2) secondary antibody is used to detect the primary antibody (eg. if the primary antibody was produced in rabbit, then the secondary antibody was raised against rabbit IgG). Again the sample is washed and finally (3) PAP complex is added. This complex consists of two antibodies and three horseradish peroxidase (HRP)

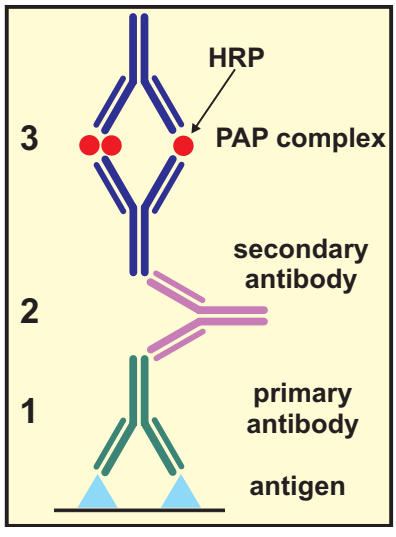


Figure 2.3. *PAP method. The numbers in the figure correspond to the text.*

molecules which are non-covalently bound together. The peculiarity of this immunohistochemical method is that the secondary antibody recognizes both primary antibody and the PAP complex (therefore the primary antibody and PAP complex have to be made in the same species). After washings, the enzyme activity of HRP is used to localize the sites that contain the studied antigen. HRP is inhibited by excess substrate (hydrogen peroxide). The complex between HRP and excess H_2O_2 (the latter provided at the final step of the staining) is catalytically inactive in the absence of an electron donor (eg. chromogenic substance). There are several electron donors that when oxidized, become colored products and are therefore called chromogens. One such is 3,3'-diaminobenzidine (DAB) that produces a brown end product (Wilson & Walker, 2000; Boenisch, 2001).

2.4.2. Chemiluminescence in immunoassays

Immunoassays are based on the specific recognition reaction between an antibody and the antigenic determinant against which the antibody has been formed (Hemmila et al., 1994). The principle of the assay developed in this study is that the microtiter wells contain monoclonal antibody that attaches CA IX to the solid surface (i.e. microtiter well). The amount of CA IX is then deduced with another monoclonal antibody that has been ligated with an attached chemiluminescent label called acridinium ester.

Chemiluminescent labels are compounds which upon chemical reaction, generally oxidative decomposition, are excited and subsequently produce a photon. The most frequently applied luminophore in chemiluminescent assays nowadays is acridinium ester. The high sensitivity obtained with acridinium ester is a result of a lower nonspecific signal in acridinium detection to the less drastic oxidative conditions needed to excite acridinium ester. The disadvantage with the direct chemiluminescent labels include flash type signal produced immediately after initiation of the reaction. Therefore the measurement cannot be repeated. The light emitted from the label is measured with a luminometer (Hemmila et al., 1994).

3. Aims of the research

This study had two specific goals. The first goal was to identify the tissues that express CA IX in mice. The aim was to study the expression both on mRNA and protein level. mRNA expression was investigated by reverse transcriptase polymerase chain reaction (RT-PCR). Protein production was studied by Western blot and in more detail by immunohistochemical methods. The second specific goal was to develop an immunoassay that could measure the concentration of CA IX in human serum and to evaluate if CA IX could have clinical potential as a tumor marker.

4. Methods

4.1. Expression of CA IX in mouse tissues

4.1.1. Tissue processing

Tissues for Western blot and immunohistochemistry were obtained from two adult mice (Balb/c). The procedures were approved by the institutional animal care committee (University of Tampere). The following samples were obtained for Western blot: stomach, colon, duodenum, jejunum, ileum, brain, cerebellum, heart, liver, kidney, pancreas, lung, spleen, thymus, psoas muscle, testis, and epididymis. The tissue samples were homogenized in PBS (150 mM NaCl, 10 mM Na_2HPO_4, 4 mM KH_2PO_4) and stored at -20 °C. Na_2HPO_4 and KH_2PO_4 were from Sigma (St Louis, MO) and NaCl from Merck (Darmstadt, Germany).

For immunohistochemistry, the following tissue samples were obtained: brain, stomach, duodenum, jejunum, ileum, colon, liver, psoas muscle, kidney, heart, lung, pancreas, spleen, thymus, testis, and epididymis. The specimens were fixed in Carnoy's fluid (absolute ethanol + chloroform + glacial acetic acid in 6:3:1 ratio) for 20 h at 4 °C. Ethanol was from Primalco (Rajamäki, Finland), and chloroform and glacial acetic acid were from Merck. After fixation the samples were dehydrated at 4 °C: 30 min in absolute ethanol, 15 min in ethanol + chloroform (1:1 ratio), and 30 min in chloroform. After dehydration, the specimens were embedded in paraffin, and finally 4 µm sections were cut and placed on microscope slides.

Commercial cDNA samples were used in RT-PCR. However, gastrointestinal tissues were missing from the cDNA kit, and therefore the following tissues were obtained from five adult mice for RNA extraction: stomach, duodenum, jejunum, ileum, and colon.

4.1.2. RNA extraction and reverse transcription

Total RNA was isolated from five murine tissues by using Trizol reagent (Invitrogen; Carlsbad, CA). 1 ml of Trizol was added per 50-100 mg of tissue, and the samples were homogenized with a needle. 200 µl of chloroform was added, and the suspension was shaken by hand. The samples were incubated for 10 min at 4 ºC, and then centrifuged

for 15 min at 13 000 rpm. The aqueous phase was transferred to a clean tube, and 500 µl of 2-propanol (Merck) was added. The tube was shaken by hand several times, and then the samples were incubated for 20 min at -20 ºC. After incubation, the tubes were centrifuged for 15 min at 13 000 rpm. The supernatant was removed and 500 µl of 75% ethanol (made in DEPC water) was added. The samples were centrifuged again for 5 min at 7500 rpm, and the supernatant was removed. 500 µl of 96% ethanol was added, and the samples were centrifuged for 5 min at 7500 rpm. The supernatant was removed and the RNA pellet was briefly dried. The isolated RNA was resuspended into 30 µl of DEPC water. The concentration and quality of isolated RNA was studied by measuring absorbance at 260 and 280 nm with a spectrophotometer (Ultrospec 3000 pro; Biochrom; Cambridge, England).

Reverse transcription to cDNA was performed using M-MuLV reverse transcriptase (Finnzymes; Espoo, Finland) and random primer method. 1 µl of random primers (400 ng/µl) were mixed with 5 µg of total RNA and DEPC water was added so that the final volume of this mixture was 15 µl. The samples were incubated for 5 min at 70 ºC and then the tubes were placed on ice. 5.75 µl of DEPC water, 2.5 µl of M-MuLV RT Buffer, 1.25 µl of 10 mM dNTP mix, and 0.5 µl of M-MuLV reverse transcriptase were added to the reaction mixture (all the reagents were from Finnzymes). The samples were incubated for 60 min at 42 ºC, and the enzyme was inactivated by incubating for 15 min at 70 ºC.

4.1.3. RT-PCR and agarose gel electrophoresis

The expression of CA IX mRNA in mouse tissues was examined using a cDNA kit (Mouse MTC Panel I) purchased from BD Biosciences (Palo Alto, CA) as well as the cDNA obtained from adult mice. The commercial kit contained first-strand cDNA preparations produced from poly-A RNAs isolated from the following mouse tissues: heart, brain, spleen, lung, liver, muscle, kidney, testis, 7-day embryo, 11-day embryo, 15-day embryo, and 17-day embryo. The cDNA preparations made from the adult mouse tissues represented mRNAs of duodenum, jejunum, ileum, and colon.

mRNA expression was studied by polymerase chain reaction (PCR). cDNA was used as a template in the PCR reaction and specific primers for CA IX were used to reveal the

tissues containing CA IX mRNA. In addition, primers for mouse β-actin were used to monitor the possible differences in cDNA concentrations.

The primers for the PCR reaction were designed by using the published information on CA IX mRNA in GenBank (accession number NM_139305). To produce an amplification product of 873 bp, the forward primer (F1) was 5'-GCTCCAAGATTGAGATC-3' (nucleotides 894-911), and the reverse primer (R1) was 5'-TCTGCCTGCATAGTAAGA-3' (nucleotides 1749-1766). The primer pair F1-R1 was used to study the commercial cDNAs. In the second set of experiments (i.e., analyzing the non-commercial cDNAs), the forward primer (F2) was 5'-GGAGGCCTGGCAGTTTTGGCT-3' (nucleotides 794-814), and the reverse primer (R2) was 5'-CTCCAGTTTCTGTCATCTCTGCC-3' (nucleotides 1336-1358). With these primers the PCR amplification product was predicted to be 565 bp. The control reaction was performed with the following primers for mouse β-actin (accession number NM_007393): the forward primer (FC) was 5'-GCCGCATCCTCTTCCTCCCT-3' (nucleotides 768-787) and the reverse primer (RC) was 5'-GTTGGCATAGAGGTCTTTACG-3' (nucleotides 948-968). The resulting control PCR product was 201 bp. The primers were produced by Sigma Genosys (Cambridge, UK).

5 ng of cDNA was used as template in the PCR reaction. The PCR reaction was as follows: 36 µl sterilized water, 5 µl 10x Titanium Taq PCR buffer, 1 µl 50x dNTP mix, 1.5 µl of 10 µM F-primer, 1.5 µl of 10 µM R-primer, 1 µl 50x Titanium Taq DNA polymerase, and 5 µl cDNA. Negative control contained sterilized water instead of cDNA samples. Positive control contained 2 µl of control primer mix supplied by the manufacturer of the cDNA panels, and the cDNA template was replaced with the control template. All the reagents were from BD Biosciences except for the dNTP mix, which was from Finnzymes.

The PCR reaction was carried out on a thermal cycler (Gene Amp PCR system 9700; Applied Biosystems; Foster City, CA) and the protocol consisted of a 94 ºC denaturation step for 1 min followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 1 min 30 s, followed by final extension at 72 ºC for 3 min. The control reaction was carried out according to the

following protocol: 94 ºC denaturation step for 1 min followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 60 ºC for 30 s, extension at 72 ºC for 40 s, followed by final extension at 72 ºC for 3 min.

The results of the PCR reaction were analyzed using a 1.2% agarose gel containing 0.1 µg/ml ethidium bromide (Sigma) with 100 bp DNA standard (100-bp DNA Ladder; New England Biolaps; Beverly, MA).

4.1.4. Sequencing of the PCR products

The PCR products from mouse muscle and kidney were sequenced to confirm the presence of the correct amplification product and to exclude unspecific binding of the primers. The corresponding DNA bands were excised from the agarose gel, and weighed. The PCR products were purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences; Poole, UK): The same amount of Capture buffer was added (in µl) than the gel slices weighed (in mg). The slices and the buffer were vortexed, and then incubated at 60 ºC until the agarose gel had melted completely. The suspensions were briefly spinned, and then transferred to a column. After 1 min incubation, the columns were centrifuged at full speed for 30 s. The flow-through was discarded, 500 µl of Wash buffer was added to the column, and the column was centrifuged at full speed for 30 s. After centrifugation the column was placed on a clean 1.5 ml eppendorf tube. The DNA was eluted into 50 µl of sterile water by pipetting the water on the top of the column filter, incubating 1 min, and finally centrifuging at full speed for 1 min.

The sequencing was performed using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reactions Kit, version 2.0 (Applied Biosystems). 5 µl of purified DNA template was mixed with 4 µl of Terminator Ready Reaction Mix and 1.6 pmol of primer was added to the solution. The sequencing was performed with both forward (F1) and reverse primers (R1). The reactions were amplified by cycle sequencing on a thermal cycler (Gene Amp PCR system 9700) according to the following protocol: 25 cycles of denaturation at 96 ºC for 1 min, annealing at 50 ºC for 5 s, and extension at 60 ºC for 4 min.

The products were purified by ethanol precipitation: 16 µl of sterilized water and 64 µl of 95% ethanol were added to the reaction mixture. The samples were vortexed, incubated for 15 min at room temperature, and centrifuged at 13 000 rpm for 15 min. The supernatant was discarded and 250 µl of 70% ethanol was added. After vortexing, the tubes were centrifuged at 13 000 rpm for 10 min. The supernatant was again discarded and the samples were resuspended into 12 µl of Template Suppression Reagent (Applied Biosystems). The samples were vortexed, spinned, and incubated at 95 ºC to denature the DNA. After vortexing and spinning the samples were stored on ice until the sequencing was performed with an ABI PRISM Genetic Analyser instrument (Applied Biosystems).

4.1.5. SDS-PAGE and Western blot

The protein concentrations of homogenized tissue samples were analyzed by measuring the absorbance at 280 nm with a spectrophotometer. The approximation principle of the concentration was that the absorbance value equals protein concentration in µg/µl. For example, if the absorbance was 0.700, the protein concentration was estimated to be 0.700 µg/µl. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the proteins in the tissue samples according to the molecular mass. 50 µg of protein from each sample was analyzed by SDS-PAGE (NuPAGE 10% Bis-Tris; Invitrogen) under reducing conditions. Laemmli sample buffer (Sigma) was added to the samples and they were incubated for 2 min at boiling water. The SDS running buffer was MOPS SDS-PAGE running buffer (Invitrogen). 10 µl of protein standard (Prestained SDS-PAGE Standards, low range; Bio-Rad; Hercules, CA) was used to estimate the protein molecular mass. The gel was run on 200 V, until the proteins had reached the bottom of the gel.

Polyvinylidene fluoride (PVDF) membranes (Macherey-Nagel; Düren, Germany) were used in the Western blot. The membrane was treated with absolute ethanol for 1 minute and then put to water to wait the blotting. The buffer used in the blotting was NuPAGE Transfer buffer (Invitrogen). The separated proteins were transferred electrophoretically (36 V, 1 h 15 min) from the gel to the PVDF membrane in a Novex Xcell II blot module (Invitrogen). The success of the blotting was analyzed by confirming that the prestained protein ladder had been transferred to the PVDF membrane.

The sample lines were detected by an ECL detection system (Amersham Biosciences). The membranes were blocked with cow colostral whey (Biotop Oy; Oulu, Finland) diluted 1:10 in TBST buffer (20 mM Tris (Sigma), 500 mM NaCl, 0.3% Tween-20 (Sigma), pH 7.5) for 25 min. The membranes were then incubated with primary antibody diluted 1:5000 in TBST buffer for 1 h and washed five times for 5 min in TBST buffer. The production of the polyclonal rabbit antibody raised against the recombinant mouse CA IX has been described by (Ortova Gut et al., 2002). Normal rabbit serum was used as a negative control. The membranes were incubated for 60 min in the secondary antibody (Anti-rabbit Ig, horseradish peroxidase linked whole antibody (from donkey); Amersham Biosciences)**,** diluted 1:25 000 in TBST. After the second antibody, the membranes were washed four times for 5 min. 7 ml of Detection reagents 1 and 2 (Amersham Biosciences; Buckinghamshire, England) were mixed and the membrane was incubated in the mixture for 1 min. After this step, the membrane was kept in the dark and an X-ray film was exposed to the membrane for 15 min. The X-ray film was then developed in Konica SRX-101A (Taiwan).

4.1.6. RT-PCR and Western blot from same tissue specimens

Because RT-PCR and Western blot results had a major discrepancy in kidney and skeletal muscle, these experiments were repeated from the tissues of one animal to exclude interanimal differences. The following tissues were obtained from an adult mouse (Balb/c): kidney, muscle, stomach, small intestine, and colon. The gastrointestinal tract did not have discrepancies, but it was taken for analysis since it is a major site of CA IX. The tissues were examined using Western blot and RT-PCR as described earlier. The primers for CA IX in this experiment were (F3) 5'-CTGGGCGTGGATGTTCTGCCC-3' (nucleotides 212-232) and (R3) 5'-CGAGATTTCTTCCAAATGGGACAG-3' (nucleotides 863-886), with the resulting PCR product of 675 bp.

4.1.7. Immunohistochemistry

Immunoperoxidase staining for mouse tissues was performed using the peroxidase-antiperoxidase (PAP) complex method that was described in section 2.4.1. The polyclonal rabbit antibody was the same as used in the Western blot. Pre-immune serum was used in the control stainings. The tissue processing has been described in section 4.1.1.

The tissue samples on the microscope slides were deparafinized by incubating them for 5 min in each of the following solutions: three times in xylene (Merck), twice in absolute ethanol, twice in 94% ethanol, once in 70% ethanol, and once in distilled water.

Immunostaining was performed according to the following protocol: (1) 3% H_2O_2 (YA Kemia; Helsinki, Finland) in methanol (Merck) for 5 min; (2) treatment with undiluted cow colostral whey (Biotop) for 30 min and rinsing in PBS; (3) incubation for 1 h with primary antibody diluted 1:100 in 1% bovine serum albumin (BSA) (Sigma) in PBS; (4) treatment with undiluted cow colostral whey for 30 min and rinsing in PBS; (5) incubation for 1 h with secondary antibody (swine anti-rabbit IgG; DAKO; Glostrup, Denmark) diluted 1:100 in 1% BSA in PBS; (6) incubation with peroxidase-antiperoxidase complex (PAP Rabbit; DAKO) diluted 1:100 in PBS for 30 min; and (7) incubation for 2 min 30 s in DAB solution (6 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 10 ml PBS plus 3.3 µl 30% H_2O_2). The sections were washed in PBS for 5 min after step (1), 3 times for 10 min after steps (3) and (5), and finally four times for 5 min after step (6). All incubations and washings were carried out at room temperature. In addition, each washing was performed on an orbital shaker. After immunostaining, the samples were counterstained with Mayer's hematoxylin solution (Sigma) for 2 min and washed with water for 2 min. Finally, the sections were mounted in Entellan Neu (Merck) and then examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss; Göttingen, Germany).

4.2. Immunoassay for CA IX

4.2.1. Coating of microtiter plates

Eighteen 96-well microtiter plates (Thermo Labsystems; Vantaa, Finland) were coated with a monoclonal antibody (V/10). The production of monoclonal antibodies against human CA IX has been described by (Zat'ovicova et al., 2003). The epitope for V/10 resides in the CA catalytic domain of CA IX.

The antibody was diluted with 0.9% NaCl so that its concentration was 2 mg/ml. Then this solution was diluted in 1:5 with 0.1 M sodium citrate buffer (0.1 M citric acid for chemiluminescence (Sigma) and adjustment of pH to 2.5 with 1 M NaOH (Sigma)). The solution was incubated for 30 min at room temperature. 0.01 M sodium phosphate buffer (7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, 0.05% NaN_3, pH 7.5, reagents from Sigma) was added so that the final concentration of the antibody was 5 µg/ml. Finally, 200 µl of this coating solution was pipetted to each well, and consequently there was 1 µg of monoclonal antibody in each well. The microtiter plates were incubated in a humid chamber overnight at room temperature.

Next day the coating solution was discarded, and the wells were washed two times with washing solution 1 (0.9% NaCl and 0.05% Tween-20) and one time with washing solution 2 (0.9% NaCl). After the washings, 275 µl of saturation solution was added to each well (50 mM Tris, 0.9% NaCl, 0.1% NaN_3, 5% sorbitol (Sigma), 0.5% BSA (Sigma), pH 7.5). The microtiter plates were incubated again in a humid chamber overnight at room temperature.

Next day saturation solution was discarded, and the plates were allowed to dry. Finally, the plates were stored at 4 °C.

4.2.2. Labeling of antibodies

Because it was not known what combination of monoclonal antibodies would function in the immunoassay, two monoclonal antibodies (M75 and IV/18) were labeled with acridinium ester. Both of these antibodies recognize the proteoglycan domain of CA IX. The labeling was performed with Chemiluminescence sub-attomole labeling kit (AssayDesigns; Ann Arbor, MI).

The amount of labeled material was 410 µg (~2.7 x 10^{-9} mol) of M75 and 590 µg (~3.9 x 10^{-9} mol) of IV/18. Ratios of acridinium ester per antibody were 3-fold and 3.66-fold, respectively: 1.0 * 10^{-8} mol of acridinium ester for M75 (6.4 µg), and 1.2 * 10^{-8} mol for IV/18 (7.4 µg). Acridinium ester was prepared by resuspending it in 50 µl of dry dimethyl formamide. The calculated amount of acridinium ester was added to the antibody solution. The mixture was stirred for 30 min at room temperature. After the incubation, 10 µl of 10% lysine solution was added, and the solution was stirred for 15 min at room temperature. The gel filtration columns were prepared by washing them with 20 ml of Column buffer. The labeled materials were applied to the columns, and by applying Column buffer 1 ml at a time, fractions were collected into tubes. 9 fractions of both M75 and IV/18 were collected.

1 µl from each fraction was pipetted into a microtiter plate well, and the results were read with Luminoskan Ascent luminometer (Thermo Labsystems). The luminometer was set to apply 50 µl of triggering solutions 1 and 2 to each well. The results were read at 120 ms after triggering the reaction, because that time point showed the highest luminescence value.

4.2.3. Production of protein standards

The goal was to produce CA IX in mouse fibroblasts that shed the protein into the cell culture medium. The generation of stable NIH 3T3 cell line producing CA IX has been described in (Pastorek et al., 1994). The cell culture medium DMEM (BioWhittaker™ DMEM; Cambrex Bio Science; Verviers, Belgium) was prepared in a way that it contained 10% fetal bovine serum (Biochrom; Berlin, Gemany) and 1% penicillin-streptomycin (Invitrogen GIBCO; Prisley, Scotland). The cell culturing was started on plates (10 ml culture) and after a week the cultures were transferred to 175 cm^2 culture dishes: The cells were washed with PBS and 1.5 ml of trypsin-EDTA (Invitrogen GIBCO) was used to detach the cells from the plates. The cells were incubated with trypsin-EDTA for 5 min and they were resuspended into 10 ml of culture medium. One plate was divided into 2 bottles that contained 50 ml of culture medium. After three days, the culture medium was collected and centrifuged at 10 000 x g for 5 min.

CA sulfonamide inhibitor (p-aminomethylbenzenesulfonamide) affinity chromatography was used to purify CA IX from the cell culture medium. The total volume of the medium was 250 ml, and the same amount of binding buffer (10mM HEPES (Sigma), pH 7.5) was added to the medium. In addition, 10 ml of CA inhibitor resin (containing 4-aminomethylbenzenesulfonamide HCl (Sigma) + N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide HCl (Sigma) in H_2O + 0.02% NaN_3 in CM Bio-Gel A Gel (Bio-Rad; Hercules, CA)) was added to the mixture. CA IX was let to bind to the inhibitor on an orbital shaker overnight at 4 ºC.

Next day the solution was drained through a column. The matrix was washed with 25 ml of washing buffer (10 mM Hepes, 150 mM NaCl, pH 7.5), and eluted with elution buffer A (0.1 M Tris-SO_4 (Sigma), 0.4 M NaN_3, 1 mM bentzamidine (Sigma), 20% glycerol (Sigma), pH 7.0). Sixteen 1 ml fractions were collected. The fractions were analyzed with SDS-PAGE to determine the samples that contained the purified protein. 25 µl of fractions 2-10 were analyzed. SDS-PAGE was carried out as described earlier. The gel was stained with (Coomassie) Colloidal blue staining kit (Invitrogen). The gel was kept in fixing solution (40 ml H_2O, 50 ml methanol, 10 ml acetic acid) for 10 min. Then the fixing solution was discarded and the gel was kept in staining solution (55 ml H_2O, 20 ml methanol, 20 ml stainer A). After 10 min incubation, 5 ml of stainer B was added to the solution. The gel was incubated in this solution for 3 h, and finally the staining solution was discarded, and the gel was transferred into water for clearance.

Because the molecular mass of the protein on the gel appeared higher than predicted for CA IX, SDS-PAGE combined with Western blot was performed to investigate if the collected fractions indeed contained CA IX. Based on the previous SDS-PAGE results, fractions 3-6 were pooled. This will be referred as CA IX pooled fraction in the results section. The following samples were pipetted into the gel: 5 µl of protein standard, 5 µl of CA IX pooled fraction, and 1 µg of albumin were used for the Coomassie staining; 2 µl and 5 µl of pooled fraction were used for the Western blot. Coomassie staining was performed as described earlier. Western blot was carried out as described in section 4.1.5 with the following exceptions: primary antibody was M75 medium diluted 1:100, secondary antibody was peroxidase labeled anti-mouse antibody (Amersham Biosciences), and the exposure time was 2 min.

Because the results implied that the CA IX produced in NIH 3T3 cells was not suitable to be used as a protein standard in the assay, another attempt was made to produce the standard protein. The aim was to produce GST-tagged protein fragments, that contained CA IX catalytic and proteoglycan domains. The generation of this GST-PGCA fusion protein has been described by (Zat'ovicova et al., 2003).

A bacterium colony containing GST-PGCA fusion protein was grown in 5 ml of LB medium (containing 50 µg/ml ampicillin (Roche; Mannheim, Germany)) on an orbital shaker (200 rpm) at 37 ºC overnight. Next day, the 5 ml bacterial suspension was transferred to 500 ml of LB medium containing ampicillin. The bacteria were grown until the OD_{600} was 0.800. After this, 1 mM IPTG (Fermentas; Vilnius, Lithuania) was added to induce the expression of GST-PGCA. After 2 h, the bacteria were pelleted by centrifuging them 3000 rpm for 5 min.

The bacteria were lysed with 80 mg of lysozyme (BioChemika; Steinheim, Switzerland) that was suspended in 20 ml PBS. The bacteria were resuspended in this solution and then incubated on ice for 45 min. Then the samples were centrifuged at 13 000 rpm for 15 min, and the supernatant was collected. The GST-purification was carried out with Bulk GST Purification module from Amersham Biosciences. 400 µl of Glutathione Sepharose 4B was prepared by washing the beads with cold PBS, and then the sepharose was added to the supernatant. The mixture was incubated on an orbital shaker for 30 min, and then transferred to the purification column. The matrix was washed with 12 ml of PBS and protein was eluted with 2 ml of glutathione elution buffer. Five 400 µl fractions were collected. The results of the purification were analyzed by SDS-PAGE followed by Coomassie staining.

The results showed several bands, and therefore, all the fractions were repurified with CA inhibitor affinity chromatography as described earlier. The only difference was that elution buffer B was used (0.1 M sodium acetate, 0.5 M sodium perchlorate (both from Sigma)) in this purification. The results were analyzed with SDS-PAGE followed by Coomassie staining and Western blot. The fractions containing protein were pooled.

DC Protein assay (Bio-Rad) was used to measure the concentration of pooled fractions obtained from CA IX and GST-PGCA proteins. The following dilutions of standard

protein BSA (Pierce; Rockford, IL) were made: 5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 125 µg/ml, and 250 µg/ml. The dilutions were made in the corresponding elution buffers (A or B) for CA IX and GST-PGCA. 5 µl of standards and protein fractions were pipetted into a microtiter plate. 25 µl of reagent A was added to each well. 200 µl of reagent B was pipetted into each well, and after 30 min, the absorbances were read at 750 nm.

4.2.4. Adjusting the parameters

Although the CA IX fraction produced in NIH 3T3 cells contained only little CA IX, it was used to adjust the parameters of the assay. Because a number of experiments were performed to adjust the parameters of the assay, only the outline of the experiments is listed here.

0.1 µl, 0.3 µl, 0.5 µl, 1.0 µl, and 2.0 µl of labeled antibodies (M75 and IV/18) were used to detect the protein. The acridinium ester –labeled antibodies were diluted into the assay buffer (7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, 0.9% NaCl, 0.05% Tween-20, 0.1% BSA, pH 6.0) A combination of 0.25 µl of M75 and 0.25 µl of IV/18 was tried as well. 50 µl, 75 µl, and 100 µl of triggering solutions 1 and 2 were used to trigger the light-producing reaction. The following dilutions of serum were used: 1:4, 1:16, 1:64, 1:200, 1:500, 1:1000, 1:2000, 1:4000, 1:8000, and 1:16000. The serum samples were diluted into the assay buffer (pH 7.5).

The first immunoassay protocol consisted of an incubation of the samples in the microtiter plate wells for 1.5 h and then washing the wells two times with the assay buffer (pH 7.5). Then the labeled antibodies were incubated in the microtiter plate wells for 1.5 h, and after the incubation the wells were washed two times with the assay buffer (pH 6.0). All the incubations were carried out on an orbital shaker. The idea behind the second protocol was to incubate the samples and labeled antibodies both at the same time for 3 h, after which the wells were washed three times with the assay buffer (pH 6.0). In both cases, the highest peak of the flash-type reaction curve (120 ms after triggering the reaction) was obtained as a result.

A standard curve obtained with the optimized parameters and CA IX protein is shown in the results section. The following amounts of protein were used: 50 pg, 250 pg, 500 pg, 1.00 ng, 2.00 ng, 4.00 ng, 8.00 ng, and 16.00 ng. These samples were pipetted in duplicates. GST-PGCA was also evaluated as standard protein. The following amounts of PGCA were used: 5 pg, 10 pg, 30 pg, 60 pg, 120 pg, 240 pg, 480 pg, 960 pg, and 1.920 ng. The GST-PGCA was tested as a standard protein in the same experiments where the assay was applied to human serum samples (see section 4.2.5). However, GST-PGCA could not be used as a protein standard, and the results of GST-PGCA are presented also in section 5.2.3.

4.2.5. Applying the assay to human serum samples

The optimized parameters were used to apply the assay to human serum samples. The samples contained 18 serum samples from control women and 15 from control men. 26 samples were obtained from renal cancer patients and 25 samples from breast cancer patients. In previous experiments the luminescence value was read at 120 ms after triggering the reaction. However, in this case the results from the luminometer were obtained by integrating the area of the curves from 0 to 400 ms.

The samples were divided into three groups for statistical analysis (controls, renal cancer patients, and breast cancer patients). Statistical analysis was performed using SPSS for Windows (version 11.0). Because the data in the groups were not normally distributed, nonparametric Mann-Whitney U test was performed to evaluate if the difference between the groups was statistically significant. The relative concentration values obtained from the groups of patients with renal or breast cancer were compared to those obtained from the control subjects. P-values < 0.05 were considered significant.

5. Results

5.1. Expression of CA IX in mouse tissues

5.1.1. RT-PCR and sequencing

The expression of CA IX mRNA in mouse tissues was studied using a commercially available cDNA kit as well as the cDNAs reverse-transcribed from mRNA that was isolated from selected mouse tissues. Negative control did not contain any band, and positive control contained the correct amplification product (data not shown). The mRNA was found in the kidney, muscle and weak bands were also seen in the brain, spleen, lung, and liver (Figure 5.1A, Table 5.1). Heart and testis, as well as 7-day-old, 11-day-old, and 15-day-old embryos were negative. Embryos of 17 days old appeared to express CA IX mRNA, which is in accordance with a recent study showing low levels of CA IX protein expression during the first postnatal day (Ortova Gut et al., 2002). In the gastrointestinal tract, mRNA was expressed in the stomach, duodenum, jejunum, ileum, and colon (Figures 5.1B and 5.3).

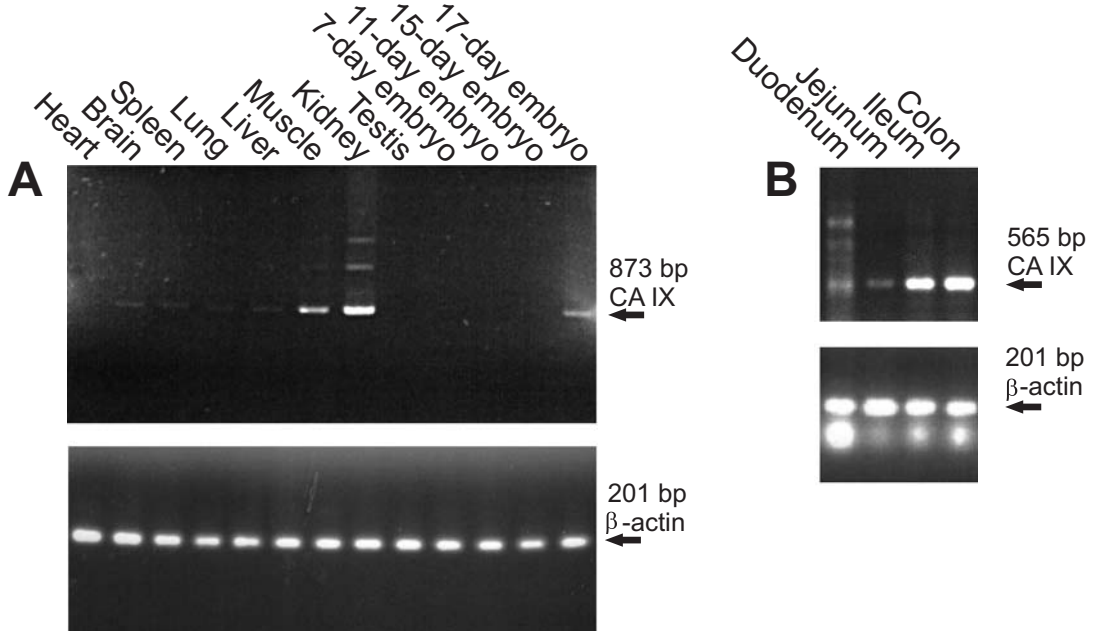


Figure 5.1. In panel A the template in the PCR reaction was obtained from the commercial cDNA kit, and in panel B the RNA was extracted in the laboratory. *(A)* The PCR product of 873 bp is amplified in the kidney and muscle, followed by brain, spleen, lung, and liver. Testis and heart are negative. The expression of CA IX mRNA becomes positive in the 17-day-old embryo. The results in panel *(B)* show that CA IX mRNA is expressed in the colon and ileum, and weaker bands are also seen in the duodenum and jejunum. The quality and quantity of cDNAs was monitored using primers for β-actin. Especially in the duodenum the mRNA has degraded, and therefore the result of this organ is somewhat questionable. Minor degradation can be observed in the ileum and colon.

Because only low amounts of CA IX mRNA have been reported in kidney and muscle (Ivanov et al., 2001; Ortova Gut et al., 2002), the PCR products from these organs were sequenced to confirm their specificity. The automated sequencing was performed using primers designed for both 5' and 3' ends, and the sequencing clearly confirmed that the obtained 873-bp band represented the correct amplification product.

5.1.2. Western blot

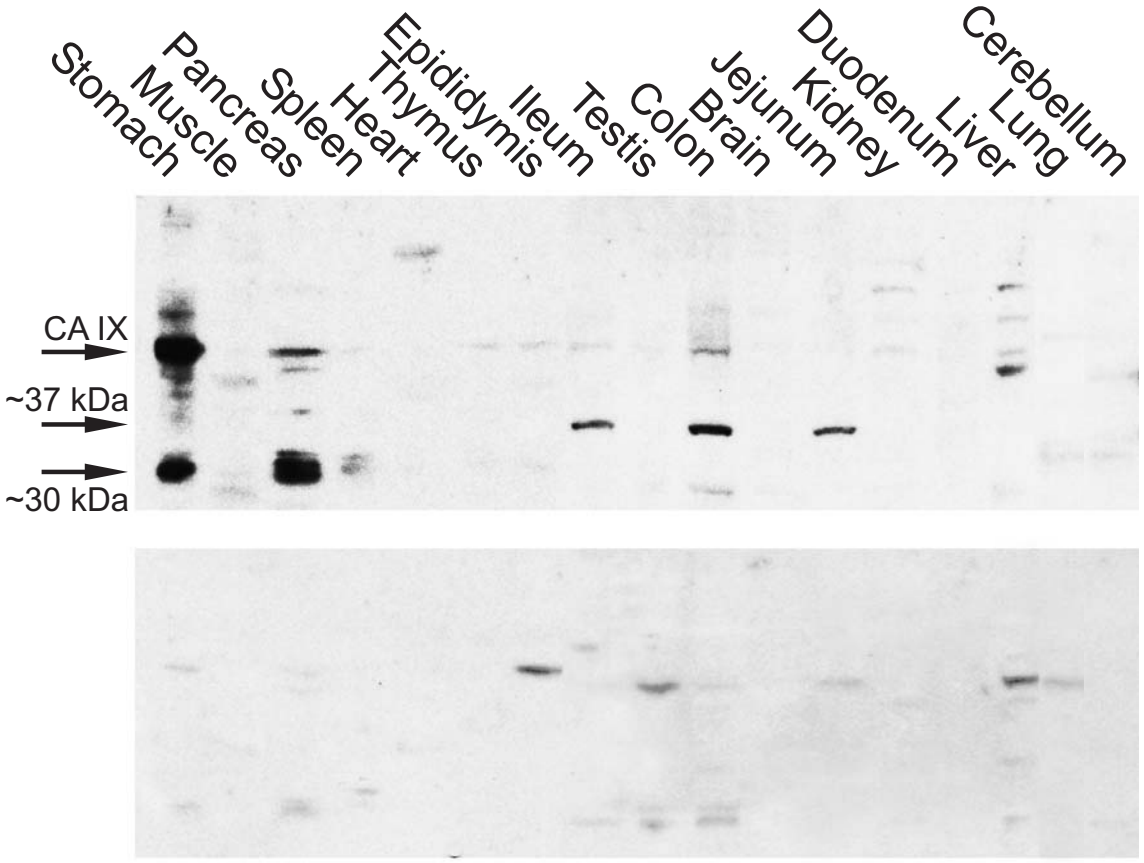


Figure 5.2. Western blotting of mouse tissues. The strongest 47-kDa polypeptide of CA IX is detected in the stomach, followed by pancreas and colon. Very faint bands are seen in the kidney, liver, thymus, testis, epididymis, ileum, and spleen. In addition, strong polypeptides of ~37 kDa are present in the ileum, jejunum, and colon and of ~30 kDa in the stomach and pancreas. Control staining with normal rabbit serum also shows some nonspecific bands but they all are of different molecular mass compared to CA IX.

Western blotting was performed to screen CA IX protein expression in 17 different mouse tissues. The molecular mass of mouse CA IX protein is 47 kDa. As expected, the highest signal for this protein was detected in the stomach (Figure 5.2, Table 5.1). The colon and pancreas showed moderate positive reactions. Very faint bands were detected

in the kidney, liver, thymus, testis, epididymis, ileum, and spleen. Jejunum, duodenum, psoas muscle, heart, lung, brain, and cerebellum showed no CA IX-specific band.

Alternative spliced form of CA IX has been reported in Swiss-Prot (accession code Q8VHB5). The molecular mass of this spliced from is 30 kDa. Strong polypeptide bands corresponding to this molecular mass were observed in the stomach and pancreas. In addition, approximately 37 kDa bands were seen in the ileum, jejunum, and colon. It is noteworthy, that this band was observed in all alimentary tissues except for duodenum. It is possible that these polypeptides are formed by degradation of CA IX. However, CA IX has an important role in the gastrointestinal tract, and thus it is likely that these polypeptide bands represent another alternative spliced form of CA IX.

5.1.3. Comparison of mRNA and protein levels in the kidney and muscle

To confirm the differential expression of CA IX mRNA versus protein in the kidney and muscle, the same tissues were processed in parallel for RT-PCR and Western blotting (Figure 5.3). Results of RT-PCR performed with a different pair of primers corresponded well to those obtained in previous analyses shown in Figure 5.1. Similarly, a profile of the proteins detected by Western blotting in the same tissues as used for RT-PCR largely agreed with the data in Figure 5.2. The CA IX-specific protein band was evident in the stomach and colon and was very weak also in the small intestine, but again completely absent from the kidney and muscle. The bands in the Western blot are not as strong as in Figure 5.2, because the exposure time of the X-ray film was shorter in this experiment.

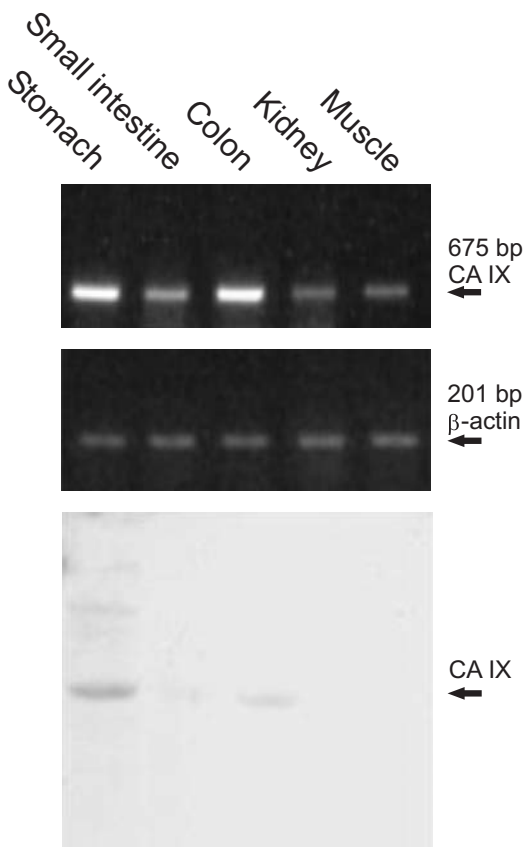


Figure 5.3. Comparison of the RT-PCR (first panel) performed for CA IX in parallel from the same tissues. Note the discrepancy in the signal obtained by RT-PCR and Western blotting in the kidney and muscle.

5.1.4. Immunohistochemistry

Immunoperoxidase staining was performed to localize the expression of CA IX in different tissues and cell types. A polyclonal rabbit antibody for mouse CA IX was used to detect this enzyme and pre-immune serum was used for negative control. Because CA IX has been reported to be present in the gastric mucosa (Ortova Gut et al., 2002), stomach specimens served as a positive control for the immunostaining. Strong reaction was seen in the basolateral plasma membranes of the mucus-producing surface epithelial cells, pepsinogen producing chief cells, and parietal cells that produce hydrochloric acid (Figure 5.4, Table 5.1). In the colon, the signal was present in the plasma membrane of the enterocytes and the strongest immunoreaction was localized to the surface epithelial cuff region. In the small intestine, the staining was very weak and present only in sporadic enterocytes. The pancreas showed moderate positive reaction in the acinar cells, in which the staining was generally diffuse, most probably due to rapid protein degradation in the tissue samples (Figure 5.5). No staining for CA IX was detected in the liver. In the skeletal muscle, only very weak immunoreactions were seen in occasional fibers, while the heart muscle was completely negative. Faint positive signal was also observed in the epithelial cells of the epididymis and in mature spermatozoa located in the seminiferous tubules. No staining for CA IX was found in the kidney, lung, thymus and spleen. The antibody also labeled some neuronal axons in the brain and Purkinje cells of the cerebellum (Figure 5.6).

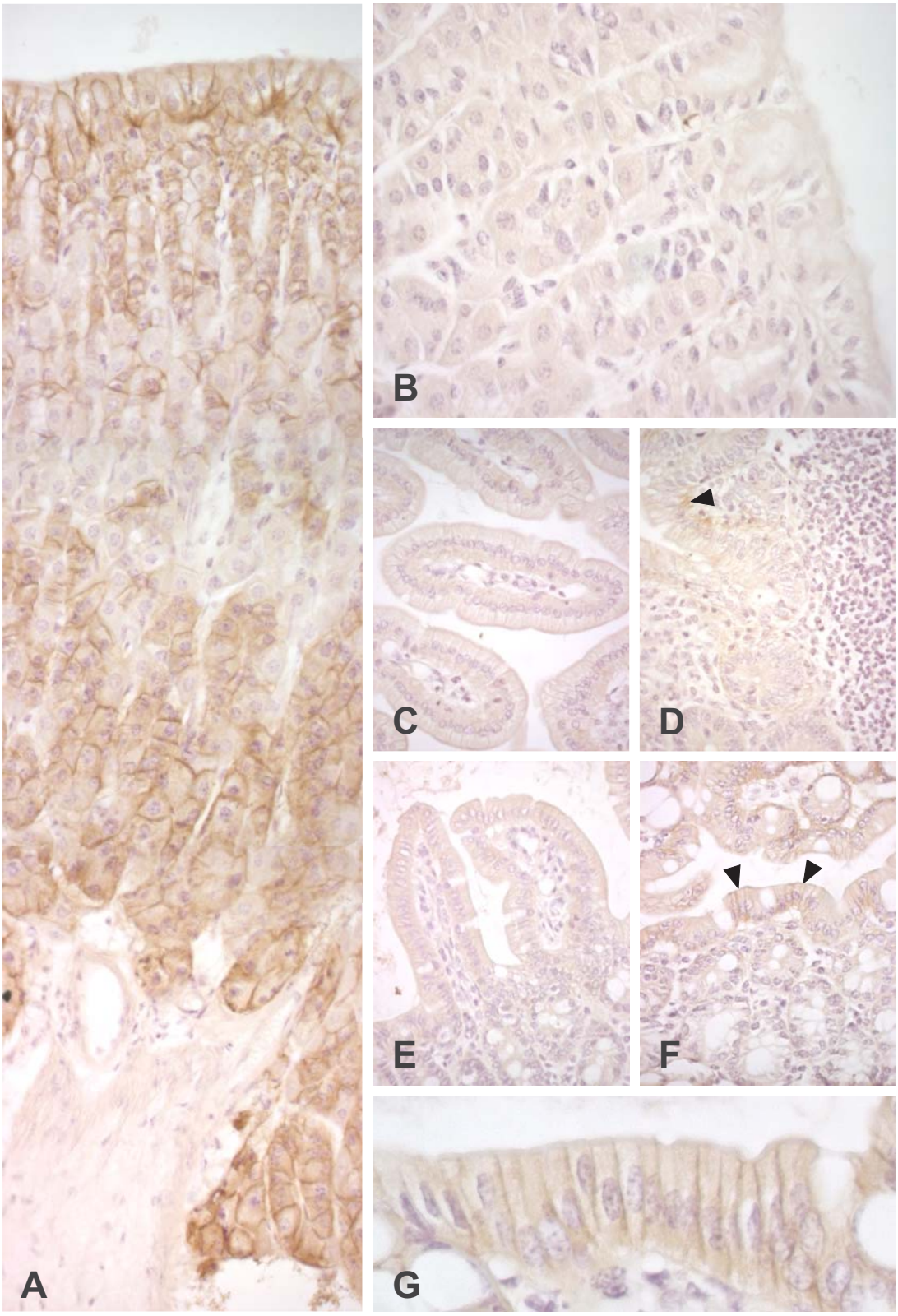


Figure 5.4. Immunohistochemical staining of CA IX in the mouse gastrointestinal tract. Stomach shows the most intense staining *(A)*. Control staining using normal rabbit serum is negative *(B)*. Duodenum *(C)*, jejunum *(D)*, and ileum *(E)* show only very weak signal in sporadic enterocytes (arrowhead in D). In colon *(F)*, moderate staining is observed in the plasma membranes of the enterocytes in the surface epithelial cuff region (arrowhead). *(G)* A higher magnification image in which the positive signal in colon is seen more clearly. Original magnifications x 630.

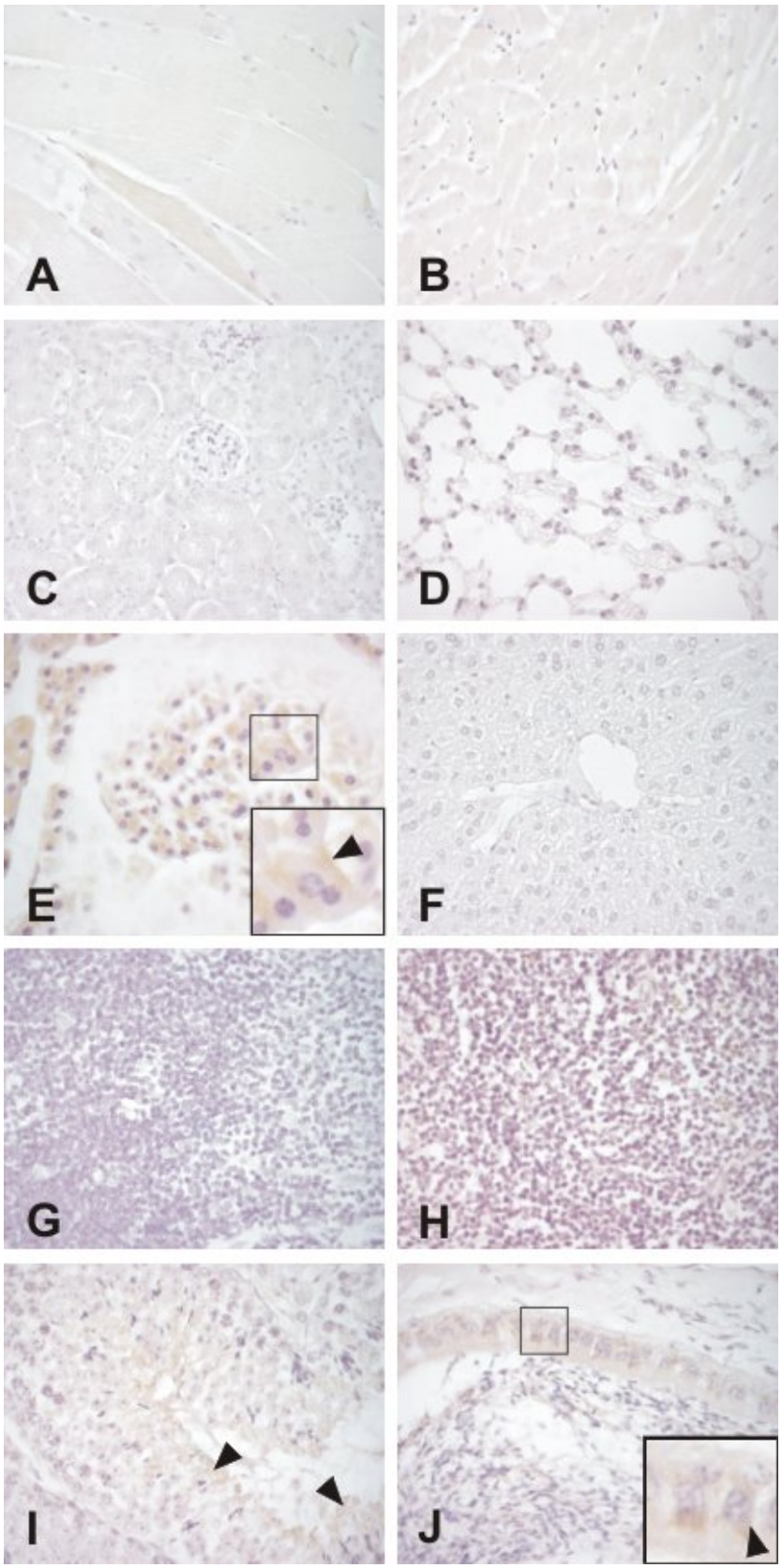


Figure 5.5. Immunohistochemical staining of CA IX in different mouse tissues. Psoas *(A)* shows occasional weakly stained muscle fibers, while heart *(B)* is completely negative. Kidney *(C)* and lung *(D)* are negative. Pancreas *(E)* shows moderate staining in acinar cells (arrowhead). Liver *(F)*, thymus *(G)*, and spleen *(H)* are negative. In testis *(I)*, faint staining is seen in the most luminal spermatozoa present in the seminiferous tubules. Weak positive reactions are also present in the epithelial cells of the epididymis (arrowhead in *J*). Original magnifications: A-E, G-J x 630; B-D, F x 400.

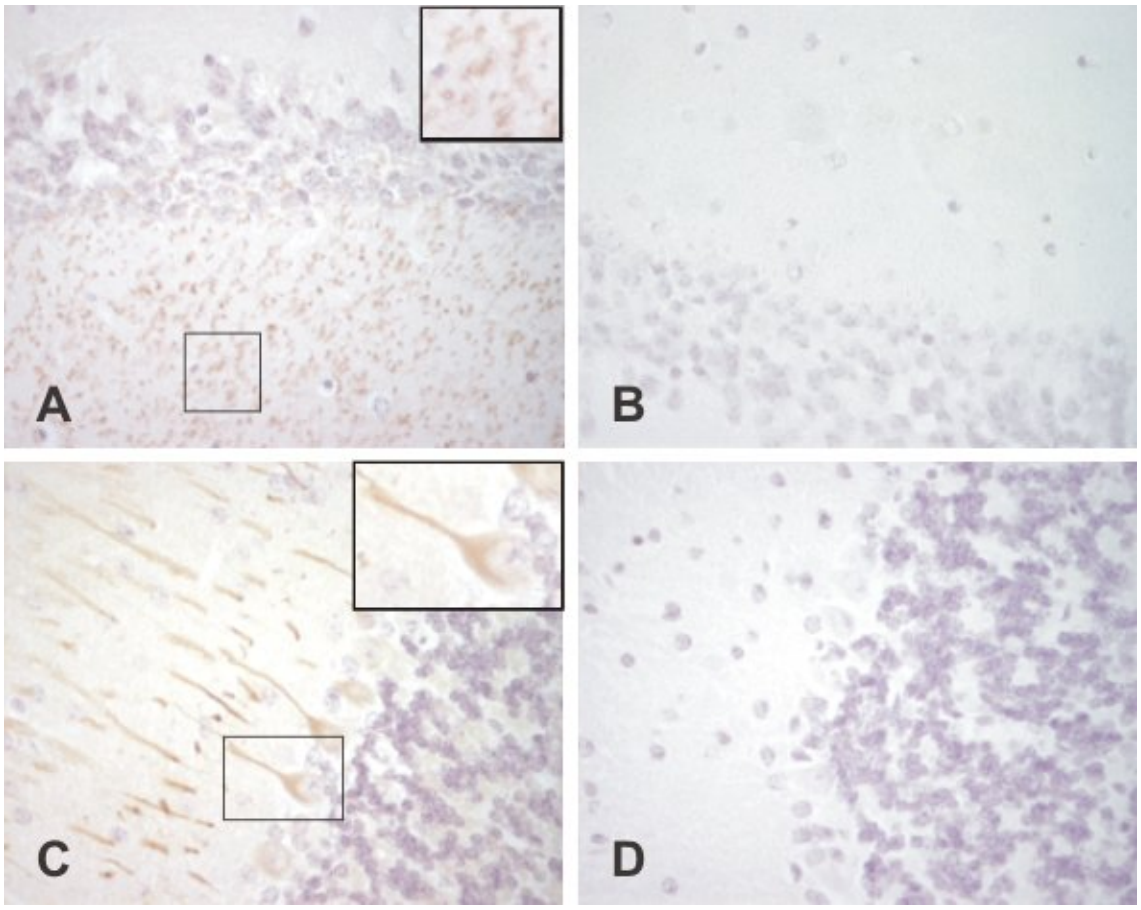


Figure 5.6. Immunostaining for CA IX in the mouse brain. Some neuronal axons are stained in the cerebrum *(A)*, while the control staining shows no reaction *(B)*. In cerebellum *(C)*, the axons originating from the Purkinje cells and the cell body show positive staining. Control staining is negative *(D)*. Original magnifications x 630.

5.1.5. Expression pattern

The expression pattern of CA IX in mouse tissues is summarized in Table 5.1. In the table, the results for Western blot are those corresponding to 47 kDa bands in Figure 5.2. Stomach is clearly the most important site of expression of CA IX. Colon and pancreas produce also CA IX but in the small intestine the expression is weak. Weak expression is also observed in the brain, cerebellum, epididymis, and testis. Skeletal muscle and kidney transcribe CA IX mRNA but the corresponding protein could not be observed by Western blot or immunohistochemistry. Liver, spleen, thymus, lung, and heart can be considered as CA IX negative tissues.

Table 5.1. Expression pattern of CA IX in mouse tissues.

Organ	RT-PCR[a]	WB[b]	IHC[b]
stomach	+	+++	+++
duodenum	+/-	-	+
jejunum	+/-	-	+
ileum	+	+	+
colon	+	++	++
liver	+/-	+	-
pancreas	ND	++	+
spleen	+/-	+/-	-
thymus	ND	+	-
lung	+/-	-	-
skeletal muscle	+	-	+/-
heart	-	-	-
brain	+/-	-	++
cerebellum	ND	-	++
epididymis	ND	+	+
testis	-	+/-	+
kidney	+	+/-	-

[a]Scores in RT-PCR: -, no band; +/-, weak band; +, strong band; ND, not done.
[b]Scores in Western blotting (WB) and immunohistochemistry (IHC): -, no reaction; +/-, very weak reaction; +, weak reaction; ++, moderate reaction; +++, strong reaction.

5.2. Immunoassay for CA IX

5.2.1. Labeling of antibodies

The aim of the immunoassay was to detect the CA IX protein in the sample with either monoclonal antibody M75 or IV/18. Therefore, acridin ester label was attached to these antibodies by using the Chemiluminescence sub-attomole labeling kit from AssayDesigns. The labeled materials were applied to columns and nine 1 ml fractions were collected from both labeling reactions. 1 µl of each fraction was applied to microtiter plates and then the results were read with a luminometer. The results are taken at 120 ms, because that point showed the highest luminescence values (RLU = relative light unit). The results are presented in Table 5.2.

Table 5.2. Acridinium ester -labeled fractions collected from the columns.

Fraction	M75 (RLU)	IV/18 (RLU)
Blank	0.01	0.01
1	0.03	0.72
2	0.03	0.59
3	0.34	0.56
4	1006.00	660.20
5	2256.00	1072.00
6	1054.00	157.50
7	638.30	104.10
8	743.60	95.09
9	740.70	93.34

The results for M75 show that fractions 4, 5, and 6 had the highest counts. Relatively high luminescence could also be observed in fractions 7, 8, and 9. However, fraction 5 was used for further studies, since it contained the highest signal. Also fractions 4 and 6 were stored. For IV/18, the highest signals were in fractions 4 and 5. Other fractions did not show high signal. The fractions 4 and 5 were pooled and this pooled fraction was used for further studies.

5.2.2. Production of protein standards

The first attempt to obtain standard protein for the assay was from a stable NIH 3T3 cell line producing CA IX. The culture medium was collected and CA inhibitor affinity chromatography was used to purify the CA IX protein. The fractions were studied with SDS-PAGE followed by Coomassie staining (data not shown). The fractions containing protein were pooled (CA IX pooled fraction). The Coomassie staining results showed that the molecular mass of the purified protein in the fractions was higher than predicted for CA IX. Because the cell culture medium contained 10% fetal bovine serum, it was suspected that the fractions could have a bovine serum albumin (BSA) contamination. Therefore both BSA and CA IX pooled fraction were analyzed with SDS-PAGE followed by Coomassie staining. To further confirm the results, another part of the gel containing CA IX pooled fraction was analyzed using Western blot. Both of these results are shown in Figure 5.7.

The results in Figure 5.7 confirm that the pooled fraction indeed had marked BSA contamination. The fraction also contained CA IX, but the concentration of CA IX was so low that it could be observed only within Western blot. The main protein component of the pooled fraction was BSA, and therefore, this protein could not be used as a protein standard in the immunoassay. Since the protein purification of CA IX has turned out to be highly challenging and the obtained fraction still contained some CA IX, it was used in the development of the pilot assay.

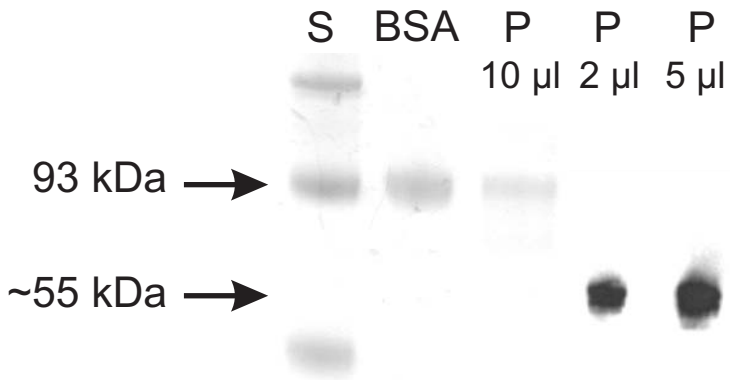


Figure 5.7. Analysis of protein purification. The first three lanes were analyzed by Coomassie staining and the fourth and fifth lanes by Western blot. The first lane shows protein standard (S): the bands correspond to molecular masses 111 kDa, 93 kDa, and 53.5 kDa. The second lane contains 1 µg of BSA which has a band exactly of the same molecular mass than the band in CA IX pooled fraction (P 10 µl). CA IX pooled fractions 2 µl and 5 µl were analyzed using Western blot: the twin band characteristic for CA IX can be observed. The twin band represents a protein with lower molecular mass than the band observed with Coomassie staining.

Because the attempt to have pure CA IX from NIH 3T3 cell line was initially failed, another approach was also explored. The ultimate goal was to purify bacterially expressed GST-tagged fusion proteins that contain CA IX proteoglycan and CA catalytic domains (GST-PGCA). The bacteria producing GST-PGCA were lysed and then purified with Bulk GST Purification module from Amersham Biosciences. Five fractions were collected from the purification column, and the results of this purification procedure are shown in Figure 5.8A. The results in panel A demonstrate that the purified fractions showed several bands. Western blot (using antibody M75) performed from the same fractions detected mainly the same bands (data not shown). This excludes the possibility that the other bands represent unspecific binding of proteins in the purification. The smaller bands may result either from premature ending of translation in bacteria or degradation of the protein, or both. Because the goal was to obtain very pure CA IX fractions, all the fractions were pooled and repurified with CA inhibitor affinity chromatography. The fractions obtained from the latter purification were analyzed with SDS-PAGE and Coomassie staining. The second purification step again produced very small amount of pure CA IX protein. Western blot was performed to study if the protein indeed was GST-PGCA. The results from fraction 3 are shown in Figure 5.8B. Panel B shows that the repurified protein was mostly full-length GST-PGCA, although some degraded protein was seen. Therefore this protein could be considered as a protein standard in the assay. However, further experiments showed that

this protein has a tendency to degrade very rapidly that further restricted its use as a standard protein. The further studies using GST-PGCA refer to the fraction 3.

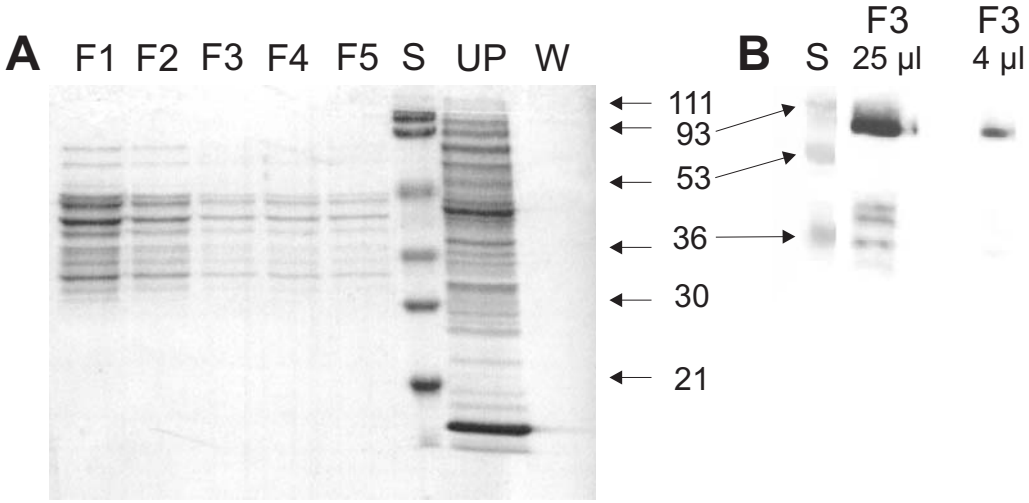


Figure 5.8. GST purification results are shown in panel (*A*). The lanes F1-F5 show the purified fractions 1-5. These lanes contain several protein bands. Sixth lane has the protein standard (S): the molecular masses (kDa) of the bands are shown between panels A and B. The seventh lane shows the unbound protein (UP) of the column and the last lane shows an aliquot taken from the last washing step (W). Because the last lane contains no visible protein, the washes can be considered sufficient. Results from the second purification step, the CA inhibitor affinity purification, are shown in panel (*B*). The first lane shows again the protein standard (S). Two volumes of purified fraction 3 (25 µl and 4 µl) were analyzed and the results show that the fraction contained much full-length GST-PGCA, and some additional smaller bands. These bands cannot be observed in 4 µl volume. The calculated mass of GST-PGCA is approximately 70 kDa.

The concentration of both final CA IX and GST-PGCA proteins was determined with DC Protein assay kit from Bio-Rad. The results showed that the protein concentration of CA IX from NIH 3T3 cell lines was 182 µg/ml. It is noteworthy that this is not the concentration of CA IX, since the solution contained much more BSA than CA IX. However, this value was used in the initial studies, because the low concentration did not allow precise protein determination. The concentration of GST-PGCA was 10.4 µg/ml. When the GST was taken off from this result, the concentration of PGCA in the fraction was 6.2 µg/ml.

5.2.3. Adjusting the parameters

The assay parameters were adjusted in a number of experiments, and detailed analysis of all of them would be beyond the scope of this thesis. Only the main results are summarized in this section. One principle in optimizing the parameters was to obtain

the highest signal-to-noise ratio with CA IX protein standard. Another principle was to minimize the intra- and interassay variation. The protein used in these experiments was the CA IX produced in NIH 3T3 cells.

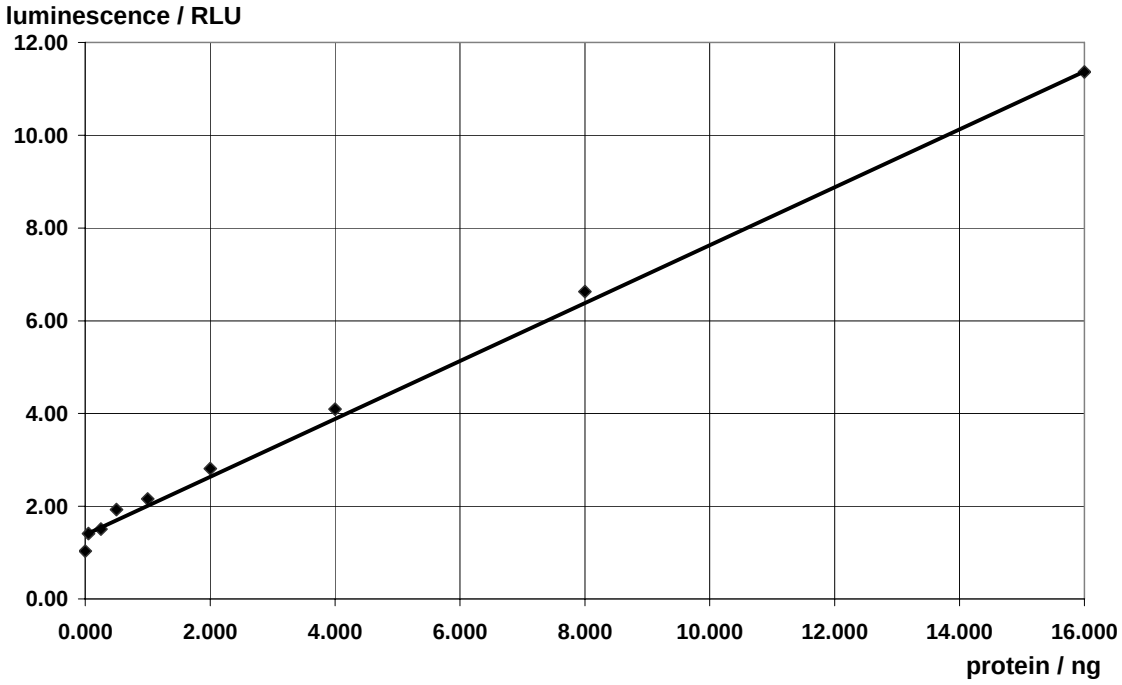
Different amounts of labeled antibodies were tested to detect CA IX in the microtiter plate well. The lowest amount was 0.1 µl / well and the highest amount 2.0 µl / well. 0.5 µl / well gave the best signal. A combination of 0.25 µl M75 + 0.25 µl IV/18 was also tested: this seemed to give even better results; especially the standard curves were more linear than with a single labeled antibody. Different amounts of triggering solutions were also tried out: 100 µl of triggering solutions 1 and 2 gave the highest signal. Also different amounts of serum were applied to the wells: 1:200 dilutions gave the most reproducible results. Because each well contained 200 µl of assay reagents, 1:200 dilution means, that each well contained 1 µl of serum. The luminescence value (RLU = relative light unit) was read at 120 ms after triggering the reaction.

Two different assay protocols were tested. The first protocol consisted of 1.5 h incubation with serum or protein samples, and then 1.5 h incubation with labeled antibodies. The second protocol consisted of one 3 h incubation step, when both protein and labeled antibodies were incubated in the well at the same time. The second protocol gave slightly better results, and thus it was used in further studies.

CA IX produced in NIH 3T3 cells was used to test if the assay detected CA IX protein. The optimized parameters were applied, i.e., the protein and 0.25 µl M75 + 0.25 µl IV/18 were incubated for 3.0 h on an orbital shaker. The amount of protein and the results obtained from the luminometer are listed in Table 5.3. Based on the results, a standard curve was constructed (shown in Graph 5.1). The results clearly illustrated that higher protein concentrations produced higher luminescence. The obtained results could be estimated quite well with a linear curve.

Table 5.3. Results with CA IX produced in NIH 3T3 cells. The first column shows the amount of protein in a well. The luminescence values of two parallel samples were obtained (A and B). The average of the two samples was used to construct the standard curve that is presented in Graph 5.1.

Protein (ng)	A (RLU)	B (RLU)	Average luminescence (RLU)
0.000	0.760	1.308	1.034
0.050	0.972	1.846	1.409
0.250	1.242	1.771	1.507
0.500	1.812	2.048	1.930
1.000	2.056	2.261	2.159
2.000	2.775	2.851	2.813
4.000	4.276	3.911	4.094
8.000	6.431	6.824	6.628
16.000	11.000	11.730	11.365



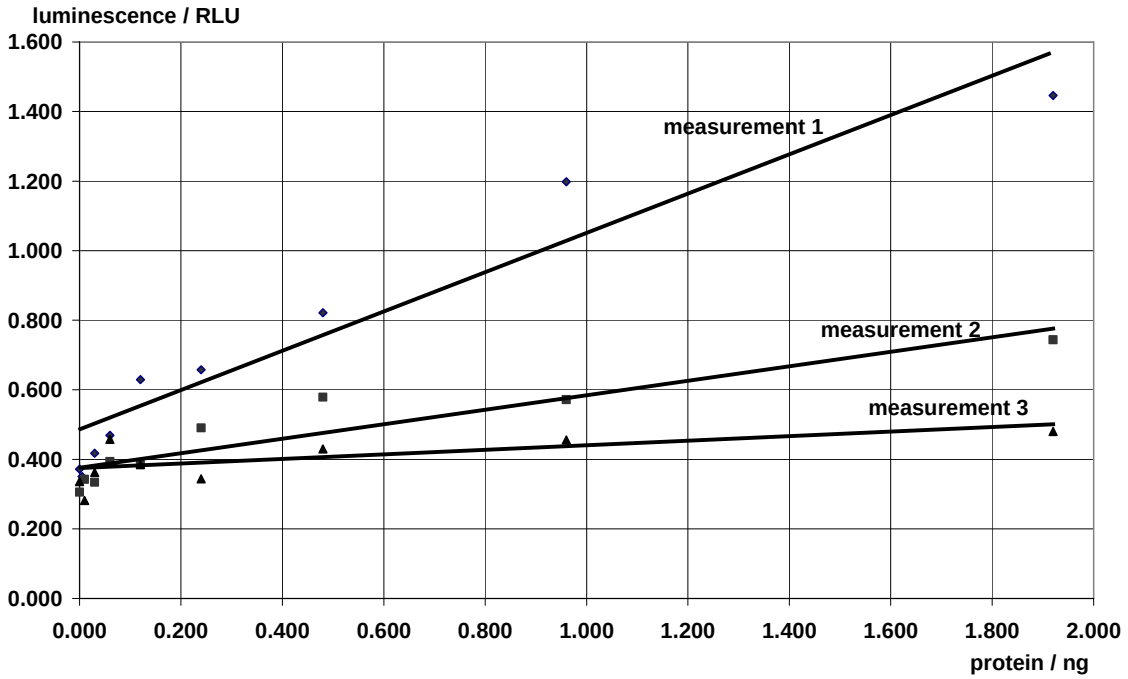
Graph 5.1. A graph where luminescence is plotted on the Y-axis and the standard protein concentration is on the X-axis. Even though the low concentrations produced a high deviation, the mean values produced a linear curve.

Also the GST-PGCA protein was used to test the assay. The parameters were the same as in the case of CA IX produced in NIH 3T3 cells. Measurements were performed on three days: measurement 1 was performed on day 1, measurement 2 on day 10, and measurement 3 on day 11. The results of these measurements can be found in Table 5.4. The graph drawn based on the values is presented in Graph 5.2. It is notable that the results have decreased on each consecutive experiment. This finding suggests that the

GST-PGCA protein has degraded while stored at 4 °C. Some aliquot was stored at -20 ºC, but the freezing and melting seemed to cause an even more dramatic degradation of the protein (data not shown). From these results it was concluded that this protein could not be used as a protein standard because of its high tendency to degradation.

Table 5.4. Three measurements with GST-PGCA. The results are plotted on Graph 5.2.

Protein / ng	Measurement 1			Measurement 2			Measurement 3		
	A (RLU)	B (RLU)	Average luminescence (RLU)	A (RLU)	B (RLU)	Average luminescence (RLU)	A (RLU)	B (RLU)	Average luminescence (RLU)
0.000	0.252	0.492	0.372	0.203	0.457	0.330	0.314	0.305	0.309
0.005	0.316	0.386	0.351	0.150	0.411	0.280	0.233	0.418	0.325
0.010	-	-	-	0.277	0.408	0.343	0.252	0.312	0.282
0.030	0.393	0.442	0.417	0.332	0.336	0.334	0.330	0.395	0.362
0.060	0.480	0.458	0.469	0.399	0.388	0.394	0.467	0.448	0.458
0.120	0.704	0.554	0.629	0.356	0.414	0.385	0.439	0.331	0.385
0.240	0.651	0.665	0.658	0.500	0.481	0.490	0.325	0.363	0.344
0.480	0.840	0.803	0.822	0.583	0.574	0.578	0.425	0.435	0.430
0.960	1.330	1.066	1.198	0.567	0.577	0.572	0.415	0.496	0.455
1.920	1.477	1.415	1.446	0.804	0.683	0.744	0.484	0.477	0.480



Graph 5.2. Luminescence plotted on the function of GST-PGCA protein concentration. The luminescence results have decreased in consecutive measurements.

The results of the measurements with proteins CA IX and GST-PGCA suggested that the assay was capable of detecting CA IX even though it showed marked fragility due to

rapid protein degradation. The assay was applied to human blood serum samples, and this will be the topic of next section.

5.2.4. Determination of relative CA IX concentrations in human serum samples

The assay was applied to serum samples in order to elucidate whether the renal or breast cancer patients had elevated concentrations of CA IX in the blood. 18 samples from healthy women and 15 samples from healthy men served as controls. 26 samples were from renal cancer patients, and 25 samples were obtained from breast cancer patients. The parameters for the assay were the optimized parameters, which were discussed in the previous section. The only difference was in the luminometer settings. In the previous experiments, the luminescence values were read at 120 ms after triggering the reaction. However, it was noticed that measuring the reaction for 400 ms, and then integrating the area within the curve, gave less inter-assay variation. Therefore, in these experiments the integrated values were used, and they appeared to be slightly lower than the one time-point values.

The original idea was to use GST-PGCA as a standard protein in these assays. The results for this protein were already discussed in the previous section. Because of the obvious degradation of the standard protein, the exact concentrations of CA IX in serum samples were not determined. Instead, relative values were obtained, which allowed a comparison of the different patient categories to each other. The obtained luminescence values are presented in Table 5.5, and those values are used to compare the relative concentrations between serum samples obtained from the controls and cancer patients. Some samples were applied to multiple microtiter plates. Those duplicates or triplicates have been omitted from these results, and instead the average values of these multiple experiments have been listed on the table.

Table 5.5. Relative CA IX concentrations in the sera of control and cancer patients. The results from control women (*A*), control men (*B*), renal cancer patients (*C*), and breast cancer patients (*D*) are listed. Each sample was measured as duplicate on a microtiter plate. Some samples were applied onto several plates, and the average values have been incorporated to the table. The median value of all controls (women and men) was 0.263. All the samples are compared to this mean value by calculating their deviation from this control value. Those samples, whose value is more than 17% higher than the median value of controls, are marked with a light grey color. Those samples having more than 30% higher value are marked with a dark grey background.

A	Serum of control women			
Sample	A (RLU)	B (RLU)	Average (RLU)	Deviation from controls
18703	0.315	0.245	0.280	6.46 %
18704	0.257	0.263	0.260	-1.14 %
18705	0.306	0.305	0.306	16.22 %
18709	0.331	0.365	0.348	32.32 %
18711	0.236	0.281	0.258	-1.88 %
18714	0.171	0.239	0.205	-22.15 %
18715	0.262	0.288	0.275	4.52 %
18718	0.257	0.232	0.244	-7.07 %
19402	0.229	0.206	0.218	-17.21 %
19404	0.303	0.286	0.294	11.96 %
19406	0.242	0.238	0.240	-8.80 %
19408	0.257	0.292	0.275	4.51 %
19412	0.222	0.221	0.221	-15.89 %
19413	0.253	0.253	0.253	-3.82 %
19416	0.265	0.287	0.276	4.83 %
19421	0.234	0.265	0.250	-5.08 %
19422	0.280	0.243	0.262	-0.49 %
19423	0.276	0.240	0.258	-1.75 %
		Median:	**0.259**	

B	Serum of control men			
Sample	A (RLU)	B (RLU)	Average (RLU)	Deviation from controls
K24	0.237	0.217	0.227	-13.75 %
K25	0.264	0.266	0.265	0.78 %
18701	0.311	0.301	0.306	16.35 %
18706	0.275	0.263	0.269	2.28 %
18707	0.311	0.290	0.301	14.28 %
18708	0.287	0.240	0.263	0.15 %
18710	0.274	0.250	0.262	-0.38 %
18712	0.254	0.260	0.257	-2.28 %
18713	0.236	0.251	0.244	-7.41 %
18716	0.309	0.301	0.305	16.05 %
19414	0.299	0.303	0.301	14.41 %
19415	0.603	0.572	0.588	123.40 %
19418	0.230	0.265	0.248	-5.76 %
19427	0.314	0.357	0.336	27.57 %
19430	0.320	0.295	0.308	16.92 %
		Median:	**0.269**	

C	Serum of renal cancer patients			
Sample	A (RLU)	B (RLU)	Average (RLU)	Deviation from controls
M1	0.260	0.265	0.263	-0.13 %
M2	0.310	0.301	0.305	16.12 %
M3	0.289	0.333	0.311	18.23 %
M8	0.288	0.280	0.284	7.89 %
M20	0.326	0.339	0.333	26.43 %
M42	0.264	0.323	0.294	11.60 %
M54	0.289	0.262	0.276	4.79 %
M57	0.816	0.758	0.787	199.35 %
M62	0.248	0.230	0.239	-9.13 %
M73	0.284	0.240	0.262	-0.38 %
M104	0.317	0.296	0.307	16.54 %
M113	0.229	0.241	0.235	-10.65 %
M114	0.320	0.342	0.331	25.86 %
M123	0.264	0.281	0.273	3.61 %
M124	0.212	0.241	0.227	-13.88 %
M126	0.271	0.311	0.291	10.65 %
M140	0.252	0.257	0.255	-3.23 %
M159	0.298	0.306	0.302	14.83 %
M167	0.279	0.320	0.300	13.88 %
M185	0.290	0.301	0.296	12.36 %
M193	0.304	0.311	0.308	16.92 %
M196	0.430	0.397	0.414	57.22 %
M214	0.420	0.429	0.424	61.27 %
M218	0.344	0.328	0.336	27.87 %
M232	0.324	0.298	0.311	18.25 %
M233	0.255	0.299	0.277	5.29 %
		Median:	**0.298**	

D	Serum of breast cancer patients			
Sample	A (RLU)	B (RLU)	Average (RLU)	Deviation from controls
2	0.292	0.262	0.277	5.32 %
3	0.231	0.217	0.224	-14.81 %
5	0.233	0.275	0.254	-3.44 %
7	0.355	0.320	0.337	28.29 %
9	0.282	0.311	0.296	12.62 %
11	0.254	0.254	0.254	-3.40 %
12	0.285	0.291	0.288	9.45 %
13	0.274	0.258	0.266	1.14 %
14	0.216	0.204	0.210	-20.15 %
15	0.182	0.176	0.179	-31.94 %
16	0.313	0.303	0.308	17.11 %
17	0.254	0.267	0.261	-0.95 %
18	0.253	0.243	0.248	-5.70 %
20	0.254	0.315	0.285	8.17 %
21	0.258	0.262	0.260	-1.06 %
22	0.296	0.306	0.301	14.37 %
23	1.009	1.084	1.047	297.91 %
25	0.297	0.296	0.297	12.76 %
28	0.184	0.190	0.187	-28.99 %
29	0.210	0.228	0.219	-16.75 %
31	0.380	0.355	0.367	39.68 %
32	0.247	0.289	0.268	1.79 %
34	0.266	0.274	0.270	2.66 %
35	0.318	0.214	0.266	1.08 %
36	0.211	0.188	0.200	-24.13 %
		Median:	**0.266**	

Because the protein standard did not give reasonable results, the luminescence values were compared with each other. The following median RLU values were obtained: control women 0.259, control men 0.269, RCC patients 0.298, and breast cancer patients 0.266. The median for controls (both men and women) was 0.263. Men had slightly higher median value than women. Breast cancer patients had nearly the same value than healthy control women. The median RLU of renal cancer patients was higher compared to all other classes. The individual samples were compared to the median of all controls (0.263) by calculating the deviation from this value in percents. A cut-off value was decided to separate normal and elevated RLUs. The cut-off was decided so that most control subjects would not have elevated concentration of CA IX. Those samples that had over 17% higher values than controls are marked with light gray color in Table 5.5. The samples having more than 30% higher values have dark grey background in the table.

The summary of these results is presented in Table 5.6. The results show that fewer renal cancer patients were in the category that had less than 17% higher results compared to controls. Most of the differences were sawn on 17-30% increase category that contained 19% of renal cancer patients but only 3% of controls. Also the over 30% category had more renal cancer patients than controls. The results from breast cancer patients were similar to those of control samples, although slight increase in values compared to controls can be observed.

Table 5.6. Summary of immunoassay results.

Deviation from control median	Controls	Breast cancer patients	Renal cancer patients
<17%	91 %	84 %	69 %
≥17%	3 %	8 %	19 %
≥30%	6 %	8 %	12 %

Mann-Whitney U test was performed to assess if the difference between cancer patient groups and controls was statistically significant. A P-value less than 0.05 was considered as statistically significant result. As expected, breast cancer patients did not have statistically significant difference compared to controls (P = 0.851). However, renal cancer patients showed statistically significant difference when compared to control population (P = 0.013).

6. Discussion

CA IX is an interesting and unique member of the CA gene family with respect to its molecular structure, distribution, and suggested functions. First, it contains two structurally distinct domains, a CA domain and a proteoglycan-like domain, which might contribute to different functions (Opavsky et al., 1996). Second, human CA IX has been reported to be present in only a few normal tissues and is overexpressed in several carcinomas. Third, its expression is induced by hypoxia and is downregulated by the product of the wild-type von Hippel-Lindau gene (Ivanov et al., 1998). Fourth, recent studies in CA IX knock-out mice have further shown that CA IX is functionally involved in normal gastric morphogenesis, and this feature in particular makes it a very distinctive isoform among the other members of the CA gene family (Ortova Gut et al., 2002).

6.1. Expression of CA IX in mouse tissues

Even though the expression of CA IX has been quite well established in human and rat tissues (Pastorekova et al., 1997; Saarnio et al., 1998b), only little has been known about its expression in mouse. The results obtained in this study provide a basic overview of CA IX distribution in mouse tissues. The findings indicate that, among a number of different tissues, gastric mucosa shows the highest expression of this enzyme. The same phenomenon has been described earlier in human and rat tissues. On the basis of both RT-PCR and Western blotting results, the colon and pancreas clearly expressed CA IX. Immunohistochemistry confirmed that colon enterocytes and pancreatic acini were indeed positive for CA IX immunostaining. Some other tissues, including kidney, liver, thymus, testis, epididymis, ileum, and spleen, also showed weak signal for CA IX in the Western blot, but the intensity was very weak, suggesting a low expression of the enzyme in those tissues. The PCR analysis included mouse tissues that were either available in the commercial cDNA kit or obtained from mice in our laboratory. Unexpectedly, the kidney and muscle showed strong positive signal for CA IX mRNA, while the control amplification using β-actin primers produced steady signal in all tissues. Furthermore, the specificity of the positive amplification products was confirmed by DNA sequencing. Immunohistochemistry and Western blotting, however, showed no or very weak reactions for CA IX in the kidney and skeletal muscle fibers. To confirm the differential expression of CA IX mRNA versus protein in the kidney

and muscle, RT-PCR and Western blotting analyses were performed from identical tissue specimens. Again the presence of CA IX mRNA and absence of the corresponding protein were found, confirming that the discrepancy was not due to interanimal differences or sampling error.

It is interesting to compare the expression of CA IX and HIF-1α, because CA IX is regulated by the VHL/HIF pathway. It could be anticipated that the tissues containing high levels of HIF-1α would also express CA IX. In normoxic mouse tissues expression of HIF-1α has been observed in the brain, kidney, liver, and heart (Stroka et al., 2001). In the brain and kidney the results for both CA IX and HIF-1α seem to point into the same direction, while in the liver and heart there is a discrepancy between the expression of these two proteins. However, very recent results have pointed out that CA IX indeed has an important role in the brain and kidney. Reanalysis of the CA IX knock-out mice showed, that in addition to gastric hyperplasia, these mice also have serious deformations in the brain and kidney (unpublished results). This suggests that CA IX is important especially in the development of these two organs. An interesting area of investigations would be to explore the expression of CA IX during mouse embryogenesis.

The discrepancy between CA IX mRNA and protein in the kidney and muscle raise two basic questions: first, why CA IX transcription is activated particularly in the muscle and kidney tissues and, second, why there is a contradiction between expression of mRNA and protein. One possible answer for the first question may be related to the presence of a functional hypoxia-response element in the promoter of the mouse *Car9* gene (unpublished results), which may allow transcription of CA IX under conditions of lowered oxygen and/or hypoglycaemia, similarly as in human tissues. It is quite conceivable that such conditions may be locally present, at least in muscle. Although this idea is indirectly supported by finding of considerably increased levels of PCR product specific for the hypoxia-regulated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in both muscle and kidney (data not shown), definite evidence requires further investigation. The response to the second question is also unclear but appears to point at a tissue-specific post-transcriptional regulation. As proven by RT-PCR amplification using different pairs of primers designed to cover whole mRNA, CA IX transcripts in kidney and muscle are intact. Therefore, the reason for the absence of

corresponding protein does not appear to reside in production of incomplete polypeptide committed to degradation or of a truncated secretory form. The results suggest that the translation efficiency of CA IX mRNA can be very low in certain cell types based on the physiological demands. It is noteworthy that the normal human kidney and muscle show no or only very weak expression of both CA IX protein and mRNA (Liao et al., 1997; McKiernan et al., 1997; Ivanov et al., 2001). Therefore, an interesting area for future investigations would be to study the regulatory mechanisms of CA IX mRNA translation in the murine kidney and muscle cells.

The present results provided a good opportunity to compare the CA IX distribution pattern among different species. It has been previously shown that CA IX is expressed in the rat stomach, duodenum, colon, and biliary tract as well as in the human stomach, colon, duodenum, jejunum, ileum, biliary tract, and pancreas (Pastorekova et al., 1997; Saarnio et al., 1998b). The distribution in the mouse gastrointestinal tract was quite similar to that of the rat and human tissues. In all species, gastric mucosa appeared to contain the highest levels of CA IX. Therefore, it is not surprising that CA IX knock-out mice showed a distinct gastric phenotype with epithelial cell hyperplasia and cystic changes in the mucosa (Ortova Gut et al., 2002). Based on the previous and present studies, CA IX is expressed in the colon of all these species. Nevertheless, CA IX knock-out mice exhibited no intestinal phenotype that might be partly explained by much lower level of expression in the gut compared with the gastric mucosa. It is also notable that CA IX immunostaining clearly shows different regional distribution in the rodent versus human colon (Pastorekova et al., 1997). In the rat and mouse colon, the enzyme located in the most superficial part of the mucosa, called the epithelial cuff region. In contrast, the human colon shows positive signal only in the crypt enterocytes. This finding may reflect important differences in colonic physiology among various species with respect to the function of CA IX. Furthermore, some lower molecular mass polypeptide bands (approximately 37 kDa) were detected by anti-mouse CA IX serum in the small intestine of mice. It would be of interest to find out, if these represent splicing variants of CA IX. The present results also showed a moderate immunoreaction for CA IX in the pancreatic acini. This finding is in line with the previous observation in the human pancreas showing positive staining in the acinar cells (Kivela et al., 2000). The major difference is that CA IX is restricted to occasional acini in the human pancreas, whereas the mouse pancreas shows a more uniform staining. The expression

in pancreatic acini was even stronger and more widespread in other immunostaining experiments using automated immunostaining system (unpublished). However, the role of CA IX in pancreas has remained completely unclear since no pancreatic phenotypic change has been reported in the CA IX deficient mice.

The immunohistochemistry also provided evidence that CA IX might be expressed in some neuronal axons and Purkinje cells. Weak signal was also observed for CA IX mRNA in the brain, while the Western blots of the brain and cerebellum remained negative, possibly due to lower detection sensitivity. The presence of CA XIV in the human and mouse brain has been previously reported (Parkkila et al., 2001). The present findings suggest that CA IX might be a second membrane-associated isozyme specifically expressed in some neurons. Similar to the present findings, CA XIV showed the highest expression in the axonal membrane. The membrane-bound CA activity has been proposed to participate in the production of an alkaline shift linked to neuronal signal transduction (Parkkila et al., 2001). Functional studies will hopefully unravel the contribution of each CA isozyme to neuronal physiology.

6.2. Immunoassay for CA IX

Normal human kidney does not produce CA IX but most renal cell carcinomas overexpress this enzyme. Renal cell carcinoma is relatively resistant to chemotherapy and radiotherapy, and therefore early diagnosis of the disease is important for the survival of the patient. Symptomatic renal cancer patients are most likely to have an advanced stage of the disease. Therefore new diagnostic approaches would be valuable for the treatment of renal cancer.

CA IX is a promising candidate for the biomarker of RCC. Previous studies have used RT-PCR for CA IX mRNA to detect circulating cancer cells in peripheral blood. The results have been convincing, since these experiments have detected many of the RCC patients, while at the same time the control samples have given only a few positive results. However, all patients do not necessarily have cancer cells circulating in their blood, so another approach would be needed to detect the disease in these subjects. It has been reported that RCC patients tend to have elevated concentration of soluble CA IX protein in the serum (Zavada et al., 2003). Normal blood cells do not express CA IX.

Thus, proteolytic cleavage from epithelial and tumor cells has been suggested as one possible explanation for the presence of CA IX protein in the blood. An immunoassay detecting elevated concentration of CA IX could possibly detect both circulating cancer cells and sCA IX in serum.

The major technical problem with CA IX assay is that even RCC patients have low levels of CA IX in their blood. One way to solve this problem would be to concentrate the serum samples, but this is not practical for routine clinical use because it is time-consuming and it also represents another factor of uncertainty of measurement. Therefore the immunoassay for clinical use should be very sensitive in order to detect this protein without additional steps. The method of choice in this study was chemiluminescence since it is known to detect very low concentrations of molecules in the sample. The Chemiluminescence sub-attomole labeling kit used in this study was from AssayDesigns, and according to the manufacturer's data sheet the assay should optimally detect as low as 10^{-18} moles of molecules in a sample. The previous studies suggested that the serum concentration of CA IX should be 20 pg/ml – 3.6 ng/ml in RCC patients. Based on the manufacturer's specifications, the chemiluminescence immunoassay should be sensitive enough for this amount of protein without concentrating the sample.

The original idea in this study was to use two monoclonal antibodies to detect CA IX protein. However, it was soon noticed that the assay sensitivity could be increased using one antibody attached to the solid surface and two labeled antibodies each detecting different epitopes of the CA IX molecule. Although several parameters were tested to gain the optimal conditions for the immunoassay, two problems could not be solved in this study: the first problem was that the interassay variation was quite high. By optimizing further the testing conditions and settings of the luminometer one could perhaps get more reliable results and less day-to-day variation.

The second major problem was linked to the production of the standard proteins. The first attempt to isolate CA IX from mammalian cells failed. This was not completely surprising, because other studies have also shown that CA IX is a very challenging protein for purification with CA inhibitor affinity chromatography. The low binding of CA IX to the inhibitor resulted in a major albumin contamination in the eluted fraction.

Therefore the concentration of CA IX could not be detected accurately, and thus this protein solution could not be used as a reference. The second attempt was to produce GST-fusion protein that contained both CA catalytic and proteoglycan domains. Both parts of the protein were needed for the immunoassay, because the epitopes detected by the antibodies were located in these domains. Indeed, the full-length protein was obtained by the CA inhibitor affinity chromatography, and the result was confirmed by Western blot. The first problem with this protein was that it tended to degrade very easily. Several storage conditions and urea were tested to maintain the integrity of the protein, but these did not provide any better results (data not shown). As a final outcome, the obtained fractions showed a number of bands in the SDS-PAGE gel. All of these polypeptides were not probably derived from the degradation, but may also represent premature ending of translation (Drs. Silvia and Jaromir Pastorek, personal communication). The ideal CA IX standard protein should combine the positive properties of these two produced proteins: it should contain some purification tag (eg. GST), in order to make purification easier, and it should be produced in mammalian cells in order to prevent the premature ending of translation and minimize the protein degradation. The development of this kind of ideal fusion protein is one aim of our future studies.

The CA IX produced in NIH 3T3 cell line, however, was used in the development of the assay. The assay also detected the bacterially produced CA IX fusion protein. The immunoassay utilized three monoclonal antibodies which were characterized and the epitopes were mapped in the laboratory of Drs. Silvia and Jaromir Pastorek. Therefore, the developed immunoassay could be considered as monospecific for CA IX.

Finally the assay was applied to human serum samples. The results were deduced straight from the luminescence values. This was because the microtiter plates contained GST-PGCA as a protein standard and the rapid degradation of the protein was noticed only at this point. Therefore, an accurate estimation of the real concentration could not be made. The values for control men were slightly higher than for control women. Serum samples of breast cancer patients gave almost the same results as obtained from the control women. The median relative concentration value in renal cancer patients was higher than in any other group. This suggests that at least some renal cancer patients may indeed have higher concentration of CA IX in the blood. One control subject, renal

cancer patient and breast cancer patient had considerable higher values than other subjects. Unfortunately, the detailed clinical information on these people was not available at this stage, so the reason for these high values cannot be definitely explained.

As a conclusion, the results indicated that some renal cancer patients may have elevated concentration of CA IX and the difference between renal cancer patients and controls was found to be statistically significant. It has to be remembered, however, that these results were quite preliminary and further studies are needed to conclude if CA IX has the potential to be used clinically as a tumor marker.

The assay needs further development, and one way to improve the assay would be to try different combinations of monoclonal antibodies in order to identify those that give the most reliable results. One approach would be to study also urine samples of RCC patients because those samples could perhaps contain even higher amounts of CA IX. In addition, carcinomas of the bladder produce CA IX, and thus, excess CA IX may be shed to the urine also in these cases. If CA IX had the potential as a tumor marker, it could be used for example to monitor the status of the patients' disease after surgical removal of the tumor. Treatment of RCC patients with monoclonal antibodies (Wilex Company) would give another reason to explore the presence of CA IX in the tumor. Because new treatment options tend to be very expensive, it is important to select those patients that indeed benefit from a certain treatment protocol. This could be one major application of CA IX immunoassay in laboratory diagnostics.

7. Conclusions

The first goal of this thesis was to study the expression of CA IX in mouse tissues. The most intense expression of this protein was observed in the gastric mucosa. The colon and pancreas were also CA IX-positive tissues, while the expression in the small intestine was weak. Minor expression was also observed in the brain, cerebellum, epididymis, and testis. Liver, spleen, thymus, lung, and heart may be considered as CA IX-negative tissues. An interesting discrepancy was observed in the kidney and muscle: they showed expression of CA IX mRNA while the corresponding protein could not be found in these tissues. An interesting area of further studies would be to explore if there are some post-transcriptional mechanisms regulating CA IX expression that are related to physiological demands in these tissues.

CA IX is a tumor-associated protein, and it is overexpressed especially in renal cell carcinomas. The second goal of this thesis was to develop an immunoassay for CA IX that could measure the concentration of CA IX in human serum. Even though the results were very preliminary and a lot of work is still required to develop a reliable immunoassay for CA IX, the results suggested that some renal cancer patients may have higher CA IX concentrations in the serum compared to those in control subjects and breast cancer patients. This result suggests that further studies are meaningful to be carried out in order to reveal if CA IX has the potential to serve as a clinical marker protein for renal cell carcinoma.

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9. Appendix: the published article

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ARTICLE

Expression of Carbonic Anhydrase IX in Mouse Tissues

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SUMMARY Carbonic anhydrase IX (CA IX) is a unique member of the CA gene family. In contrast to the other isozymes, it has been implicated in regulation of cell proliferation, adhesion, and malignant cell invasion. In a recently described knockout mouse model for CA IX deficiency, the only phenotypic abnormalities were limited to the gastric mucosa, while no changes were observed in the other tissues known to express CA IX in rats and humans. Here we investigated the expression of CA IX mRNA and protein in mouse tissues. Immunohistochemical (IHC) analysis showed strong staining in the gastric mucosa. Moderate reactions were seen in the colon enterocytes and pancreatic acini. The expression pattern of CA IX was similar in certain human and rodent tissues, although some differences existed, especially in the gut epithelium. Reverse transcriptase PCR analyses surprisingly revealed strong signals for CA IX mRNA in the kidney and skeletal muscle, while the IHC and Western blotting showed no or weak signals for the corresponding protein. This result suggests a tight tissue-specific post-transcriptional control for CA IX expression, possibly related to the physiological demands. **(J Histochem Cytochem 52:1313–1321, 2004)**

KEY WORDS
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Carbonic anhydrases (CAs) form a family of zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide according to the following reaction: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$. The first reaction is catalyzed by CAs and the second part of the reaction occurs instantaneously (Breton 2001). CAs participate in a number of biological processes such as pH regulation, CO_2 and HCO_3^- transport, bone resorption, production of biological fluids, ureagenesis, gluconeogenesis, and lipogenesis (Sly and Hu 1995). The CA isozymes are expressed in different tissues and their kinetic properties and sulfonamide inhibition profiles also vary (Supuran and Scozzafava 2000). They differ in subcellular localizations: CA I, II, III, VII, and XIII are cytoplasmic (Sly and Hu 1995; Lehtonen et al. 2004), CA IV, IX, XII, and XIV are anchored to cell membranes (Sly and Hu 1995; Pastoreková et al. 1997; Türeci et al. 1998; Parkkila et al. 2001), CA VA and VB are found in mitochondria (Fujikawa-Adachi et al. 1999), and CA VI is the only secretory form present in saliva and milk (Kivelä et al. 1999; Karhumaa et al. 2001).

CA IX is composed of four domains: it has an N-terminal proteoglycan domain, a CA catalytic domain, a transmembrane region, and a short cytoplasmic tail (Opavský et al. 1996). CA IX is a sulfonamide-sensitive isozyme with high enzymatic activity, and it is also considered a cell adhesion molecule (Závada et al. 2000; Wingo et al. 2001; Svastová et al. 2003; Vullo et al. 2003). The distribution of CA IX has been studied in human and rat alimentary tracts (Pastoreková et al. 1997). In the human alimentary tract, strong expression of CA IX was detected in stomach and gallbladder. In addition, ileum, colon, liver, and pancreas showed positive expression. Rat alimentary tract showed a slightly different distribution: stomach, colon and duodenum appeared to be positive and liver gave weak positive staining. One of the interesting features of CA IX is its overexpression in human epithelial tumors derived from tissues that normally do not express this isozyme, including carcinomas of uterine cervix, lung, kidney, and breast (Liao et al. 1994; McKiernan et al. 1997; Vermylen et al. 1999; Bartosová et al. 2002). In contrast, tumors originating from CA IX-positive tissues, such as stomach, tend to have

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lowered expression of CA IX (Leppilampi et al. 2003). It is overexpressed in von Hippel-Lindau (VHL)-defective tumors and under hypoxic conditions (Wykoff et al. 2000; Loncaster et al. 2001; Mandriota et al. 2002). In tumors, CA IX has been proposed to maintain extracellular acidic pH, which is conducive to tumor growth and spread (Ivanov et al. 2001).

The aim of this study was to investigate the expression of CA IX in mouse tissues by reverse transcriptase polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry (IHC). This has become an important area of investigation because a knockout mouse model with CA IX deficiency has been recently described (Ortova Gut et al. 2002). The study showed that CA IX deficiency leads to gastric hyperplasia of the glandular epithelium with many cysts. The results suggested that CA IX participates in signal transduction and has an important role in control of cell proliferation, differentiation, and protection of integrity of the stomach mucosa. On the other hand, the lack of CA IX did not have any major physiological consequences on gastric pH regulation or acid secretion, suggesting that other isozymes may compensate for a single enzyme deficiency. However, except for basic data provided therein, no thorough information on the CA IX distribution in mouse tissues is available thus far.

Materials and Methods

Polymerase Chain Reaction (PCR) Method

The expression of CA IX mRNA in mouse tissues was examined using a cDNA kit (MTC panel I) purchased from BD Biosciences (Palo Alto, CA). The mouse MTC panel I contained first-strand cDNA preparations produced from poly A RNAs isolated from 12 different mouse tissues. The first-strand cDNA was the template in the PCR reaction and specific primers for CA IX were used to reveal the tissues containing CA IX mRNA. In addition, primers for mouse β-actin were used to monitor the possible differences in cDNA concentrations.

mRNA was isolated using TRIZOL reagent (Invitrogen; Carlsbad, CA) from the tissues absent from the panel (stomach and gut) as well as from muscle and kidney. Reverse transcription was performed with Mo-MuLV reverse transcriptase (Finnzymes; Espoo, Finland) using random primers (500 μg/ml).

The primers for the PCR reaction were designed by using the published information on CA IX mRNA in GenBank (accession number NM_139305). To produce an amplification product of 873 bp, the forward primer (F1) was 5′-GCTCCAAGATTGAGATC-3′ (nucleotides 894–911) and the reverse primer (R1) 5′-TCTGCCTGCATAGTAAGA-3′ (nucleotides 1749–1766). In the second set of experiments, the forward primer (F2) was 5′-GGAGGCCTGGCAGTTTTGGCT-3′ (nucleotides 794–814) and the reverse primer (R2) 5′-CTCCAGTTTCTGTCATCTCTGCC-3′ (nucleotides 1336–1358). With these primers the PCR amplification product was predicted to be 565 bp. To confirm the results, we also used a third set of primers: (F3) 5′-CTGGGCGTGGATGTTCTGCCC-3′ (nucleotides 212–232) and (R3) 5′-CGAGATTTCTTCCAAATGGGACAG-3′ (nucleotides 863–886), with the resulting PCR product of 675 bp. The primers were produced by Sigma Genosys (Cambridge, UK) and all the other reagents for the PCR reaction were from BD Biosciences except for the dNTP mix, which was from Finnzymes. Five ng of cDNA was used as template. The PCR reaction was carried out on a thermal cycler (Gene Amp PCR system 9700; Applied Biosystems, Foster City, CA) and the protocol consisted of a 94C denaturation step for 1 min followed by 30 cycles of denaturation at 94C for 30 sec, annealing at 55C for 30 sec, and extension at 72C for 1 min 30 sec, followed by a final extension at 72C for 3 min. The control PCR reaction was performed with the following primers for mouse β-actin: the forward primer was 5′-GTTGGCATAGAGGTCTTTACG-3′ and the reverse primer was 5′-GCCGCATCCTCTTCCTCCCT-3′. The control reaction was carried out according to the following protocol: 94C denaturation step for 1 min followed by 30 cycles of denaturation at 94C for 30 sec, annealing at 60C for 30 sec, and extension at 72C for 40 sec, followed by final extension at 72C for 3 min. The results of the PCR reaction were analyzed using a 1.2% agarose gel containing 0.1 μg/ml ethidium bromide with DNA standard (100-bp DNA Ladder; New England Biolabs, Beverly, MA).

Sequencing of the PCR Products

The PCR products from mouse muscle and kidney were sequenced to confirm the presence of the correct amplification product. The PCR products were purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences; Poole, UK) following the protocol of the manufacturer. The sequencing was performed using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reactions Kit, version 2.0 (Applied Biosystems). Five μl of DNA template was mixed with 4 μl of Terminator Ready Reaction Mix (Applied Biosystems) and 1.6 pmol of primer was added to the solution. The sequencing was performed with both forward and reverse primers. The reactions were amplified by cycle sequencing on a thermal cycler (Gene Amp PCR system 9700; Applied Biosystems) according to the manufacturer's instructions. The products were purified by ethanol precipitation, resuspended in Template Suppression Reagent (Applied Biosystems), and denatured according to the manufacturer's protocol. The sequencing was performed with an ABI PRISM Genetic Analyser instrument (Applied Biosystems).

Western Blotting

Samples of stomach, colon, duodenum, jejunum, ileum, brain, cerebellum, heart, liver, kidney, pancreas, lung, spleen, thymus, psoas muscle, testis, and epididymis were obtained from adult mice (Balb/c). The procedures were approved by the institutional animal care committee (University of Tampere). The tissue samples were homogenized in PBS and 50 μg of protein from each sample was analyzed by SDS-PAGE (NuPAGE 10% Bis-Tris; Invitrogen) under reducing conditions.

The separated proteins were transferred electrophoretically from the gel to a polyvinylidene fluoride (PVDF) mem-

brane (Macherey-Nagel; Düren, Germany) in a Novex Xcell II blot module (Invitrogen). The sample lanes were detected by an ECL detection system (Amersham Biosciences). The membranes were blocked with cow colostral whey (Biotop Oy; Oulu, Finland) diluted 1:10 in TBST buffer (20 mM Tris, 500 mM NaCl, 0.3% Tween-20, pH 7.5) for 25 min. The membranes were then incubated with primary antibody diluted 1:5000 in TBST buffer for 1 hr and washed five times for 5 min in TBST buffer. The production of polyclonal rabbit antibody raised against the recombinant mouse CA IX has been described by Ortova Gut et al. (2002). Normal rabbit serum was used for control purposes. The second antibody was horseradish peroxidase-labeled and the rest of the procedure was performed according to the manufacturer's instructions (Amersham Biosciences).

Immunohistochemistry

Immunoperoxidase staining for mouse tissues was performed using the peroxidase–antiperoxidase (PAP) complex method. A polyclonal rabbit antibody against the recombinant mouse CA IX was used to detect CA IX in tissue sections. Normal rabbit serum was used in the control stainings.

Tissue specimens from brain, stomach, duodenum, jejunum, ileum, colon, liver, psoas muscle, kidney, heart, lung, pancreas, spleen, thymus, testis, and epididymis were obtained from two adult mice (Balb/c). The specimens were fixed in Carnoy's fluid (absolute ethanol + chloroform + glacial acetic acid in 6:3:1 ratio) for 20 hr at 4C. The samples were then dehydrated, embedded in paraffin, and 4-μm sections were cut and placed on microscope slides.

Immunostaining was performed according to the following protocol: (a) 3% H_2O_2 in methanol for 5 min; (b) treatment with undiluted cow colostral whey (Biotop) for 30 min and rinsing in PBS; (c) incubation for 1 hr with primary antibody diluted 1:100 in 1% bovine serum albumin (BSA) in PBS; (d) treatment with undiluted cow colostral whey for 30 min and rinsing in PBS; (e) incubation for 1 hr with second antibody (swine anti-rabbit IgG; DAKO, Glostrup, Denmark) diluted 1:100 in 1% BSA in PBS; (f) incubation with peroxidase–antiperoxidase complex (PAP Rabbit; DAKO) diluted 1:100 in PBS for 30 min; (g) incubation for 2 min 30 sec in DAB solution (6 mg 3,3′-diaminobenzidine tetrahydrochloride; Sigma, St Louis, MO) in 10 ml PBS plus 3,3 μl 30% H_2O_2; and (h) counterstaining with Mayer's hematoxylin solution (Sigma). The sections were washed in PBS for 5 min after the step a, 3 times for 10 min after steps c and e, and finally four times for 5 min after step f. All incubations and washings were carried out at room temperature. In addition, each washing was performed on an orbital shaker. Finally, the sections were mounted in Entellan Neu (Merck; Darmstadt, Germany) and then examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss; Göttingen, Germany).

Results

PCR and Sequencing

The expression of CA IX mRNA in mouse tissues was studied using a commercially available cDNA kit as well as the cDNAs reverse-transcribed from mRNA that we isolated from selected mouse tissues. The mRNA was found in the kidney and muscle and weak bands were also seen in the brain, spleen, lung, and liver (Figure 1; Table 1). Heart and testis, as well as 7-day-old, 11-day-old, and 15-day-old embryos were negative. Embryos of 17 days appeared to express CA IX mRNA, which is in accordance with a recent study showing low levels of CA IX protein expression during the first postnatal day (Ortova Gut et al. 2002). In the gastrointestinal tract, mRNA was expressed in the stomach, duodenum, jejunum, ileum, and colon (Figures 1 and 3).

Because only low amounts of CA IX mRNA have been reported in kidney and muscle (Ivanov et al. 2001; Ortova Gut et al. 2002), the PCR products from these organs were further sequenced to confirm their specificity. The automated sequencing was performed using primers designed for both 5′ and 3′ ends and the sequencing clearly confirmed that the obtained 873-bp band represented the correct amplification product.

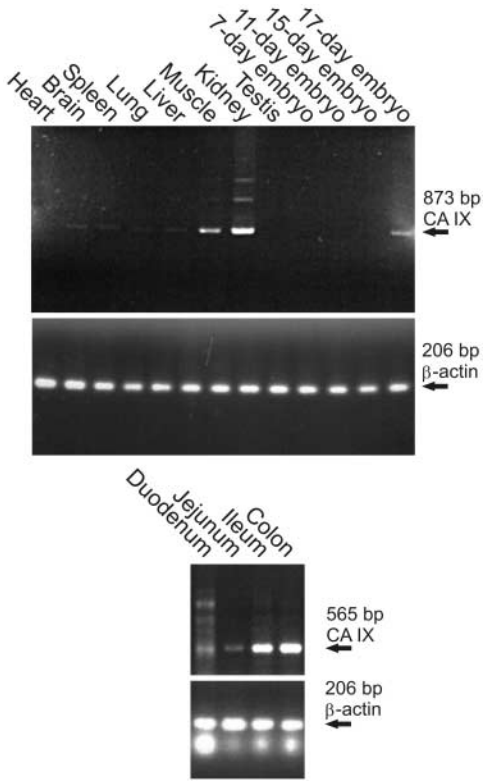

Figure 1 The PCR product of 873 bp is amplified in the kidney and muscle, followed by brain, spleen, lung, and liver. Testis and heart are negative. The expression of CA IX mRNA becomes positive in the 17-day-old embryo. The results in the lower panel show that CA IX mRNA is expressed in the colon and ileum, and weaker bands are also seen in the duodenum and jejunum. The amount of cDNAs produced from each tissue was monitored using primers for β-actin.

Table 1 Expression pattern of CA IX in different mouse tissues[a,b]

Organ	RT-PCR	WB	IHC
Stomach	+	+++	+++
Duodenum	+	−	+
Jejunum	+	−	+
Ileum	+	+	+
Colon	+	++	++
Liver	+	+	−
Pancreas	ND	++	+
Spleen	+	+/−	−
Thymus	ND	+	−
Lung	+	−	−
Skeletal muscle	+	−	+/−
Heart	−	−	−
Brain	+	−	++
Cerebellum	ND	−	++
Epididymis	ND	+	+
Testis	−	+/−	+
Kidney	+	+/−	−

[a]Scores in RT-PCR: − or +; ND, not done.
[b]Scores in Western blotting (WB) and immunohistochemistry (IHC): −, no reaction; +/−, very weak reaction; +, weak reaction; ++, moderate reaction; +++, strong reaction.

Western Blotting

Western blotting was performed to screen CA IX protein expression in 17 different mouse tissues. As expected, the highest signal for this protein was detected in the stomach (Figure 2; Table 1). Colon and pancreas showed moderate positive reactions. Very faint bands were detected in the kidney, liver, thymus, testis, epididymis, ileum, and spleen. Jejunum, duodenum, psoas muscle, heart, lung, brain, and cerebellum showed no CA IX-specific band. Smaller polypeptide bands were observed in the ileum, jejunum, and colon (~37 kD) and in stomach and pancreas (~30 kD). These polypeptides may represent CA IX fragments produced by degradation.

To confirm the differential expression of CA IX mRNA vs protein in the kidney and muscle, the same tissues (excised from two mice) were processed in parallel for RT-PCR and Western blotting (Figure 3). Results of RT-PCR performed with a different pair of primers corresponded well to those obtained in previous analyses and shown in Figure 1. Similarly, a profile of the proteins detected by Western blotting in the same tissues as used for RT-PCR largely agreed with the data in Figure 2. The CA IX-specific protein band was evident in the stomach and colon and was very weak also in the small intestine, but was completely absent from the kidney and muscle.

Immunohistochemistry

Immunoperoxidase staining was performed to localize the expression of CA IX in different tissues and cell types. A polyclonal rabbit antibody for mouse CA IX was used to detect this enzyme and pre-immune serum was used for control purposes. Because CA IX has been reported to be present in the gastric mucosa (Ortova Gut et al. 2002), stomach specimens served as a positive control tissue for the immunostaining. Strong reaction was seen in the basolateral plasma membranes of the mucus-producing surface epithelial cells, chief cells, and parietal cells (Figure 4; Table 1). In the colon, the signal was present in the plasma membrane of the enterocytes and the strongest immunoreaction was localized to the surface epithelial cuff region. In the small intestine, the staining was very weak and present only in sporadic enterocytes. The pancreas showed moderate positive reaction in the acinar cells, in which the staining was generally diffuse, most probably due to rapid protein degradation in the tissue samples (Figure 5). No staining for CA IX was detected in the liver. In the skeletal muscle, only very weak immunoreactions were seen in occasional fibers, while the heart muscle was negative. Faint positive signal was also observed in the epithelial cells of the epididymis and in mature spermatozoa located in the seminiferous tubules. No staining for CA IX was found in the kidney, lung, thymus, and spleen. The antibody also labeled some neuronal axons in the brain and Purkinje cells of the cerebellum (Figure 6).

Discussion

CA IX is an interesting and unique member of the CA gene family with respect to its molecular structure, distribution, and suggested functions. First, it contains

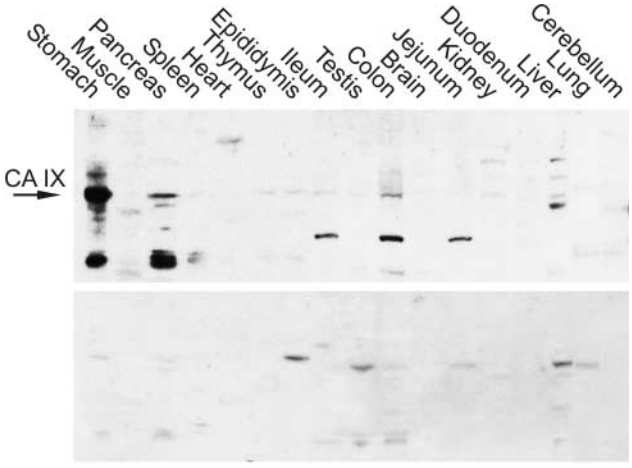

Figure 2 Western blotting of mouse tissues. The strongest 47-kD polypeptide of CA IX is detected in the stomach, followed by pancreas and colon. Very faint bands are seen in the kidney, liver, thymus, testis, epididymis, ileum, and spleen. In addition, strong polypeptides of ~37 kD are present in the ileum, jejunum, and colon and of ~30 kD in the stomach and pancreas. Control staining with normal rabbit serum also shows some nonspecific bands but they all are of different molecular weight compared with CA IX.

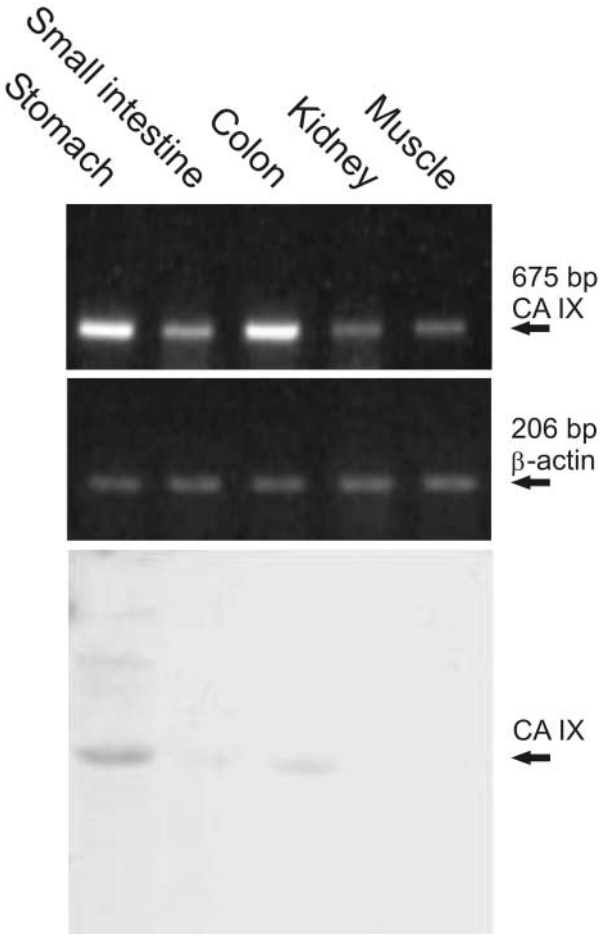

Figure 3 Comparison of the RT-PCR (first panel) and Western blotting (third panel) performed for CA IX in parallel from the same tissues. Note the discrepancy in the signals obtained by Western blotting and RT-PCR in the kidney and muscle.

two structurally distinct domains, a CA domain and a proteoglycan-like domain, which might contribute to different functions (Opavský et al. 1996). Second, human CA IX has been reported to be present in only a few normal tissues and is overexpressed in some carcinomas (Závada et al. 1993). Third, its expression is induced by hypoxia and is downregulated by the product of the wild-type von Hippel-Lindau gene (Ivanov et al. 1998; Wykoff et al. 2000). Fourth, recent studies in CA IX knockout mice have further shown that CA IX is functionally involved in normal gastric morphogenesis, and this feature in particular makes it a very distinctive isoform among the other members of the CA gene family (Ortova Gut et al. 2002).

Even though the expression of CA IX has been quite well established in human and rat tissues (Pastoreková et al. 1997), only little has been known about its expression in mouse. The results obtained in this study (summarized in Table 1) provide a basic overview of CA IX distribution in mouse tissues. Our findings indicate that, among a number of different tissues, gastric mucosa shows the highest expression of this enzyme. The same phenomenon has been described earlier in human and rat tissues. On the basis of both RT-PCR and Western blotting results, the colon and pancreas clearly expressed CA IX. IHC confirmed that colon enterocytes and pancreatic acini were indeed positive for CA IX immunostaining. Some other tissues, including kidney, liver, thymus, testis, epididymis, ileum, and spleen, also showed weak signals for CA IX in the Western blot, but the intensity was very weak, suggesting a low expression of the enzyme in those tissues. The PCR analysis included mouse tissues that were either available in the commercial cDNA kit or obtained from mice in our laboratory. Unexpectedly, the kidney and muscle showed strong positive signal for CA IX mRNA, while the control amplification using β-actin primers produced steady signal in all tissues. Furthermore, the specificity of the positive amplification products was confirmed by DNA sequencing. IHC and Western blotting, however, showed no or very weak reactions for CA IX in the kidney and skeletal muscle fibers. To confirm a differential expression of CA IX mRNA vs protein in the kidney and muscle, we performed RT-PCR and Western blotting analyses from identical tissue specimens. We again found the presence of CA IX mRNA and absence of the corresponding protein, confirming that the discrepancy was not due to inter-animal differences.

These results raise two basic questions: first, why CA IX transcription is activated particularly in the muscle and kidney tissues and, second, why there is a contradiction between expression of mRNA and protein. One possible answer for the first question may be related to the presence of a functional hypoxia-response element in the promoter of the mouse Car9 gene (unpublished results), which may allow transcription of CA IX under conditions of lowered oxygen and/or hypoglycemia, similarly as in human tissues. It is quite conceivable that such conditions may be locally present, at least in the muscle. Although this idea is indirectly supported by our finding of considerably increased levels of PCR product specific for the hypoxia-regulated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in both muscle and kidney (data not shown), definitive evidence requires further investigation. The response to the second question is also unclear but appears to point at a tissue-specific post-transcriptional regulation. As proved by RT-PCR amplification using different pairs of primers designed to cover whole mRNA, CA IX transcripts in kidney and muscle are intact. Therefore, the reason for the absence of corresponding protein does not appear to reside in production of incomplete polypeptide committed to degrada-

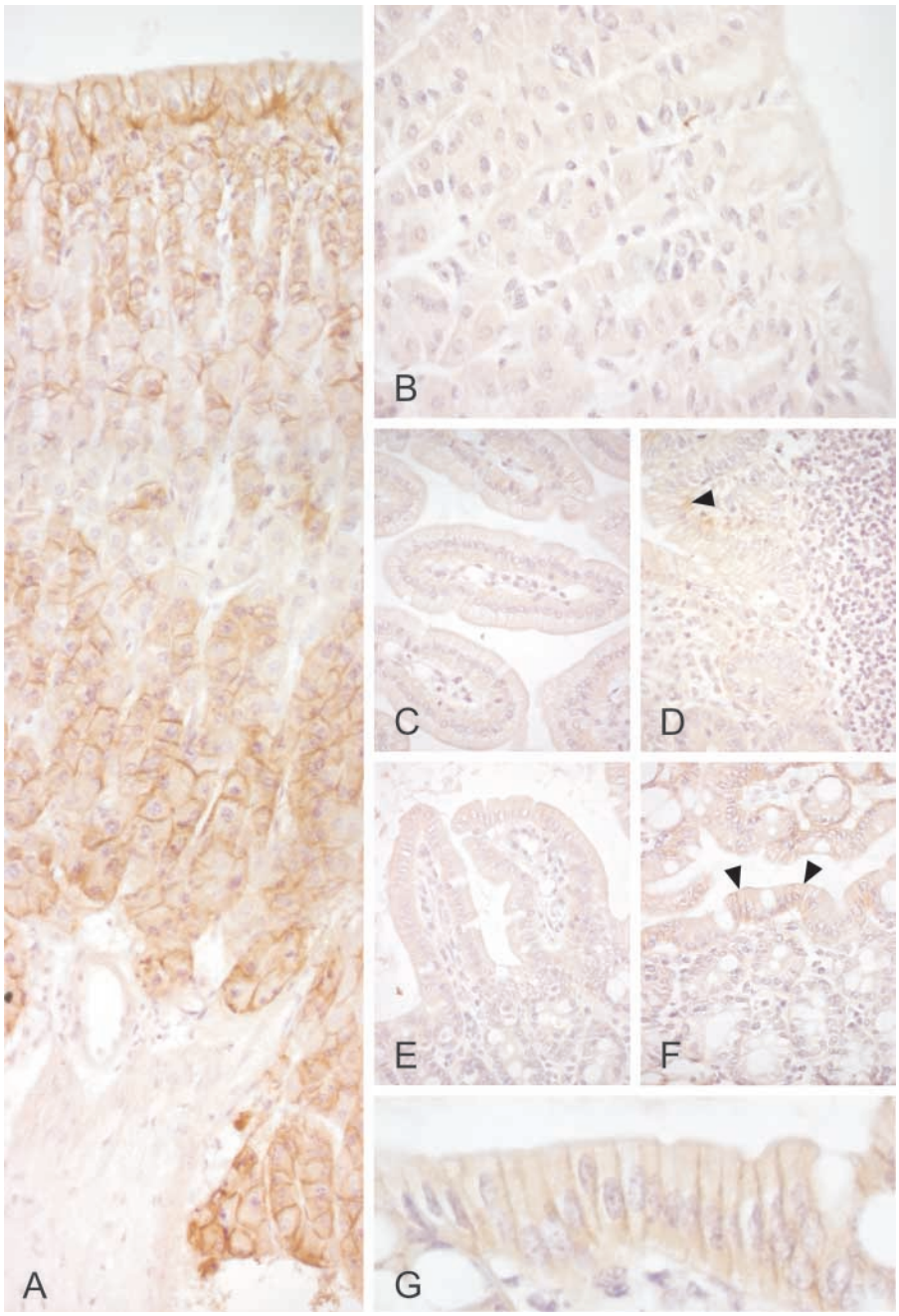

Figure 4 Immunohistochemical staining of CA IX in the mouse gastrointestinal tract. Stomach shows the most intense staining (**A**). Control staining using normal rabbit serum is negative (**B**). Duodenum (**C**), jejunum (**D**), and ileum (**E**) show only very weak signal in sporadic enterocytes (arrowhead in **D**). In colon (**F**), moderate staining is observed in the plasma membranes of the enterocytes in the surface epithelial cuff region (arrowhead). (**G**) A higher-magnification image in which the positive signal is seen more clearly. Original magnifications ×630.

tion or of a truncated secretory form. Our results suggest that the translation efficiency of CA IX mRNA can be very low in certain cell types based on the physiological demands of each tissue. It is noteworthy that normal human kidney and muscle show no or only very weak expression of both CA IX protein and mRNA (Liao et al. 1997; McKiernan et al. 1997; Ivanov et al. 2001). Therefore, an interesting area for future investigations would be exploration of the regulatory mechanisms of CA IX mRNA translation in the murine kidney and muscle.

The present results provided a good opportunity to compare the CA IX distribution pattern among different species. We have previously shown that it is expressed in the rat stomach, duodenum, colon, and biliary tract as well as the human stomach, duodenum, jejunum, ileum, colon, biliary tract, and pancreas (Pastoreková et al. 1997; Saarnio et al. 1998). The distri-

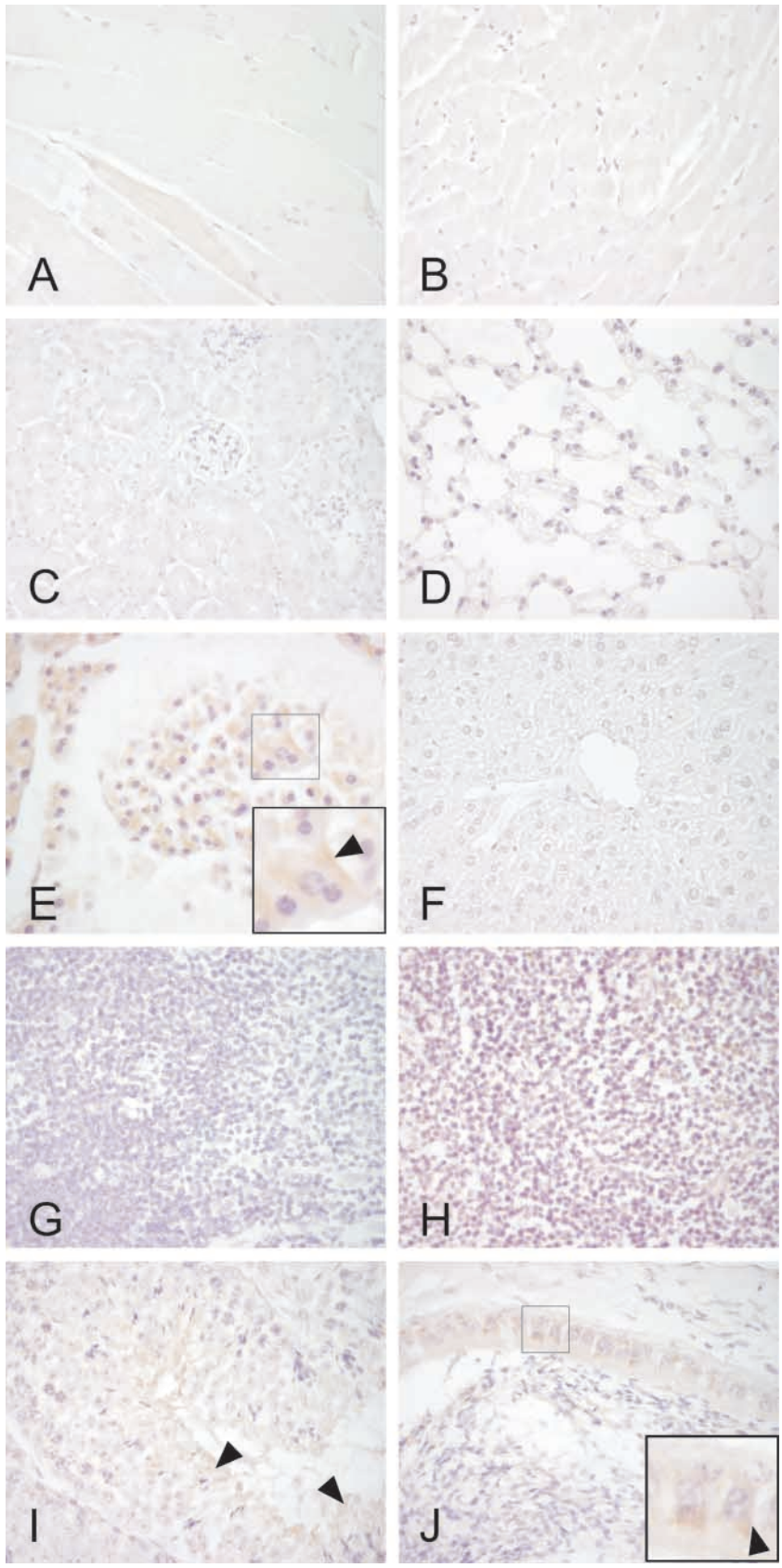

Figure 5 Immunohistochemical staining of CA IX in different mouse tissues. Psoas (**A**) shows occasional weakly stained muscle fibers, while heart (**B**) is completely negative. Kidney (**C**) and lung (**D**) are negative. Pancreas (**E**) shows moderate staining in acinar cells (arrowhead). Liver (**F**), thymus (**G**), and spleen (**H**) are negative. In testis (**I**), faint staining is seen in the most luminal spermatozoa present in the seminiferous tubules. Weak positive reactions are also present in the epithelial cells of the epididymis (arrowhead in **J**). Original magnifications: **A,E,G–J** ×630; **B–D,F** ×400.

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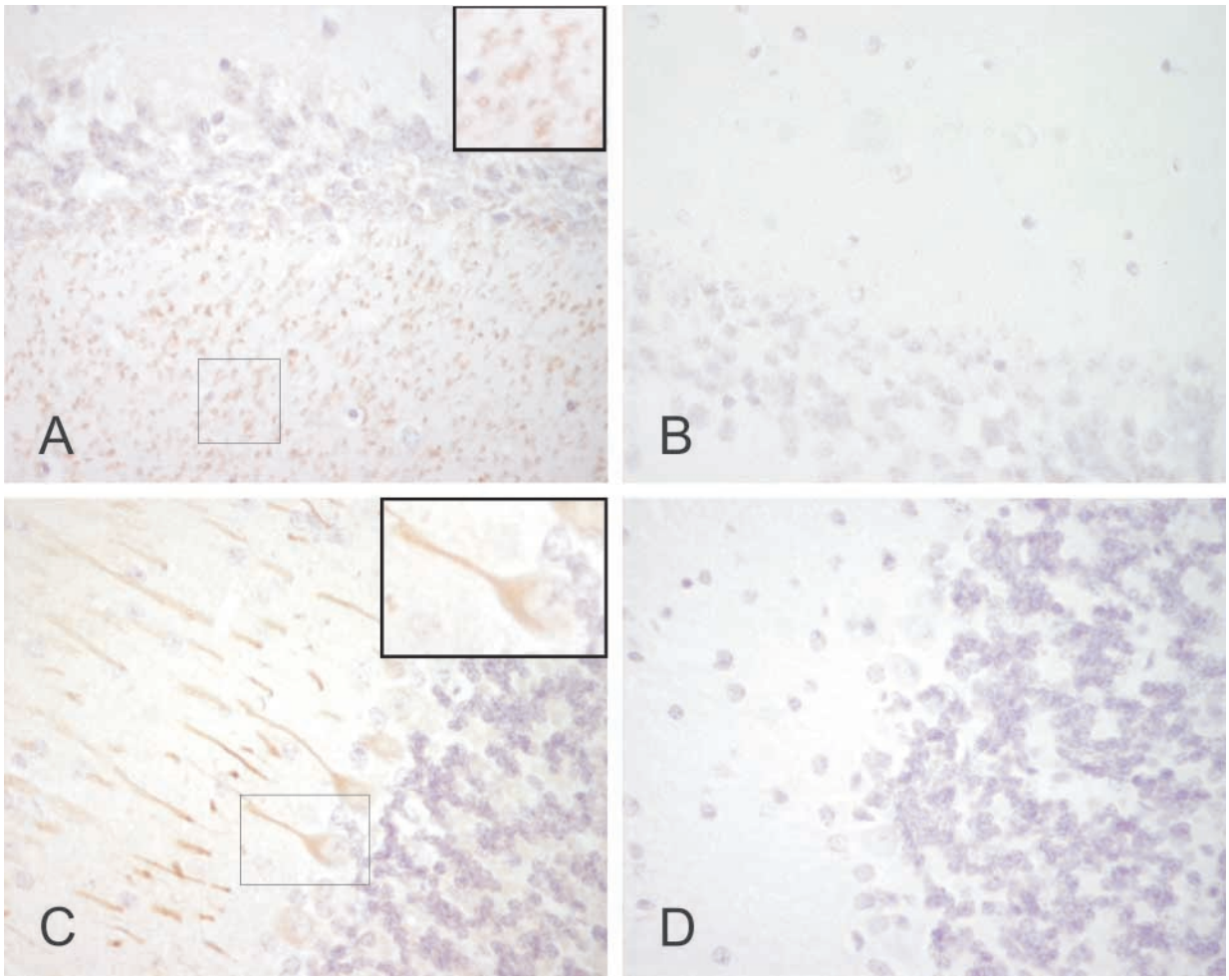

Figure 6 Immunostaining of CA IX in the mouse brain. Some neuronal axons are stained in the cerebrum (**A**), while the control staining shows no reaction (**B**). In cerebellum (**C**), the axons originating from the Purkinje cells and the cell body show positive staining. Control staining is negative (**D**). Original magnifications ×630.

bution in the mouse gastrointestinal tract was quite similar to that of the rat and human tissues. In all species, gastric mucosa appeared to contain the highest levels of CA IX. Therefore, it is not surprising that CA IX knockout mice showed a distinct gastric phenotype with epithelial cell hyperplasia and cystic changes in the mucosa (Ortova Gut et al. 2002). Based on the previous and present studies, CA IX is expressed in the colon of all these species. Nevertheless, CA IX knockout mice exhibited no intestinal phenotype that might be partly explained by much lower level of expression in the gut compared with the gastric mucosa. It is also notable that CA IX immunostaining clearly shows different regional distribution in the rodent vs human colon (Pastoreková et al. 1997). In the rat and mouse colon, the enzyme is located in the most superficial part of the mucosa, called the epithelial cuff region. In contrast, the human colon shows positive signal only in the crypt enterocytes. This finding may reflect important differences in colon physiology among various species with respect to the function of CA IX. The present results also showed a moderate immunoreaction for CA IX in the pancreatic acini. This finding is in line with the previous observation in the human pancreas showing positive staining in the acinar cells (Kivelä et al. 2000). The major difference is that CA IX is restricted to occasional acini in the human pancreas, whereas the mouse pancreas shows a more diffuse staining.

The IHC also provided evidence that CA IX might be expressed in some neuronal axons and Purkinje cells. Weak signal was also observed for CA IX mRNA in the brain, while the Western blots of the brain and cerebellum remained negative, possibly due to lower detection sensitivity. We have previously demonstrated the presence of CA XIV in the human

and mouse brain (Parkkila et al. 2001). The present findings suggest that CA IX might be a second membrane-associated isozyme specifically expressed in some neurons. Similar to our present findings, CA XIV showed the highest expression in the axon membrane. The membrane-bound CA activity has been proposed to participate in the production of an alkaline shift linked to neuronal signal transduction (Parkkila et al. 2001). It can be hoped that future studies will unravel the contribution of each CA isozyme to neuronal function.

Acknowledgments

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