

Carbonic anhydrase gene expression in CA II-deficient (*Car2*^{-/-}) and CA IX-deficient (*Car9*^{-/-}) mice

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Using real-time PCR and immunohistochemistry, we have examined the expression of carbonic anhydrase isozymes (CA) I, II, III, IV, IX, XII, XIII and XIV in the brain, kidney, stomach and colon of the wild-type, CA II-deficient (*Car2*^{-/-}), and CA IX deficient (*Car9*^{-/-}) mice. The expression of *Car4*, *Car12*, *Car13* and *Car14* mRNAs did not show any significant deviations between the three groups of mice, whereas both groups of CA deficient mice showed decreased expression levels of *Car1* in the colon and *Car3* in the kidney. The *Car2* mRNA level was greatly reduced but not completely abolished in all four tissues from the *Car2*^{-/-} mice in which no CA II protein was expressed. Sequencing the *Car2* cDNA isolated from C57BL6 *Car2*^{-/-} mice revealed two nucleotide differences from the wild-type C57BL6 mice. One is a silent polymorphism found in *Car2* mRNA from wild-type DBA mice, which is the strain that provided the original mutagenized chromosome. The second change is a mutation that causes prematurely terminated translation at codon 155 (Gln155X). *Car9* mRNA and CA IX protein expression levels were up-regulated about 2.5- and 3.6-fold, respectively, in the stomach of the *Car2*^{-/-} mice. These results suggest that the loss of function of cytosolic CA II in the stomach of *Car2*^{-/-} mice leads to up-regulation of an extracellular CA, namely CA IX, which is expressed on the cell surface of the gastric epithelium.

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Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that catalyse the reversible hydration of carbon dioxide in the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$, and participate in various biological processes, including CO_2 transport, regulation of pH homeostasis, bone resorption, ureagenesis, gluconeogenesis, production of body fluids, and fertilization (Sly & Hu, 1995; Parkkila, 2000; Lehtonen *et al.* 2004; Kivela *et al.* 2005a). The mammalian α -CA family is comprised of 16 different isoforms, among which 13 (CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV and XV) are enzymatically active, whereas the other three (CA-RP VIII, X and XI) appear to lack CA activity because of substitutions in one or more of the functionally important histidine residues (Sly & Hu, 1995; Tashian *et al.* 2000; Lehtonen *et al.* 2004; Hilvo *et al.* 2005; Kivela *et al.* 2005a). In addition, the receptor-type protein-tyrosine phosphatases (RPTP) β and γ have also been reported to contain 'CA-like' domains. The 13 active CA isozymes differ in their subcellular localizations: CA

I, II, III, VII and XIII are cytosolic, CA IV, IX, XII, XIV and XV are membrane associated, CA VA and VB are mitochondrial, and CA VI is secreted.

The expression of CAs in mammalian tissues has been intensively studied during the past three decades by means of reverse transcription polymerase chain reaction (RT-PCR), Northern blot, Western blot and immunohistochemical staining (Kumpulainen & Korhonen, 1982; Fleming *et al.* 1995; Ivanov *et al.* 1998; Saarnio *et al.* 1998; Parkkila, 2000; Ivanov *et al.* 2001; Wykoff *et al.* 2001; Leppilampi *et al.* 2003; Kivela *et al.* 2005b). These studies have provided important background information to understanding the roles of different isozymes in each type of tissue. The most distinctive features of CA expression are: (1) CA II appears to be the most widely expressed isozyme which is present in all major mammalian organs. As a high-activity isozyme, it may represent the most important isozyme for several fundamental biological processes (Sly & Hu, 1995; Parkkila & Parkkila, 1996); (2) two CA isozymes, CA IX and XII, are called

cancer-associated isozymes due to their overexpression in certain carcinomas (Pastorekova *et al.* 2004; Pastorekova & Zavada, 2004); (3) multiple isozymes are often expressed in a particular mammalian tissue or organ, e.g. the murine kidney expresses at least CA II, IV, XII, XIII, XIV and XV (Brion *et al.* 1997; Kaunisto *et al.* 2002; Kyllonen *et al.* 2003; Lehtonen *et al.* 2004; Hilvo *et al.* 2005). The co-expression of several isozymes in different cell types and in different subcellular localizations in one organ suggests different functions for the different isozymes, which may or may not be redundant in a given tissue. Loss of one may lead to a compensatory up-regulation of another. Such compensatory mechanisms have never been studied thoroughly. In this study, we used quantitative real-time RT-PCR and immunohistochemical staining to assess the expression levels of CA isozymes I, II, III, IV, IX, XII, XIII and XIV in the brain, kidney, stomach and colon of the wild-type, CA II-deficient (*Car2*^{-/-}), and CA IX-deficient (*Car9*^{-/-}) mice.

Methods

The experimental mice

The brain, stomach, colon and kidney samples were collected from five wild-type (WT), three *Car2*^{-/-}, and four *Car9*^{-/-} mice. The C57BL/6 *Car2*^{-/-} mice were originally generated by chemical mutagenesis (Lewis *et al.* 1988; Spicer *et al.* 1989). The *Car9*^{-/-} mice were recently produced by targeted disruption of *Car9* gene as described in Ortova Gut *et al.* (2002). These mice were introduced into the animal facility of Oulu University by embryo transfer, backcrossed 10 generations (F10) to the C57BL/6J strain, and then heterozygous mice were intercrossed to produce mice homozygous for the targeted gene. The mice were fed with standard feed (R36 for mice and rats, Lactamin, Stockholm, Sweden), and housed in pathogen-free conditions. The study protocols were approved by the Animal Care Committee of the Oulu University.

Determination of *Car9* genotype

DNA was isolated from mice ear markings using NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) DNA extraction kit. *Car9* gene was amplified with PCR using Reddy Mix PCR Master Mix (ABgene, Epsom, UK). The template DNA for each reaction was 150 ng. The primers were: 5'-CCAGTCAGCTGCATGGCC-3' and 3'-AGGAGCCTCGGGAGTCGA-5' for the WT allele, and 5'-AGGAGCAAAGCTGCTATTGG-3' and 3'-AGGAGCCTCGGGAGTCGA-5' for the targeted *Car9* allele. The primers were chosen from the first exon that was disrupted in the knockout mice (Ortova Gut *et al.* 2002). The PCR program was: 96°C for 5 min, 35 cycles of: 96°C

for 30 s, 56°C for 60 s, 72°C for 60 s. The PCR products were characterized in 1.2% agarose (LE, analytical grade, Promega Madison, WI, USA) gel containing 0.005% nucleic acid gel stain GelStar (BMA, Rockland, ME, USA) and visualized by UV light.

Determination of *Car2* genotype

The *Car2* genotypes were determined by phenotypic analysis of the mice. CA activity was assayed from the blood sample (treated with EDTA, diluted 1 : 5000) with the imidazole-tris technique (Brion *et al.* 1988). Since CA II constitutes the major fraction of CA activity of the blood, the test is considered reliable in monitoring CA II activity, and therefore, the *Car2* genotype.

RNA extraction and first strand cDNA synthesis

All tissue samples used for quantitative real-time PCR were stabilized in RNAlater (Ambion, Austin, TX, USA) and stored at -80°C until use. Total RNA was isolated from tissue samples using RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm. RNAs were pooled for each group (wild-type, *Car2*^{-/-} and *Car9*^{-/-}) to eliminate the potential individual variation. Three micrograms of total RNA was converted into first strand cDNA using First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada), utilizing random hexamers, according to the protocol recommended by the manufacturer.

Quantitative real-time PCR

The amount of mouse *Car1*, *Car2*, *Car3*, *Car4*, *Car9*, *Car12*, *Car13* and *Car14* transcripts in different tissues was assessed by real-time RT-PCR using the Lightcycler detection system (Roche, Rotkreuz, Switzerland). Real-time PCR primers were designed based on the complete cDNA sequences deposited in GenBank (accession numbers: NM_009799 for *Car1*, NM_009801 for *Car2*, NM_007606 for *Car3*, NM_007607 for *Car4*, NM_139305 for *Car9*, NM_178396 for *Car12*, NM_024495 for *Car13*, and NM_011797 for *Car14*). To avoid amplification of contaminating genomic DNA, the two primers of all primer sets were placed in different exons. β -Actin was used as an internal RNA control to normalize the RNA samples for differences (Chtanova *et al.* 2001). The primer sequences are shown in Table 1.

Every PCR was performed in a total reaction volume of 20 μ l containing 0.5 μ l of first strand cDNA, 1 \times QuantiTect SYBR Green PCR Master Mix (Qiagen), and 0.5 μ M of each primer. Amplification and detection were

Table 1. Primer sequences for real-time RT-PCR (T_m: annealing temperature)

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	PCR product size (bp)	T _m (°C)
β -actin	AGAGGGAAATCGTGCCTGAC	CAATAGTGATGACCTGGCCGT	138	57
Car1	TTGATGACAGTAGCAACC	CCAGTGAAGTAAGTGAAG	161	51
Car2	CAAGCACAACGGACCAGA	ATGAGCAGAGGCTGTAGG	122	56
Car3	GCTCTGCTAAGACCATCC	ATTGGCGAAGTCGGTAGG	160	54
Car4	CTC CTT CTT GCT CTG CTG	GAC TGC TGA TTC TCC TTA	145	55
Car9	CTGAAGACAGGATGGAGAAG	GCAGAGTGCGGCAGAATG	221	57
Car12	CCTATGTTGGTCCTGCTG	CGTTGTAACCTTGGAACTG	143	53
Car13	AATACGACTCCTCACTCC	TGCCGCAACTGTAGTTC	116	52
Car14	TGTTGTTCTTCGCTCTCTCTG	CACTGTCTGTCTGGATATTG	161	53

carried out as follows. After an initial activation step of 15 min at 95°C, amplification was performed in a three-step cycling procedure: denaturation at 95°C, 15 s, ramp rate 20°C s⁻¹; annealing at T_m, 20 s, ramp rate 20°C s⁻¹; and elongation at 72°C, 20 s, ramp rate 20°C s⁻¹ for 45 cycles and a final cooling step. The melting curve analysis was always performed after the amplification to check PCR specificity. To quantify the concentration of β -actin and the eight CA transcripts in the four tissues of this study, a standard curve for each gene was established using 5-fold serial dilutions of known concentrations of purified PCR products generated from the same primer sets. Each cDNA sample was tested in duplicate and the crossing point (C_p) value obtained allowed the determination of the amount of the starting message using the specific standard curve. The final real-time PCR result was indicated as the copies of target gene per 1000 copies of β -actin.

Sequencing of Car2 full-length cDNA

Primers were designed to amplify the full-length cDNA from stomach samples of wild-type and CA II-deficient mice according to the sequence of Car2 cDNA (accession numbers: NM_009801). The primer sequences were as follows: F: 5'-CAAGCACAACGGACCAGA-3', R2: 5'-TGTTGGTATGTTATCTGAAGG-3'. Amplification was carried out in a 50 μ l reaction containing 1 \times Extensor Hi-Fidelity PCR Master Mix (Abgene), 0.2 μ M of each primer, and 1 μ l of cDNA. The PCR profile was: 94°C denature for 2 min followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 57°C for 30 s, and extension at 68°C for 1 min, followed by a final extension at 68°C for 2 min. The 1386 bp PCR fragment was purified with the GFX PCR DNA and Gel Band purification Kit (Amersham Biosciences, Buckinghamshire, UK) and cloned into the pGEM-T vector (Promega) according to the instructions of the manufacturer. The plasmid DNA was purified using QIAprep Spin Miniprep kit (Qiagen) and then used as a template in the sequencing PCR. T7 (5'-TAATACGACTCACTATAGGCG-3'), Sp6

(5'-ATTTAGGTGACACTATAGA-3') and Car2-specific primers (F1: 5'-TGAAGAAGCGATGGTGGGA-3', R1: 5'-TACAGAGAGGCGGTCACA-3') were used for the sequencing PCR. The sequencing PCR reaction contained 4 μ l of ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Mix v. 3.1 (Applied Biosystems, Foster City, CA, USA), 0.16 μ M of primer, and 200–500 ng of plasmid DNA, and the PCR profile was 25 cycles of denaturation at 94°C for 10 s, annealing at 50°C for 5 s, and extension at 68°C for 10 min.

Immunohistochemistry and digital image analysis

The tissue specimens from the wild-type, *Car2*^{-/-} and *Car9*^{-/-} mice were fixed in 4% neutral-buffered formaldehyde at +4°C for 8–27 days. The samples were then dehydrated in an alcohol series, treated with xylene, embedded in paraffin wax, and 4 μ m sections were cut and placed on Superfrost microscope slides.

The immunohistochemical staining was performed for CA II, IV, IX, XII and XIV, but only CA II, IX and XII antigens were preserved well in the fixation conditions used. The rabbit polyclonal antibodies for each isozyme have been described and used previously in the following studies: anti-CA II (Lehtonen *et al.* 2004), anti-CA IV (Brion *et al.* 1997), anti-CA IX (Ortova Gut *et al.* 2002), anti-CA XII (Kyllonen *et al.* 2003), and anti-CA XIV (Parkkila *et al.* 2001).

After removal of paraffin, the sections were immunohistochemically stained using automated Laboratory Vision Autostainer 480 and Power Vision⁺ Poly HRP IHC Kit (both from ImmunoVision Technologies, Co., Brisbane, CA, USA) reagents according to the following procedure: (1) rinsing with 1 \times tris-buffered saline (TBS) containing 0.05% Tween; (2) blocking with 3% H₂O₂ for 5 min and rinsing with 1 \times TBS containing 0.05% Tween; (3) incubation in 5 times diluted cow colostrum whey for 30 min and rinsing with 1 \times TBS containing 0.05% Tween; (4) incubation with primary antibody diluted 1: 2000 for 30 min and rinsing with 1 \times TBS containing 0.05% Tween for 3 \times 5 min; (5) incubation

with Poly HRP anti-mouse/rabbit IgG polymer for 30 min and rinsing with $1 \times$ TBS containing 0.05% Tween for 3×5 min; (6) treatment with $1 \times 3,3'$ -diaminobenzidine tetrahydrochloride (DAB) solution for 6 min and rinsing with distilled water. The slides were then examined and photographed using a Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany). The images photographed with $200 \times$ magnification were subjected to digital image analysis. The staining extent was analysed with analySIS software (Soft Imaging System GmbH, Münster, Germany). From the stomach, eight and six rectangular regions were scored for the wild-type and *Car2*^{-/-} mice, respectively, using colour threshold values of 145. Each analysed region ($700 \mu\text{m} \times 550 \mu\text{m}$) covered the whole thickness of the gastric mucosa from the surface to the base of the glands. The relative area value obtained indicates the mean percentage of stained area within the analysed regions. An unpaired Student *t* test was used to evaluate the significance of differences in protein expression in different groups of mice detected by immunohistochemical staining and digital image analysis.

Results

Expression of CA transcripts in tissues of wild-type mice

In this report, the expression levels of Car1, Car2, Car3, Car4, Car9, Car12, Car13 and Car14 mRNAs were examined by quantitative real-time RT-PCR. As shown in Fig. 1, each of the CA isozymes exhibited a distinct expression profile between different tissues of the wild-type mice. Car2 mRNA was expressed in all four tissues studied and represented the most abundant CA mRNA in the brain and stomach. This similar broad expression pattern was found for the Car13 mRNA. It was expressed in all four tissues (high levels in the colon and less in the brain, kidney and stomach). Car4 also showed a broad expression profile. Its mRNA was detected in the

brain, colon and kidney (higher levels in the colon and kidney). Car1, Car3 and Car9 mRNAs exhibited clearly different expression patterns. Car1 mRNA was detected only in the colon where it was actually the highest CA transcript. Car3 mRNA was highly expressed in the kidney and less in the stomach. Car9 mRNA showed the strongest signal in the stomach and was negligible in the brain, kidney and colon. Positive expression of Car12 mRNA was detected in three tissues. The signal was highest in the kidney and very low in the brain and colon. Car14 mRNA showed a very high signal in the kidney, and in fact, it was the most abundant CA mRNA in this particular organ. Car14 mRNA was also expressed in the brain and stomach, although the expression levels were markedly lower in these tissues than in the kidney.

Expression of CA transcripts in tissues of *Car2*^{-/-} and *Car9*^{-/-} mice

The expression of Car1, Car2, Car3, Car4, Car9, Car12, Car13 and Car14 mRNAs in tissues of *Car2*^{-/-} and *Car9*^{-/-} mice in comparison with wild-type mice is shown in Fig. 2. Interestingly, the Car1 (Fig. 2A) and Car3 (Fig. 2C) mRNA levels were down-regulated in the colon and kidney, respectively, of *Car2*^{-/-} and *Car9*^{-/-} mice. It is noteworthy that all the transcripts showed some variability between different groups of mice analysed. However, the Car2 mRNA was very clearly decreased in all tissues of *Car2*^{-/-} mice as compared with the wild-type and *Car9*^{-/-} mice (Fig. 2B). It is also notable that the expression of Car9 mRNA was markedly up-regulated in the stomach of *Car2*^{-/-} mice (Fig. 2E), whereas this effect was not observed in the brain, colon or kidney.

Car2 mRNA in *Car2*^{-/-} mice

CA II-deficient mice were originally produced by random mutagenesis induced by *N*-ethyl-*N*-nitrosourea treatment

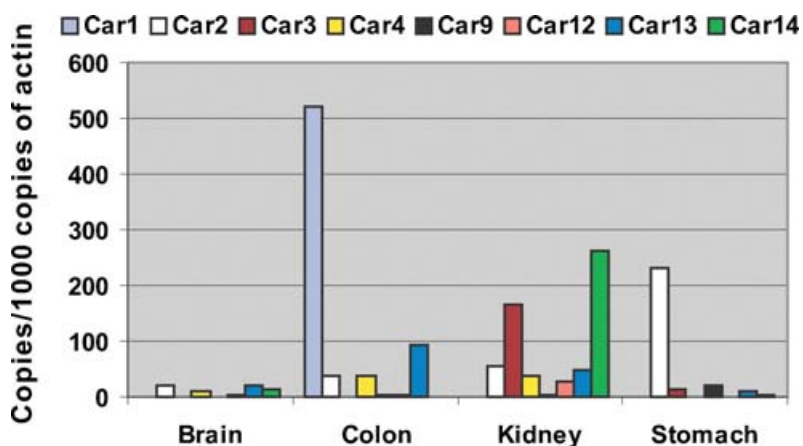


Figure 1. Expression of Car1, 2, 3, 4, 9, 12, 13 and 14 mRNAs in the wild-type mice detected by real-time RT-PCR

Note: Car1 mRNA in colon is expressed as copies/100 copies of actin.

of male DBA mice (Lewis *et al.* 1988). CA II-deficient mice produced by this method have been shown to lack CA II activity and protein. However, the only report of the mutational basis of this deficiency is a review citing unpublished data (Sly & Hu, 1995). In the present study, real-time RT-PCR analysis revealed a significantly decreased *Car2* mRNA expression in the brain, kidney, stomach and colon of the *Car2*^{-/-} mice as compared with the wild-type and *Car9*^{-/-} mice (Fig. 2B). Immunohistochemical staining confirmed the absence of CA II protein in all the tissues of *Car2*^{-/-} mice observed in the present study (data not shown).

As *Car2* mRNA expression was not completely absent in the CA II-deficient mice, full-length cDNAs could be amplified from the mRNAs obtained from the stomach samples of the wild-type and *Car2*^{-/-} mice. The cDNAs were cloned and the resultant plasmids sequenced. Comparing the sequences revealed two nucleotide variations in the *Car2*^{-/-} mice from the sequence obtained from *Car2* mRNA from C57BL6 mice. The first was at nucleotide position 145 (G→C), which caused no change in amino acid sequence. Sequencing the mRNA from the DBA mouse strain revealed that this is a polymorphism present in the DBA mouse and not an

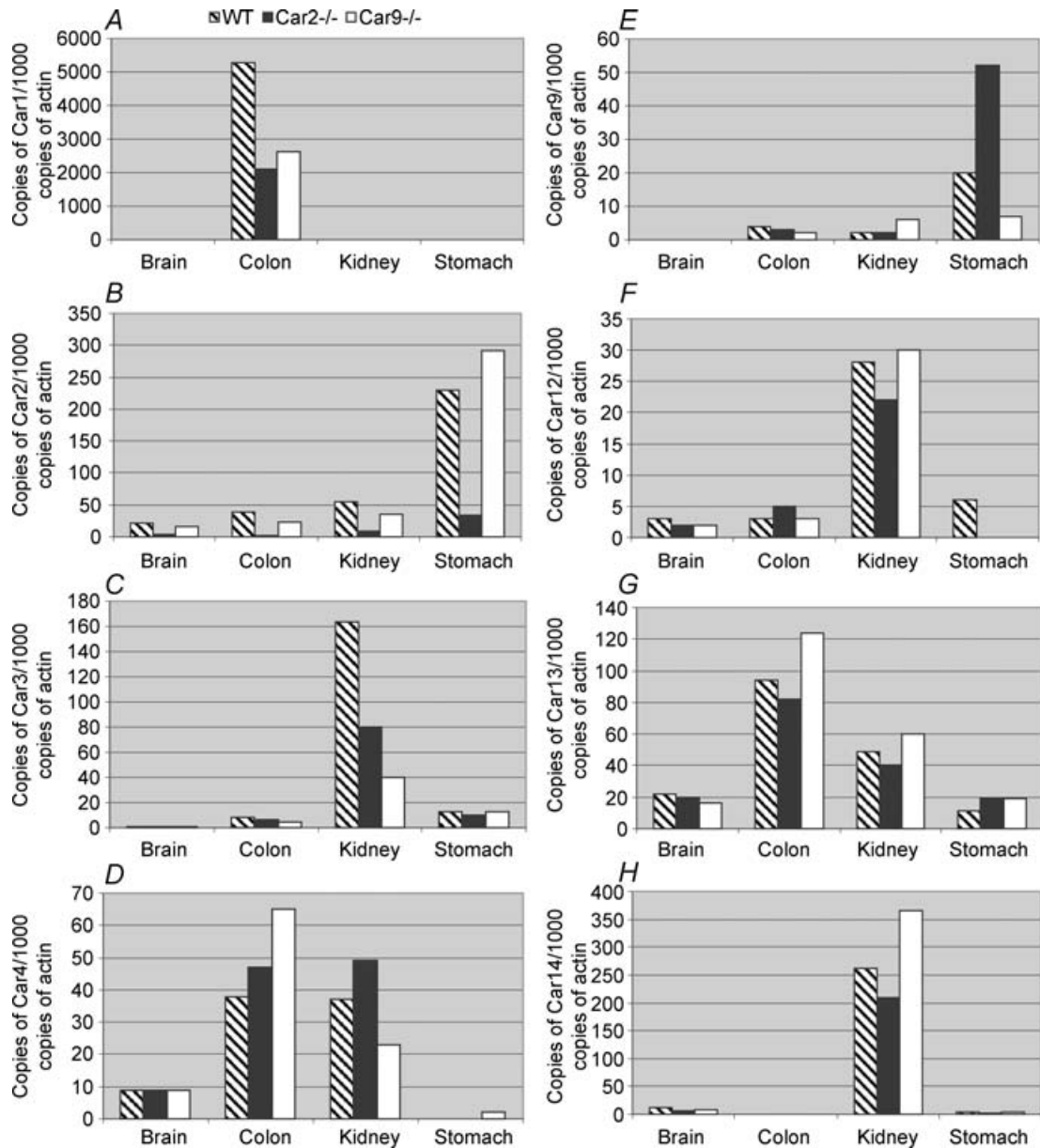


Figure 2. Expression of *Car1*, *2*, *3*, *4*, *9*, *12*, *13* and *14* mRNAs in the wild-type, *Car2*^{-/-} and *Car9*^{-/-} mouse tissues

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WT Car2  KHNGPENWHKDFPIANGDRQSPVDIDTATAHHPALQPLLI SYDKAASKSIVVNGH SFNV
M Car2   KHNGPENWHKDFPIANGDRQSPVDIDTATAHHPALQPLLI SYDKAASKSIVVNGH SFNV

WT Car2  EFDDSDQNAVLKGGPLSDSYRLIQFHFHWGS SDGQ GSEHTVNK KKYAAELHLVHWNTKYG
M Car2   EFDDSDQNAVLKGGPLSDSYRLIQFHFHWGS SDGQ GSEHTVNK KKYAAELHLVHWNTKYG

WT Car2  DFGKAVQQPDGLAVLGIFLKIGPASQGLQKVLEALHSIKTKGKRAAFANFDP C SLLPGNL
M Car2   DFGKAVQQPDGLAVLGIFLKIGPAS*****

WT Car2  DYWTYPGSLTTPPLLECVTWIIVLREPI TVSSEQMSHFRTLNFNEEGDAEEAMVDNWRPAQ
M Car2   *****

WT Car2  PLKNRKIKASFK
M Car2   *****

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Figure 3. Alignment of the wild-type and mutant mouse CA II sequences
WT, wild-type mice; M, CA II-deficient mice. Asterisks indicate untranslated amino acids.

induced mutation. The second difference was at position 508 (C→T), which produced a prematurely terminated protein at codon 155 (Gln155X). Figure 3 shows the alignment result of the amino acid sequences translated from the two groups.

Car9 mRNA and CA IX protein content is up-regulated in the stomach of *Car2*^{-/-} mice

The results summarized in Fig. 4 indicate that the expression of Car9 mRNA was increased about 2.5-fold in the stomach of *Car2*^{-/-} mice in comparison with wild-type. To determine whether the elevated mRNA expression resulted in an increased expression of CA IX protein, immunohistochemical staining was carried out for the same tissue specimens. The results presented in Fig. 5A and B show that CA IX protein was, indeed, more widely expressed in the *Car2*^{-/-} mice compared with the wild-type mice. In the *Car2*^{-/-} mice, the strongly positive area covered the whole mucosa from the deep

gastric glands to the most superficial part. In the wild-type mice, the expression was high in the superficial part of the mucosa involving the mucus-producing epithelial cells, while weaker reactions were detected in the gastric glands. Figure 5C shows the digital image analysis results based on the immunohistochemical staining of CA IX. These results confirmed that the extent of CA IX protein staining was significantly ($P = 0.0022$) increased (about 3.6-fold) in the stomach of the *Car2*^{-/-} mice as compared with the wild-type mice.

Discussion

A number of studies have examined the expression of different CA isozymes in mammalian tissues (Fleming *et al.* 1995; Kivela *et al.* 2005a; Purkerson & Schwartz, 2005). These investigations provided important background information for later functional studies using CA inhibitors and various cell culture and animal models. Previous studies made clear that several isozymes could be identified in the same mammalian tissue or organ. In many cases, the different isozymes were expressed in different cell types in these tissues and/or in different subcellular localizations. Examples of tissues which express multiple CA isozymes include the four tissues selected for the present investigation.

The purpose of this study was to determine whether loss of function of CA II or CA IX in the respective knockout mouse models leads to compensatory changes in other CAs. The mRNA expression of Car4, Car12, Car13 and Car14 did not show any significant changes in the brain, colon, stomach and kidney of either the *Car2*^{-/-} or *Car9*^{-/-} mice. The unchanged level of CA XII protein expression in these tissues as examined by immunohistochemical staining confirmed the lack of compensatory change in this isozyme (data not shown). In a previous study, CA IV protein levels were reported to be up-regulated in the central nervous system of the *Car2*^{-/-}

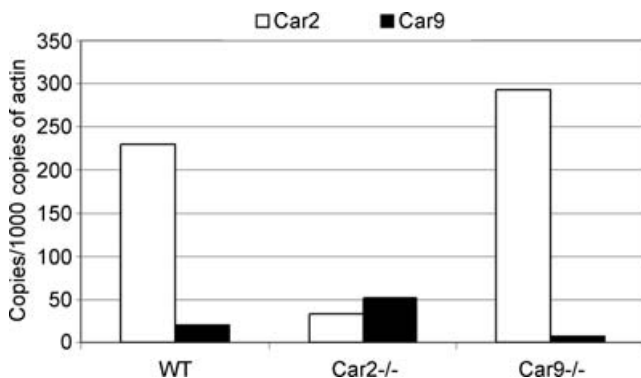


Figure 4. Expression of Car2 and Car9 mRNAs in the stomach of the wild-type, *Car2*^{-/-} and *Car9*^{-/-} mice

Car9 mRNA expression was increased in the stomach of the *Car2*^{-/-} mice. Also the signal for Car2 mRNA was slightly elevated in the *Car9*^{-/-} mice.

mice (Brion *et al.* 1994). However, no such alteration in the expression of Car4 mRNA in the brain of *Car2*^{-/-} mice was detected in the present study. We were unable to document the protein levels of CA IV by immunohistochemistry since the antigenicity for CA IV was lost in these samples due to fixation.

The expression of Car1 mRNA in the colon of wild-type mice was extremely high in comparison with other isozymes. Car3 mRNA level was also high in the kidney of wild-type mice. Interestingly, both groups of CA-deficient mice showed a down-regulation of Car1 and Car3 mRNAs in the colon and kidney, respectively. The mechanism for this change is unclear, although it may simply reflect a disturbed pH regulation in these organs due to CA II or CA IX deficiency.

A somewhat surprising finding was the high basal expression of Car14 mRNA in the kidney relative to the mRNAs for the other CAs in the wild-type mice. The relative copy number of Car14 mRNA was approximately 5 or 10 times higher than those of Car2 or Car12, respectively. This finding suggests an important role of CA XIV in mouse kidney function. Recent studies have also indicated that CA XIV has even higher CA activity than murine CA II (Whittington *et al.* 2004). The same report indicated that CA XIV is nearly five times as active as murine CA IV. These results add further weight to the idea that CA XIV is probably a very important enzyme for renal physiology in the mouse.

Comparing the full-length Car2 cDNAs from the wild-type C57BL and *Car2*^{-/-} mice revealed two nucleotide changes in the cDNA from the *Car2*^{-/-} mice. One is the chemically induced mutation that causes an early stop codon in the Car2 transcript. This nonsense mutation at codon 155 (Gln155X) would account for the absence of CA II protein in the *Car2*^{-/-} mice. It is also likely that the reduced level of mRNA in the *Car2*^{-/-} mice is explained by the nonsense-mediated decay mechanism, which has been well characterized (Conti & Izaurralde, 2005). The original report of the *Car2*^{-/-} mouse detected no difference in the expression of Car2 mRNA between the *Car2*^{-/-} and wild-type mice (Lewis *et al.* 1988). However, the real-time RT-PCR method used in the present study is more sensitive to quantitative differences.

CA II has been considered the major isozyme in the stomach, where it participates in the production of gastric acid by proton secretion from the parietal cells, and on the other hand, protects the epithelium from acid digestion by bicarbonate secretion from the mucus-producing epithelial cells. To date, no gastrointestinal symptoms have been reported in CA II-deficient humans or mice (Ohlsson *et al.* 1986; Lewis *et al.* 1988). CA IX is another isozyme expressed in the gastric epithelium. The absence of functional CA IX enzyme has been linked to abnormal morphogenesis of the gastric mucosa (Ortova Gut *et al.* 2002). In the present study, the expression of Car9

mRNA and CA IX protein in the *Car2*^{-/-} mice was found to be significantly increased compared with levels in the wild-type mice. This result is interesting in that it apparently reflects a compensatory up-regulation of a CA expressed on the cell surface of the gastric mucosal cells in response to the loss of the cytosolic isozyme CA II. Comparison of the gastric function in *Car2*^{-/-} and *Car9*^{-/-} knockout mice may provide clear insights into

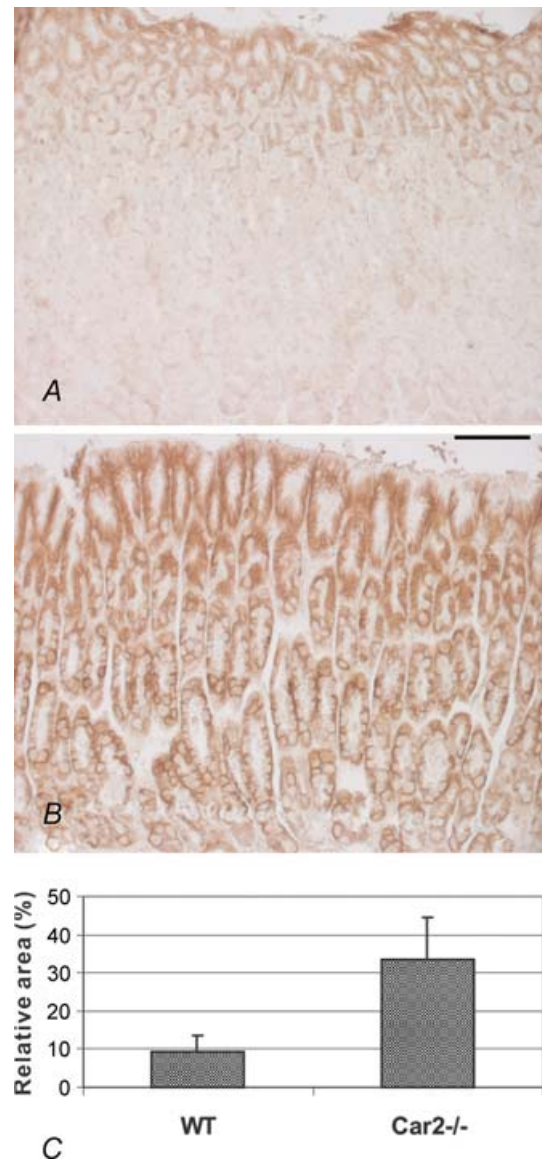


Figure 5. Immunohistochemical staining of CA IX protein in the stomach of the wild-type and CA II-deficient mice

In the wild-type mouse, the staining is mainly located in the mucus-producing epithelial cells of the outer part of the mucosa (A). In the *Car2*^{-/-} mouse, the signal was widely spread, covering intensively the whole thickness of the gastric mucosa (B). Original magnifications $\times 200$. C shows digital image analysis results, comparing the staining extent of CA IX protein in the stomach of the wild-type and *Car2*^{-/-} mice ($P = 0.0022$). Scale bar, 100 μm , and applies to A and B.

the relative contribution of each to gastric acidification and indicate whether the apparent compensatory increase in CA IX in CA II deficiency is effective.

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