Type IV carbonic anhydrase is present in the gills of spiny dogfish (Squalus acanthias)

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Type IV carbonic anhydrase is present in the gills of spiny dogfish (Squalus acanthias). Am J Physiol Regul Integr Comp Physiol 292: R556–R557, 2007. First published September 14, 2006; doi:10.1152/ajpregu.00477.2006.—Physiological and biochemical studies have provided indirect evidence for a membrane-associated carbonic anhydrase (CA) isoform, similar to mammalian type IV CA, in the gills of dogfish (Squalus acanthias). This CA isoform is linked to the plasma membrane of gill epithelial cells by a glycosylphosphatidylinositol anchor and oriented toward the plasma, such that it can catalyze the dehydration of plasma HCO₃⁻ ions. The present study directly tested the hypothesis that CA IV is present in gill tissue and specifically localized to cell membranes and branchial epithelial cells that flanked the pillar cells. Immunohistochemistry using a polyclonal antibody raised against rainbow trout type IV CA revealed a similar pattern of CA IV immunoreactivity and demonstrated a limited degree of colocalization with Na⁺/K⁺-ATPase immunoreactivity. The presence and localization of a type IV CA isoform in the gills of dogfish is consistent with the hypothesis that branchial membrane-bound CA with an extracellular orientation contributes to CO₂ excretion in dogfish by catalyzing the dehydration of plasma HCO₃⁻ ions.

Several lines of evidence suggest that a membrane-associated carbonic anhydrase (CA) isoform similar to the mammalian type IV CA is present in the gills of spiny dogfish (Squalus acanthias) and probably other chondrichthians (17). Mammalian type IV CA is a high-activity enzyme that is linked to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor such that the enzyme itself projects into the extracellular environment where it can catalyze the reversible dehydration/hydration reactions of CO₂ (Refs. 1, 32, 33, 74; reviewed in Ref. 47). Mammalian CA IV can be released from its membrane association by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), and this feature, together with its unusual resistance to the detergent SDS, a property conferred by the presence of one or more stabilizing intramolecular disulphide bonds, is often used as diagnostic in characterizing an unknown CA activity as CA IV like (Refs. 33, 66, 74; reviewed in Ref. 47).

In dogfish, the presence of plasma-accessible CA activity in the gills has been inferred from several studies that have examined the effects of selectively inhibiting extracellular CA activity (Refs. 14–16, 53, 57, 71; but see also Ref. 55 for an opposing point of view). For example, Swenson et al. (53, 57) found that the branchial clearance of an infused HCO₃⁻ load was significantly reduced following treatment of dogfish with a CA inhibitor, polyoxymethylene-aminobenzolamide (F3500), that is restricted to the extracellular environment. Similar use of F3500 or the sulphonamide inhibitor benzoamide, which only slowly permeates cell membranes, revealed a role for extracellular CA activity in CO₂ excretion (15, 16) as well as in determining the pH equilibrium status of postbranchial blood in dogfish (14). The plasma of elasmobranch fish, unlike most vertebrates, contains CA (14, 17, 19, 73) and therefore a difficulty with these in vivo approaches was that they were unable to distinguish between the contributions of plasma and branchial membrane-associated CA activities. The use of an in situ saline-perfused dogfish gill preparation provided clear evidence that branchial CA activity was available to catalyze perfusate CO₂ reactions (71). In particular, Wilson et al. (71) reported that passage through an in situ dogfish gill preparation of a saline solution exhibiting a pH disequilibrium was sufficient to establish an equilibrium condition, but that inclusion of the CA inhibitor acetazolamide in the perfusate eliminated this effect.

In addition to the physiological evidence supporting the presence in dogfish gills of membrane-associated CA activity with an extracellular orientation, biochemical evidence suggests that this CA activity can be released from its membrane linkage by PI-PLC (16). Differential centrifugation of gill homogenates from rainbow trout and dogfish revealed that the CA activity of the microsomal or membrane fraction was significantly higher in dogfish than in trout (16, 19). Subsequent incubation of microsomal fractions with PI-PLC resulted in the significant transfer of CA activity to the supernatant in dogfish but not in trout, suggesting that the membrane-associated CA activity of dogfish gills is a type IV-like enzyme (16).

A strong case can therefore be made from the available physiological and biochemical evidence that a type IV-like CA isoform is present in the gills of dogfish. However, the available evidence is indirect, and recent work on fish red blood cell cytosolic CAs has underlined the fact that CAs of similar biochemical properties may have distinct phylogenetic origins (7, 8, 64). Thus the objective of the present study was to directly test the hypothesis that a type IV-like CA is present in...
the gills of dogfish by cloning dogfish CA IV and examining its mRNA and protein distribution in gill tissue.

MATERIALS AND METHODS

Experimental animals. Pacific spiny dogfish (Squalus acanthias) were collected by net during trawls by local fishermen and transported to a holding facility at Bamfield Marine Station where they were held in a 75,000-liter circular tank supplied with full-strength seawater at 13°C. To obtain tissue samples, dogfish were euthanized by immersion in an anesthetic solution (ethyl-p-aminobenzoate; 1 g/l). Blood samples were withdrawn by caudal puncture, frozen in liquid N₂, and stored at −80°C until use. Gill, kidney, intestine, rectal gland, liver, heart, brain, and white muscle tissues were then harvested, frozen in liquid N₂, and stored at −80°C until use. In addition, gill tissue was immersion fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH = 7.4) overnight, then transferred to 15% sucrose followed by 30% sucrose for 2 h in each case. These tissues were kept at 4°C throughout the fixation procedure and were then stored in 30% sucrose at 4°C until use. Cryoprotected gill tissue pieces were frozen in Shandon Cryomatrix embedding medium (Fisher). Thin sections (10 μm) from the trailing edge of the gill, obtained using a cryostat (CM 1850; Leica) at −15°C, were collected onto electrostatically charged slides (SuperFrost Plus; VWR International), air-dried for 30 min, and stored at −20°C until use.

Molecular cloning of dogfish CA isoforms. Total RNA was extracted from gill tissue or whole blood using Trizol (Invitrogen) according to the instructions of the manufacturer. Spectrophotometry (Eppendorf BioPhotometer; VWR International) was used to verify RNA concentrations, and cDNA was then synthesized from 5 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. PCR was performed using 1 μl of cDNA template in 25-μl reaction mixtures containing 3.5 mM MgCl₂, 200 μM of each dNTP, 250 nM of each primer, and 1 U of Taq polymerase (Invitrogen) in PCR buffer supplied with the enzyme. All PCR reactions involved an initial denaturation at 94°C for 3 min followed by 40 cycles of 94°C for 30 s; annealing temperature for 60 s; 72°C for 30 s, and ending with a final extension for 10 min at 72°C. For gill tissue, the template for the PCR reactions consisted of 1 μl of gill cDNA, the annealing temperature was 49°C, and a degenerate primer pair was designed from regions ofvertebrate CA IV sequences exhibiting a high degree of amino acid conservation; this pair consisted of the forward primer 5'-CAR WSI CCN ATH AAY ATH GT-3' and reverse primer 5'–RTT IAC DAT RTG IAR YTC CAT-3'. Using this primer pair, a 290-bp cDNA fragment was amplified. In addition, a 361-bp cDNA fragment was amplified using as a template for the PCR reactions, 1 μl of blood cDNA, an annealing temperature of 49.5°C, and a primer pair consisting of the forward and reverse primers 5'-CAG TTC CAY TTC CAY TGG G-3' and 5'-RAC GAT CCA KGT GAC RCT CTC-3', respectively; this primer pair was designed on the basis of consensus amino acid sequences for fish cytosolic CAs. These initial PCR products were ligated into PCR 2.1 vectors (TOPO TA cloning kit; Invitrogen) and sequenced. A search of GenBank protein databases using BLASTX revealed that the 290-bp cDNA fragment cloned from gill tissue exhibited highest amino acid identity with known CA IV sequences, whereas the 361-bp cDNA fragment cloned from blood exhibited highest amino acid identity with known CA XIII and CA II sequences.

Based on the sequences of the cDNA fragments cloned, primers were designed to extend the initial fragment lengths by 3' and 5' rapid amplification of cDNA ends (RACE). For 3' RACE, total gill or blood RNA was isolated using Trizol reagent (Invitrogen) and reverse transcribed to cDNA using a 3' RACE adapter primer (GIBCO) and Superscript II reverse transcriptase (Invitrogen). Semi-nested PCR was then performed on the cDNA using abridged universal amplification primers (GIBCO) with 3' RACE primers. For blood, the first round primer 5'-AGC ACC TGA TGG TCT GGC TGT AA-3' (56°C annealing temperature) was followed by a second round with primer 5'-GCT GCC TTG TCT CAA T-3' (59°C annealing temperature). For gill, additional rounds were required. Two initial rounds with primer 5'-TGA GGG GTA TGG GTA TCG TCT GAG GTA A-3' followed by a third round with primer 5'-GCT CAC TCG GTG CAG GTA A-3'. A final round of PCR with primer 5'-ACA AGG CAG TTC AGT TCC AC-3' resulted in a product of the correct size that was extracted from a gel (PureLink Gel Extraction Kit; Invitrogen) before cloning and sequencing (see below). The procedures used for 5' RACE differed for gill tissue and blood. cDNA for 5' RACE was synthesized from total gill RNA using the FirstChoice RLM-RACE kit (Ambion), and PCR was carried out according to the kit instructions using the gene-specific primer 5'-CCA GGA TCC ACC TTT GTT C-3' and an annealing temperature of 57°C. cDNA for 5' RACE was synthesized from total blood RNA using an oligo dT primer and Superscript II reverse transcriptase (Invitrogen), purified using a PCR purification kit (Sigma), and then tailed with dCTP using a terminal transferase TdT (Invitrogen) with final reaction conditions 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.5 mM MgCl₂, 200 μM dCTP, 1 μl dCTP, and 1 μl TdT. The tailed cDNA was then used for two rounds of nested PCR. In the first round of PCR, the gene-specific primer 5'-TCC ATG TGA CGC TCT CAA AC-3' was used with an abridged anchor primer (Invitrogen) at an annealing temperature of 63°C, whereas the second round utilized the gene-specific primer 5'-ATG CAG TCC AGA GAA GAA AGC-3' with an abridged universal amplification primer (Invitrogen) at an annealing temperature of 58°C. All RACE PCR products were cloned into pCR2.1 vectors (TOPO TA cloning kits; Invitrogen) and sequenced, and the sequenced RACE products were then confirmed by overlap with the appropriate initial cDNA fragment. Repeated bidirectional sequencing of RACE products together with multiple sequence alignment (DNA-MAN; Lynnon Biosoft) permitted the construction of consensus sequences for dogfish CA IV (dCA IV) and dogfish blood cytosolic CA (dCAb).

Phylogenetic analyses. Dogfish CA deduced amino acid sequences were aligned with GenBank sequences of CA I, II, III, IV, VII, IX, XII, XIII, XIV, and XV from selected vertebrates, as well as fish cytoplasmic CA sequences, using ClustalX version 1.83 (60) with penalties for gap opening and gap extension set to 30 and 0.75, respectively, for pairwise alignments, and 15 and 0.3, respectively, for multiple alignments. The PHYLIP package was then used to carry out neighbor-joining phylogenetic analysis (41) on a matrix of mean character distances, with a bootstrapping resampling option to assess the support for nodes (100 pseudoreplicates). In general, default parameters were used with the exceptions that, where the option to specify an outgroup existed, Drosophila CA was specified (9), and the input order of species was randomized where possible. The accession numbers for sequences used in the phylogenetic analysis are presented in the legend for Fig. 2.

Analysis of dCA IV mRNA expression by real-time PCR. For the quantification of dCA IV mRNA expression across a range of tissues by real-time PCR, total RNA was extracted from 30-μg aliquots of frozen tissue samples using Trizol (Invitrogen) and treated with DNase I for 10 min at 37°C to ensure the removal of any remaining genomic DNA. Its quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf BioPhotometer; VWR International), and 2 μg of RNA was then used for cDNA synthesis by means of random hexamer primers and Stratascript reverse transcriptase (Stratagene; Cedarlane Laboratories).

Real-time PCR was then carried out on a Stratagene MX-4000 multiplex quantitative PCR system using a Fullvelocity QPCR Master Mix Kit (Stratagene; Cedarlane Laboratories). The PCR conditions for a 25-μl final reaction volume consisted of 0.5-μl cDNA template, 300 nM forward and reverse primers, 12 μl of 2× Master Mix, and 1:30,000 final dilution of ROX, with annealing at 58°C for 30 s and extension at 72°C for 30 s over 40 cycles. The primer pair used to assess dCA IV mRNA levels was designed using Primer3 software.
and consisted of the forward primer 5'-CGT TTG CTG GCT TTG ATG A-3' with the reverse primer 5'-CAA GCA CAG GAA GGG CAA A-3'. The specificity of the primers was verified by the cloning (TA 2.0 cloning kit, Invitrogen) and sequencing of amplified products. In addition, the PCR products of initial experiments were analyzed by gel electrophoresis to ensure that SYBR Green was not being incorporated into primer-dimers or nonspecific amplicons, and single bands of the expected size were obtained in all cases. For every real-time PCR run, SYBR Green dissociation curves were constructed after completion of 40 PCR cycles and revealed the presence of single amplicons for each primer pair. No template controls, in which reverse transcriptase was omitted during cDNA synthesis, were also included in every real-time PCR run to ensure that residual genomic DNA was not being amplified. Relative levels of dCA IV mRNA expression in different tissues were standardized to the amount of RNA present. Amplification efficiencies were determined from standard curves generated by serial dilutions of pooled RNA from all tissues (N = 3 fish per tissue), and β-actin was used as a control to ensure that all cDNA syntheses were carried out with similar efficiencies (the primer pair used to assess dogfish β-actin mRNA levels consisted of the forward primer 5'-ATG CCA ACA CTG TCC TTG C-3' together with the reverse primer 5'-GAC AGG GAA GGC AGG AT-3').

Analysis of dCA IV mRNA localization by in situ hybridization. In situ hybridization was used to localize dCA IV mRNA expression within the branchial epithelium. An antisense riboprobe for dCA IV was generated from gill cDNA using a primer pair designed to yield a PCR product of ~650 bp, specifically the forward primer 5'-ATG CAC TCA CTT ATT CTC TTG CT-3' coupled with the reverse primer 5'-ATG AGC CAT GAT AGC GGT AG-3'. The PCR product was cloned into the pcR2.0 vector (TA cloning kit; Invitrogen), and the plasmid was sequenced to confirm the identity and orientation of the product. To label the probe, first 2 μg of plasmid DNA was digested using XbaI (Invitrogen) and the conditions recommended by the manufacturer. The resultant product was phenol/chloroform purified and resuspended in 10 μl of DEPC H2O. Following verification of the digest concentration by spectrophotometry (Eppendorf BioPhotometer; VWR International), a probe-labeling assay was carried out using T7 RNA polymerase (New England Biolabs) and a digoxigenin (DIG) RNA labeling mix (Roche), as described by the manufacturer. To prepare tissue sections for in situ hybridization, sections on slides were hydrated (2 × 15 min) in 1× PBS containing 0.1% Tween-20 (PBST), then treated with 20 μg/ml proteinase K (GBICO) in PBST for 20 min at room temperature. The tissues were rinsed (2 × 10 min) in 1× PBST, refixed in 4% formaldehyde in PBS for 4 min, rinsed again (2 × 10 min; 1× PBST) and air-dried at 58°C for 15 min. To prepare the probe for hybridization, ~900 pg of probe was denatured for 3 min at 94°C in a solution containing 250 μg/ml salmon sperm DNA and 250 μg Poly A tacked to 12.5 μl with DEPC H2O. Denaturation was halted by quick chilling the probe on ice and centrifuging for 1 min at 7,500 g. Hybridization buffer [100 μl of 50% deionized formamide, 1× Denhardt's, 0.2% SDS, 5% dextran sulphate, 0.75 M NaCl, 25 mM EDTA, and 25 mM PIPES; 1× Denhardt's consists of 0.1% Ficoll 400,000, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin (BSA)] was then added to the probe, and the solution was mixed by vortexing. The probe (in hybridization buffer) was placed on the prepared tissue sections, which were then incubated overnight at 58°C in a humid chamber. Following hybridization, sections were thoroughly washed in 2× SSC (2 × 15 min, 58°C), 0.2× SSC (2 × 15 min, 58°C), 0.1× SSC (1 × 10 min, room temperature) and 0.1 M PBS (1 × 10 min, room temperature), where SSC consisted of 0.9 M NaCl and 0.09 M sodium citrate at pH 7.0. To detect hybridization signals, sections were first incubated for 1 h at room temperature with a blocking solution consisting of 1% goat serum and 2 mg/ml BSA in 0.1 M PBS containing 0.3% Triton-X. The sections were then incubated overnight at 4°C with anti-DIG conjugated to alkaline phosphatase (Roche Molecular Biochemicals), diluted 1:1,000 in the blocking solution. Following a series of washes (2 × 15 min in 0.1 M phosphate buffer at room temperature, brief rinse in water, 2 × 5 min at room temperature in coloration buffer consisting of 100 mM Tris at pH 9.5, 50 mM MgCl₂, 100 mM NaCl, and 0.1% Tween-20), color visualization was achieved by dissolving nitroblue tetrazolium and 5-bromocresyl-3-indolyl phosphate tablets (Sigma) in 10 ml of H2O and layering this solution over the sections. Color was allowed to develop for at least 4 h at room temperature in a dark, humid chamber. When satisfactory coloration was achieved, the slides were washed with 0.1 M PBS (2 × 15 min), mounted with 60% glycerol, and coverslipped.

Sections (9–12 sections per fish for each of 3 fish) were viewed using a Zeiss Axioshot light microscope equipped with a Hamamatsu C5985 chilled charge-coupled device camera, and images were captured using the Metamorph v4.01 imaging system. To assess the specificity of hybridization, probe was omitted from the hybridization buffer or sections were pretreated with excess unlabeled probe. For the latter, sections were incubated, before hybridization, for 3 h at 58°C with ~5× unlabeled probe in hybridization buffer, and then hybridized with ~900 pg of probe together with 5× unlabeled probe according to the protocol outlined above.

Analysis of dCA IV protein localization by immunohistochemistry. Localization of dCA IV within the branchial epithelium of dogfish was accomplished using a custom rabbit polyclonal antibody (AAbbent) raised against a synthetic peptide antigen conjugated to keyhole limpet hemocyanin. The antiseraum was purified by protein G affinity chromatography followed by peptide affinity purification (AAbbent). The peptide TTRYTLPDERTFPTFTTYG was used to raise the antibody to amino acids 57–74 of the rainbow trout CA IV protein sequence (GenBank accession no. AAR99330); the corresponding region of the dogfish CA IV amino acid sequence, TRNAQSVPNHLTPIIEFG (amino acids 56–73), was identical to the trout CA IV peptide at 8 of 18 amino acids.

Western blot analysis was used to test the ability of the trout CA IV antibody to detect dogfish CA IV. Proteins were prepared from frozen dogfish gill tissue samples by first grinding the tissue under liquid N2 with a precooled mortar and pestle, and then homogenizing the powdered tissue in RIPA buffer (50 mM Tris-Cl, pH = 8.0, 150 mM NaCl, 1% Nonidet P-40 substitute, 0.1% SDS, 0.5% sodium deoxycholate) containing protease inhibitors (complete Mini protease inhibitor cocktail tablets; Roche Molecular Biochemicals) and 2 μg/ml pepstatin A (Sigma) by passing the suspension through a needle and syringe a number of times. Samples were stored on ice for 15 min and centrifuged at 7,500 g for 10 min at 4°C, and the supernatant was then flash frozen and stored at −80°C until subsequent analysis of the soluble proteins. Total protein concentration was assayed using the bicinchoninic acid method (Pierce Biotechnology Micro BCA protein assay kit; Fisher Scientific) with BSA as the standard. Samples containing 120 μg of protein were then separated by SDS-PAGE using 10.5% tris-tricine polyacrylamide gels. Proteins were then transferred to 0.45-μm nitrocellulose membranes (Bio-Rad) using a wet transfer unit. Membranes were blocked in 5% PBST-milk for 1 h at room temperature. One membrane was then probed with a 1:1,000 dilution of the rabbit anti-trout CA IV for 1 h at 37°C, whereas a second blot was incubated simultaneously with the CA IV antibody in the presence of an excess (20 μg) of the peptide against which the antibody was raised. Both membranes were then incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Life Sciences) for 1 h at room temperature. After several washes, the proteins were visualized using the Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer). The protein size marker was used was obtained from Fermentas Life Sciences.

To localize dCA IV by immunohistochemistry, a hydrophobic barrier was created around each gill tissue section on a slide with a PAP pen (electron microscopy suppliers). The sections were first incubated (3 × 5 min) in a blocking buffer containing 2% normal goat serum.
serum, 0.1 M PB, 0.9% Triton-X, 1% gelatin, and 2% BSA, and were then incubated for 2 h at room temperature in a humidified chamber with primary antibody diluted in blocking buffer. The primary antibody was the rabbit anti-trout tCA IV (1:200), or this antibody together with 5-antibody (1:100), a mouse monoclonal antibody against the α5-subunit of chicken Na$^+$/K$^+$-ATPase. The α5-antibody developed by D. M. Fambrough was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. The α5-antibody has been used to localize Na$^+$/K$^+$-ATPase in a wide range of tissues and organisms, including gill tissue of elasmobranch fish (38). Negative control sections were incubated with blocking buffer, from which primary antibodies had been omitted, or with the rabbit anti-trout tCA IV antibody in the presence of an excess (20 μg) of the peptide against which the antibody was raised. Detection was accomplished using Alexa Fluor 488-coupled goat anti-rabbit IgG (Fisher) for tCA IV or Alexa Fluor 546-coupled goat anti-mouse IgG (Fisher) for α5-antibody. Following incubation with the primary antibody, sections were washed in 0.1 M PB (3 × 5 min) and incubated for 1 h at room temperature in a humidified chamber with a 1:400 dilution in 0.1 M PB of one or both secondary antibodies, as appropriate. The sections were again washed (3 × 5 min in 0.1 M PB), then mounted using a medium (Vector Laboratories) containing 4,6-diamidino-2-phenylindole for the visualization of nuclei. A conventional epifluorescence microscope (Zeiss Axioskop) fitted with a charge-coupled device camera (Hamamatsu C5985) was used to view the sections (at least 9–12 sections per fish for each of 3 fish), and images were captured using Metamorph v4.01 imaging software. In some instances, sections were also examined using a confocal microscope (Olympus Fluoview BX50W1).

RESULTS

Molecular cloning of dogfish CA isoforms. Using homology cloning strategies, two dogfish CA isoforms were cloned. From dogfish gill tissue, an 1,127-bp cDNA was assembled (GenBank accession no. DQ092628). This cDNA product included a complete coding sequence for a deduced protein of 306 amino acids (Fig. 1A) that most closely resembled CA IV, sharing 40% identity and 57% similarity to human CA IV (GenBank accession no. NP_000708), and 44–51% identity and 57–62% similarity to CA IV sequences for other fish species (pufferfish, Tetraodon nigroviridis, GenBank accession nos. CAG08972 and CAF98532; zebrafish, Danio rerio, XP_682741 and AAH78387; rainbow trout, Oncorhynchus mykiss, AAR99330). Web-based protein prediction tools suggested the existence of an NH2-termi-
nal signal peptide 19 amino acids in length that, together with a COOH-terminal GPI modification site at amino acid 282 or 283 (of the immature protein), would yield a mature GPI-linked protein of ~263 amino acids (Fig. 1A). In addition, this predicted amino acid sequence included four cysteine residues (at positions 5, 13, 25, and 209 of the mature protein) that were deemed likely to form two disulphide linkages (cys 5-cys 13 and cys 25-cys 209), and a single N-glycosylation site (Fig. 1A). The presumed active site of this dogfish sequence strongly resembled those of known or putative vertebrate CA IVs, particularly those of other fish species and Xenopus (Table 1). Notably, all residues thought to constitute the active site itself were conserved between the dogfish sequence and those of Xenopus and other fish species, and all but one between the dogfish sequence and mammalian CA IVs (Table 1). In addition, phylogenetic analysis of vertebrate CA sequences grouped this dogfish gene with other vertebrate CA IVs, within a broad grouping of membrane-associated CA isoforms that was distinct from the cytoplasmic CA grouping (Fig. 2). Hence, this dogfish gene was tentatively identified as CA IV (dfCA IV).

A second cDNA, of 1,107 bp, was assembled from dogfish blood (dfCAb) and included a complete coding sequence for a deduced protein of 260 amino acids (Fig. 1B). The deduced protein was most similar both to fish CAs, sharing 57–60% identity and 70–73% similarity with fish CAs cloned largely from blood (gar, Lepisosteus osseus, GenBank accession nos. AAM94169; tilapia, Oreochromis mossambicus, AAQ89896; lamprey, Petromyzon marinus, AAZ83742; Japanese dace, Tribolodon hakonensis, BAB83090; and rainbow trout, AAP73748), and mammalian CA XIII, sharing 57–58% identity and 73–74% similarity with mouse, rat, and human CA XIII (mouse, GenBank accession nos. NP_078771; rat XP_574890; human NP_940986). The presumed active site of this dogfish sequence was comparable to those of fish cytosolic CAs, as well as those of consensus mammalian CA VII (5 amino acid differences), CA XII (7 amino acid differences), and CA I (11 amino acid differences). Unlike virtually all other CA sequences examined, however, a serine rather than a histidine residue was present as the proton shuttling ligand (Tables 1 and 2). In addition, the dogfish sequence was unusual in exhibiting a tyrosine rather than a tryptophan residue at position 192 (Table 2). In phylogenetic analyses, this dogfish gene joined the broad cytoplasmic CA grouping (Fig. 2), a grouping distinct from that of membrane-associated CA isoforms and including CA VII sequences, as well as tetrapod CA I, II, III, and XIII, and fish cytoplasmic CAs. However, within the broad cytoplasmic CA clade, the dogfish blood sequence did not join the monophyletic clade of fish blood-specific and cytoplasmic CA isoforms (Fig. 2). Rather, the dogfish sequence was basal (although note the low bootstrap value) to both the fish cytoplasmic CA clade and the tetrapod CA I, II, III, and XIII isoforms. The dogfish blood CA sequence was in this respect similar to lamprey CA, although lamprey CA groups more closely with CA VII sequences (10), and the dogfish blood CA sequence was clearly distinguishable from this grouping (Fig. 2); notably, the CA VII grouping includes a partial sequence for dogfish CA VII (GenBank accession no. CX196604).

**Table 1. A comparison of the amino acid residues comprising the presumed active site pocket across vertebrate CA IV (and/or putative CA IV) sequences**

| Residue Number | 7 | 29 | 61 | 62 | 64 | 65 | 66 | 69 | 91 | 92 | 94 | 96 | 106 | 107 | 117 | 119 | 121 | 131 | 141 | 143 | 145 | 192 | 194 | 198 | 199 | 200 | 201 | 202 | 204 | 206 | 207 | 209 | 211 | 214 | 246 |
|----------------|---|----|----|----|----|----|----|----|----|----|----|----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Trout          | Y | S | N | N | T | V | Q | D | I | Q | H | H | E | E | E | E | H | H | V | I | L | V | G | F | F | Y | L | T | T | P | P | E | A | V | W | W | T | R |
| Zebrasfish     | Y | S | N | H | T | V | N | Q | Q | H | H | E | E | E | E | H | H | V | L | V | L | V | G | F | F | Y | L | T | T | P | G | A | A | W | I | T | R |
| Pufferfish     | Y | S | N | N | T | V | Q | D | L | Q | H | H | E | E | E | E | H | H | V | I | V | V | G | F | F | Y | L | T | T | P | D | A | A | W | L | T | R |
| Pufferfish     | Y | S | N | H | S | V | Q | D | Q | Q | H | H | E | E | E | E | H | H | V | I | V | V | G | F | F | Y | L | T | T | P | D | A | A | W | L | T | R |
| Xenopus        | Y | S | N | H | S | A | Q | D | I | Q | H | H | E | E | E | E | H | H | V | T | L | V | G | F | Y | Y | L | T | T | P | P | N | T | W | V | L | N |
| Cow            | Y | S | N | N | T | V | M | L | T | Q | H | H | E | E | E | E | H | H | V | A | I | V | A | F | Y | L | T | T | T | P | D | K | V | W | V | N | R |
| Human          | Y | S | N | N | S | V | M | L | Q | H | H | E | E | E | E | H | H | V | V | V | V | A | F | Y | L | T | T | T | P | D | K | V | W | V | N | R |

Carbonic anhydrase (CAIV) sequences were individually aligned against human CA I (GenBank accession no. AAH27890) to identify the residues of the active site. Residue numbering is based on the human CA I sequence, and residues thought to constitute the active site itself (23, 58, 59). Shading designates residues that are largely invariant across all CA IV sequences examined, whereas bold print denotes residues that are conserved across fish and amphibian sequences and differ from those in chicken or mammalian sequences. GenBank accession nos.: Dogfish, Squalus acanthias AA Z03744; Trout, Oncorhynchus mykiss AAR99330; Zebrasfish, Danio rerio AAH78387; Pufferfish, Tetraodon nigroviridis 1CAG08972 and 2CAF98532; Xenopus laevis AAH54242; Chicken, Gallus gallus XP_415893; Rat, Rattus norvegicus NP_0652047; Mouse, Mus musculus AAH12704; Rabbit, Oryctolagus cuniculus P48283; Dog, Canis familiaris XP_537711; Cow, Bos Taurus NP_776322; human, Homo sapiens NP_000708.
cells, also exhibited positive hybridization signals. These positive signals were eliminated when gill tissue was pretreated with excess unlabeled probe (Fig. 4C) or incubated in the absence of probe (negative control; data not shown).

A trout CA IV antibody (12) was used to probe the pattern of dfCA IV protein expression in dogfish gill tissue (Fig. 5). Use of the trout CA IV antibody for this purpose was validated by western analysis of dogfish gill tissue, which revealed that the trout CA IV antibody detected a protein of 40-kDa mass, slightly higher than the predicted molecular mass for dfCA IV of 35 kDa (Fig. 5B). This immunoreactive band was eliminated by preabsorption of the antibody with an excess of the peptide against which it was raised (blocking peptide, Fig. 5B), confirming the specificity of the trout CA IV antibody for dfCA IV. The pattern of dfCA IV protein expression in the dogfish gill was similar to that of its mRNA expression (Fig. 5A). Cells exhibiting immunoreactivity were again identified as pillar cells on the basis of their location (Fig. 5A, a–c). Frequently, but not universally, the lamellar epithelial cells flanking the pillar cells were immunoreactive for CA IV, and occasionally this CA IV protein was colocalized with Na\(^+\)-K\(^+\)-ATPase (Fig. 5Ab). CA IV immunofluorescence was prevented by omission of the primary (tCA IV) antibody (Fig. 5Ad) or when sections were preincubated with an excess of the peptide against which the antibody was raised (Fig. 5Ae). The membrane localization of CA IV immunofluorescence was evident when sections were examined by confocal microscopy (Fig. 6; supplementary data).

**DISCUSSION**

Previous work provided indirect physiological and biochemical evidence to support the presence in dogfish gills of a type IV CA that is accessible to blood CO\(_2\) reactions (15, 16, 71). In

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**Fig. 2.** A phylogenetic tree to illustrate the relationships between the two dogfish (*Squalus acanthias*) CA isoforms cloned in the present study (and which are highlighted in black), dfCA IV (dogfish IV, GenBank protein accession no. AAZ03744) and dfCAb (dogfish CAb), and selected vertebrate cytoplasmic and membrane-associated CA isoforms. The phylogenetic tree was constructed using neighbor-joining analysis (see MATERIALS AND METHODS for more details). The tree was ordered using *Drosophila* CA (AAAY56645) as a monophyletic outgroup. Horizontal branch lengths are scaled to represent the relative number of amino acid substitutions occurring along a branch, and support values at the nodes are indicated as a percentage from bootstrap analysis using 100 pseudoreplicates. GenBank protein accession numbers for the sequences used in the tree were as follows: CA I, human AAH27890, mouse AAH11223, rat XP_226922; CA II, human AAS51909, mouse AAS73357, rat CAA41227, *Xenopus* CAJ83242; CA III, human AA52293, mouse NP_031632, rat AAA40846; CA IV, rainbow trout AAR99330, *zebrafish* AAH78387, *fugu* (Tetradon nigroviridis) CAG08972, *Xenopus* AAY54242, human NP_000708, mouse AAC52569, rat NP_062047; CA VII, dogfish CX196604 (this GenBank nucleotide accession number was used to deduce the amino acid sequence), *zebrafish* NP_957107, *Xenopus* CAJ83128, human AAL78167, mouse AAG16230, rat XP_226204; CA IX, human CAI13455, mouse AAL14193, rat XP_233380; CA XII, *Xenopus* CAJ81489, human AAP53502, mouse AAL13185, rat AX50191; CA XIII, human NP_940986, mouse NP_078771, rat XP_222295; CA XIV, human Q9ULX7, mouse Q9WVT6, rat XP_342298; CA XV, mouse AA16671; fish cytoplasmic CAs, lamprey AAZ83742, gar AAM94196, tilapia AAQ89896, rainbow trout blood isoenzyme (CAb) AAP73478, rainbow trout cytoplasmic isoenzyme (CaC) AA09329, *zebrafish* NP_571185 (denoted CAb as it grouped with other blood-specific isoforms) and NP_954685 (denoted CaC as it grouped with other cytoplasmic isoforms). Japanese dace BAB83090, carp AA93743, salmon BG933892 (this GenBank nucleotide accession number was used to deduce the amino acid sequence).
the present study, a type IV CA was successfully cloned from dogfish gill. Moreover, the dogfish CA IV was demonstrated to be present within the dogfish gill in a location consistent with it being available to catalyze blood CO2 reactions, i.e., associated with the plasma membrane of pillar cells (Fig. 7). This new information, in conjunction with the results of previous work in which extracellular CA was demonstrated to contribute to CO2 excretion in dogfish (15, 16), offers convincing support for a model in which dfCA IV-catalyzed dehydration of plasma HCO3 makes a substantial contribution to CO2 excretion in dogfish.

Type IV CA is distinguished from other CA isoforms by a collection of characteristics that were present in dfCA IV, contributing to its tentative identification as a CA IV isozyme. CA IV belongs to the grouping of membrane-associated isozymes (IV, IX, XII, XIV, and XV) and specifically to the subgroup (IV and XV) that is bound to the plasma membrane. As with other GPI-anchored proteins, posttranslational modification occurs in the endoplasmic reticulum before the delivery of the mature protein to the plasma membrane. Specifically, an 18- to 20-residue NH2-terminal signal peptide that functions in membrane targeting and a COOH-terminal hydrophobic domain are removed by endoproteolytic cleavage (32, 33). Removal of the COOH-terminal hydrophobic domain allows attachment of the GPI anchor to a highly conserved serine residue. In keeping with these key characteristics of CA IV, prediction software suggested that the dfCA IV sequence contains a 19 residue NH2-terminal leader peptide together with a 25 residue COOH-terminal hydrophobic domain that would result in attachment of the GPI anchor to the conserved serine residue (Fig. 1). Most CA IVs (human CA IV is an exception) contain carbohydrate (67), and the dfCA IV sequence exhibits a potential glycosylation site. Human CA IV exhibits two disulphide linkages (cys 6–cys 11 and cys 23–cys 203) that act to stabilize the mature conformation of the protein and, in particular, an active site loop (48, 49), thereby conferring on CA IV its unusual resistance to solubilization by the detergent SDS (66, 69). In the dfCA IV sequence, cysteine residues are present at the appropriate locations and are predicted to form two disulphide bridges (Fig. 1). Finally, CA IV is a high-activity isoform (1).

![Graph](image-url)
Fig. 4. Representative light micrographs illustrating the localization by in situ hybridization of dogfish type IV carbonic anhydrase (dfCA IV) mRNA expression in the gills of dogfish (*Squalus acanthias*). A and B: two different regions of gill lamellae viewed at medium and high magnification, respectively. Strong signals for dfCA IV are evident in what appear to be pillar cells, as well as the lamellar epithelial cells flanking the pillar cells. These signals were eliminated by incubation of sections in the presence of excess unlabeled probe (C). Scale bar = 5 μm.

Fig. 5. A: localization by immunohistochemistry of dogfish type IV carbonic anhydrase (dfCA IV) protein in the gills of dogfish (*Squalus acanthias*). The images are overlays of images collected individually for dfCA IV immunoreactivity (green), α5 (Na⁺-K⁺-ATPase) immunoreactivity (red), and nuclei visualization (blue). Areas of overlap of dfCA IV and α5 immunoreactivity are indicated in yellow/orange. Nuclei were visualized using 4',6'-diamidino-2-phenylindole. Medium (a) and high (b and c) magnification images representative of the sections examined indicate that cells tentatively identified as pillar cells on the basis of their location consistently displayed immunoreactivity for only dfCA IV. The cells of the lamellar epithelium that displayed immunoreactivity for dfCA IV typically were found flanking the pillar cells. Colocalization with α5 occurred to a variable extent in some of these cells (arrows in b); in other cases, cells expressed only the α5 signal. Omission of primary antibodies eliminated all immunofluorescence (d). Preabsorption of the CA IV antibody using the peptide against which the antibody was raised eliminated dfCA IV immunoreactivity without affecting α5 immunoreactivity (e). Scale bars = 5 μm. B: Western blot of dogfish gill tissue depicting an immunoreactive band at ~40 kDa (control) that was eliminated after preabsorbing the primary antibody with excess peptide antigen (blocking peptide).
Although no catalytic data for dfCA IV are available, the similarity of the active site pocket of dfCA IV to those of vertebrate CA IV sequences of known high activity (Table 1) suggests that dfCA IV is also a high-activity isozyme.

In mammals, CA IV has been localized to endothelial surfaces of capillary beds (e.g., lung; Refs. 11, 69) and/or epithelial cell plasma membrane surfaces (e.g., kidney; Refs. 4, 72) in a variety of tissues, including the gas exchange surface (lung), kidney, brain, gastrointestinal tract, skeletal muscle, and heart (35, 47). CA IV in these locations has an apical or luminal orientation and is thought to play roles in both local and systemic acid-base regulation, primarily through its impact on buffering and/or HCO$_3^-$ ion reabsorption (e.g., Refs. 35, 42, 44, 51, 68). The tissue distribution of dfCA IV mRNA expression was consistent with patterns of CA IV distribution in tetrapods, with the gas-exchange surface (gill), kidney, intestine, and brain standing out as sites of dfCA IV mRNA expression (Fig. 3). Interestingly, dogfish rectal gland was also included in this group (Fig. 3). The elasmobranch rectal gland is a specialized salt-secreting tissue (45). Although current models do not implicate CA in rectal gland salt secretion mechanisms (46), CA activity is present in the gland (25), where it is thought to facilitate the elimination of metabolically produced CO$_2$ (54). Using histochemical approaches, Lacy (25) reported CA localization along basolateral membranes of rectal gland epithelial cells, a location that would be consistent with CA IV expression.

Western blot analysis corroborated the real-time PCR data for dfCA IV mRNA expression in gill tissue by demonstrating dfCA IV protein expression (Fig. 5B). The presence of dfCA IV protein in gill tissue is in agreement with the findings of earlier studies on dogfish gills of a membrane-linked CA activity that could be released from its membrane association by PI-PLC (16, 19). The available biochemical evidence suggests that the gills of other chondrichthyan fish may also possess a type IV CA activity (17). By contrast, most biochemical evidence (e.g., Refs. 13, 16, 17, 19, 20, 22, 43; but see Ref. 65) as well as the available molecular data (12) suggest that the gills of teleost fish lack CA IV-like activity. An explanation for this difference between cartilaginous and teleost fish remains elusive.

The localization of dfCA IV within dogfish gill tissue was examined using in situ hybridization (for mRNA expression) and immunohistochemistry (for protein expression). Similar distribution patterns of positive hybridization signals (Fig. 4) and immunoreactivity (Fig. 5) were apparent, supporting the localization of dfCA IV to the plasma membranes (Fig. 6) of pillar cells (Fig. 7) and, to a lesser extent, the lamellar epithelial cells flanking the pillar cells. Pillar cells consist of two parts; a nucleated body that acts as a post to separate the two epithelial layers forming the lamella and cytoplasmic processes or flanges that spread out to meet the flanges of adjacent pillar cells, thereby delimiting and lining the blood space (26, 34, 70). Although pillar cells are unique to fish gills, they are in some respects the functional equivalents of the endothelial cells that line the pulmonary vasculature of tetrapods (70). Thus localization of dfCA IV to pillar cell membranes is
consistent with the capillary endothelial location of pulmonary CA IV in mammals (11) and suggests that dfCA IV activity would be accessible to plasma CO2 reactions. Underlying the pillar cells is a basal lamina on which sits the lamellar epithelium. The lamellar epithelium, in turn, is made up largely of squamous pavement cells typically organized into one to three cell layers (27, 70). Dogfish CA IV localized to the lamellar epithelial cells flanking pillar cells could conceivably be oriented toward either the interstitial space or the external environment. Because CO2 reactions in water flowing over the gills of dogfish do not appear to have access to CA activity (37), it is likely that dfCA IV localized to the lamellar epithelium is oriented toward the interstitial space.

The role of dfCA IV that has emerged from studies employing physiological approaches is to contribute to CO2 excretion by catalyzing the dehydration of plasma HCO3\(^-\) ions (15, 16), and the pillar cell localization of dfCA IV is certainly consistent with this function (Fig. 7). Although pulmonary capillary endothelial CA IV in mammals also appears well placed to contribute to CO2 excretion by catalyzing plasma HCO3\(^-\) dehydration, neither model simulations of lung gas exchange (2, 3, 6) nor experimental measurements of CO2 excretion following CA inhibition (5, 52, 56) support a significant role for extracellular CA activity in CO2 excretion (reviewed in Refs. 21, 51). Pulmonary capillary CA IV may be limited in its contribution to CO2 excretion by two key factors, namely the 100-fold greater CA activity and 10-fold greater buffering capacity of the red cell environment over that of plasma (21, 51). Higher levels of CA activity coupled with greater proton availability for HCO3\(^-\) dehydration tend to drive the majority of HCO3\(^-\) dehydration in mammals through the red cell, minimizing the contribution of extracellular CA to CO2 excretion. By contrast, several factors may conspire to increase the importance of plasma HCO3\(^-\) dehydration in dogfish. Whereas plasma buffer capacity in most mammals contributes little to whole blood buffering (e.g., 22% in humans; Ref. 28), plasma buffering in elasmobranch fish accounts for 40–70% of whole blood buffer values (17). Coupled with the absence of a Haldane effect (73), the relatively high plasma buffer capacity in dogfish will tend to diminish differences between the plasma and red cell compartments in proton availability for HCO3\(^-\) dehydration (16). In addition, dogfish lack an endogenous plasma CA inhibitor (19). The presence of endogenous CA inhibitors in the plasma of some mammalian species (24, 39, 40) may lower the effective activity of CA IV, thereby contributing to limitations in the involvement of CA IV-catalyzed HCO3\(^-\) dehydration in CO2 excretion (18). Finally, red cell CA activity in dogfish is low relative to that of teleost fish or mammals (19, 31). The low red cell CA activity of dogfish contrasts with the availability of a high-activity CA IV isozyme to catalyze plasma CO2 reactions and will again serve to diminish differences between the plasma and red cell compartments. Moreover, although both mammalian CA II and CA IV are considered to be high-activity isozymes, human CA IV is markedly more efficient than CA II in HCO3\(^-\) dehydration (1). A similar or even more marked disparity between dfCAb and dfCA IV would further level out differences between the red cell and plasma compartments, accounting for the enhanced role of plasma HCO3\(^-\) dehydration in CO2 excretion in dogfish.

The cloning of a dogfish blood CA isoform in the present study provided some insight into the low red cell CA activity reported for dogfish (19, 31). Analysis of the active site pocket of dfCAb revealed the substitution of a serine residue for histidine at the proton shuttling ligand (Table 2). In the catalytic mechanism of CA (reviewed in Refs. 30, 36, 48, 50), a captive CO2 molecule is attacked by a highly reactive Zn-bound hydroxide ion, resulting in the formation of a HCO3\(^-\) ion that is coordinated to the Zn. The HCO3\(^-\) ion is then replaced by a water molecule. To regenerate the reactive Zn-bound hydroxide, a proton must be transferred from the active site to the environment. It is this function that is carried out by the proton shuttle, which is typically a histidine residue for efficient proton transfer. Proton transfer is the rate-limiting step in the catalytic mechanism, and enzyme activity can be greatly reduced by replacement of the histidine with residues that cannot transfer protons (30, 48, 63). Thus the substitution of serine for
histidine as the proton shuttle would be expected to lower the catalytic activity of dICab, and could account for the low turnover number reported for dogfish red cell CA (31), a turnover number that is approximately an order of magnitude lower than values reported for teleost species (8, 31). In addition, Esbaugh and Tufts (10) pointed out that the determination of turnover number using red cell lysates is influenced by the sensitivity of the CA to the sulphonamide acetazolamide, since this inhibitor is used to estimate enzyme concentration, and dogfish red cell CA was reported to exhibit an unusually low sensitivity to acetazolamide (31). As the elements of the active site pocket with which acetazolamide interacts (29) are conserved in dICab, an explanation for the high resistance of dogfish red cell CA to acetazolamide inhibition remains to be determined. Interestingly, the inhibition constant against acetazolamide of a cytoplasmic CA in dogfish gills was reported to be similar to that of other fish species and hence much lower than that of dogfish red cell CA (16), implying the existence of more than one cytoplasmic CA isoform in dogfish.

The low activity of dogfish red cell CA (or presumed low activity of dICab) contrasts with the high activity of other cytoplasmic CA isoforms from fish (or presumed high activity based on active site comparisons), including that of the lamprey (10). The existence of a high-activity red cell CA isoform in lamprey, together with the placement of lamprey red cell CA in phylogenetic analyses (Fig. 2; Ref. 10), implies the appearance early in vertebrate evolution of a high-activity CA, the catalytic structure of which was then strongly conserved throughout the vertebrate lineage. Given this context, the apparent loss of high activity in dogfish blood CA is puzzling and warrants further investigation. In particular, the potential existence of more than one red cell CA isoform, as is found in trout (8) and many mammals (51), should be explored.

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