The Basis of Prostaglandin Synthesis in Coral

MOLECULAR CLONING AND EXPRESSION OF A CYCLOOXYGENASE FROM THE ARCTIC SOFT CORAL GERSEMA FRUTICOSA

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In vertebrates, the synthesis of prostaglandin hormones is catalyzed by cyclooxygenase (COX)-1, a constitutively expressed enzyme with physiological functions, and COX-2, induced in inflammation and cancer. Prostaglandins have been detected in high concentrations in certain corals, and previous evidence suggested their biosynthesis through a lipoxygenase-allene oxide pathway. Here we describe the discovery of an ancestor of cyclooxygenases that is responsible for prostaglandin biosynthesis in coral. Using a homology-based polymerase chain reaction cloning strategy, the cDNA encoding a polypeptide with ~50% amino acid identity to both mammalian COX-1 and COX-2 was cloned and sequenced from the Arctic soft coral Gersema fruticosa. Nearly all the amino acids essential for substrate binding and catalysis as determined in the mammalian enzymes are represented in coral COX: the arachidonate-binding Arg120 and Tyr355 are present, as are the heme-coordinating His207 and His388; the catalytic Tyr385; and the target of aspirin attack, Ser530. A key amino acid that determines the sensitivity to selective COX-2 inhibitors (Ile523 in COX-1 and Val523 in COX-2) is present in coral COX as isoleucine. The conserved Glu524, implicated in the binding of certain COX inhibitors, is represented as alanine. Expression of the G. fruticosa cDNA afforded a functional cyclooxygenase that converted exogenous arachidonic acid to prostaglandins. The biosynthesis was inhibited by indomethacin, whereas the selective COX-2 inhibitor nimesulide was ineffective. We conclude that the cyclooxygenase occurs widely in the animal kingdom and that vertebrate COX-1 and COX-2 are evolutionary derivatives of the invertebrate precursor.

Prostaglandins have been found in a diverse range of vertebrates and invertebrates (1, 2). In vertebrates, they are synthesized by prostaglandin-H₂ synthase, known also as cyclooxygenase (COX) (3–5). COX is a hemoprotein with two distinct catalytic activities: the cyclooxygenase activity involved in the formation of PGG₂ from arachidonic acid and the peroxidase activity that catalyzes the reduction of PGG₂ to PGH₂ (3). There are two COX isozymes called COX-1 and COX-2 (6, 7). COX-1 is expressed constitutively in nearly all mammalian tissues and forms prostaglandins with housekeeping functions. COX-2, although absent from most cells, can be rapidly induced in many cell types upon treatment with inflammatory cytokines, growth factors, and tumor promoters (8). These two isoforms share ~60% amino acid sequence identity. They have similar structural topology and an identical catalytic mechanism (4, 9). The three-dimensional x-ray crystal structures of COX-1 and COX-2 are virtually superimposable. The residues that form the substrate-binding channel, catalytic sites, and residues immediately adjacent are all identical except for some small variations (9–12). These small differences in sequence lead to clear biochemical differences in substrate selectivity and sensitivity to various nonsteroidal anti-inflammatory drugs (4, 6).

The mechanism of prostaglandin biosynthesis in invertebrates, particularly in the prostaglandin-containing corals (13, 14), has been the object of intense studies and speculations over the years. A proposal that coral uses a fundamentally different mechanism from the mammalian pathway, i.e., a lipoxygenase-allene oxide synthase route similar to the jasmonic acid pathway in plants (15–17), has not found experimental support more recently. The highly active peroxidase-lipoxygenase fusion protein identified in Plexaura homomalla that catalyzes conversion of arachidonic acid into allene oxide is not involved in prostaglandin synthesis (17). Our previous biochemical studies showed that (i) crude enzyme preparations of the Arctic soft coral Gersema fruticosa convert exogenous arachidonic acid into a mixture of prostaglandins with typical mammalian stereochemistry; (ii) the biosynthetic pathway involves a common hydroperoxyperoxide intermediate, PGG₂; and (iii) the synthesis is inhibited by nonsteroidal anti-inflammatory drugs (18–20). These findings provide strong evidence that a relative of mammalian cyclooxygenases is responsible for prostaglandin synthesis in coral. However, no enzyme has been isolated, cloned, or otherwise characterized from coral or any other invertebrate.

Here we report investigations aimed at characterization of this enzyme. Using a homology-based PCR strategy, we cloned and sequenced the cDNA encoding the functional cyclooxygenase that catalyzes transformation of arachidonic acid into prostaglandin; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; bp, base pair(s); ORF, open reading frame; PBS, phosphate-buffered saline.
taglinids in the progestational-containing Arctic coral G. fruticosa. In expression studies, the coral enzyme was located in the endoplasmic reticulum and nuclear envelope of COS-7 cells, and the putative N-terminal signal peptide of the enzyme was cleaved. Our results indicate that the first cloned COX from non-vertebrates is an ancestor of vertebrate cyclooxygenase isozymes.

MATERIALS AND METHODS

Preparation of Total RNA

For preparation of total RNA, the method of Chomczynski and Sacchi (21) gave the crude product with a remarkable amount of low molecular mass decomposition products as determined by denaturing RNA gel electrophoresis. The best results were obtained with the method used by Su and Gilmore (22) for RNA isolation from marine red or green algae. Stage A gave an almost colorless pellet of RNA that was successfully used for cDNA synthesis. Approximately 3 g of G. fruticosa stored at −70 °C was pulverized to a fine powder in liquid nitrogen. 10 ml of lysis buffer (150 mM Tris-HCl (pH 7.5), 2% SDS, and 1% β-mercaptoethanol) was added immediately to the coral powder and vortexed for 2 min. Then 1 ml of 5 g guanidine-HCl was added, and the mixture was vortexed for 1 min. The solution was extracted with 1 volume of chloroform/isoamyl alcohol (49:1, v/v), and the phases were separated by centrifugation at 10,000 × g for 20 min. The aqueous phase was collected and re-extracted with 1 volume of phenol/chloroform/isoamyl alcohol (50:49:1, by volume), and the sample was then centrifuged again. To remove the traces of phenol, the aqueous phase was re-extracted with chloroform/isoamyl alcohol (49:1, v/v). Precipitation of the total RNA was carried out by addition of 0.33 volume of 12 M LiCl and β-mercaptoethanol (final concentration of 1% (v/v)) at −20 °C for 24 h. The total RNA was pelleted by centrifugation at 20,000 × g for 90 min and washed with 75% ethanol, followed by centrifugation at 20,000 × g for 15 min. All operations were carried out at 0 to +4 °C. The pellet of RNA was dissolved in 1 ml EDTA and quantified by UV spectroscopy. Approximately 2 mg of total RNA was recovered using this protocol.

mRNA purification for 5'-RACE was carried out with an oligo(dT)-cellulose column and purification kit (Amersham Pharmacia Biotech) and yielded 10 μg of poly(A)-rich RNA from 1.45 mg of G. fruticosa total RNA. For Northern blot analysis, the mRNA was purified using an Oligotex spin column (Oligotex mRNA midi kit, QIAGEN Inc.). Approximately 20 μg of mRNA was recovered from 330 μg of total RNA using this protocol.

cDNA Synthesis and PCR Cloning

cDNA reactions were run as described previously (23) using total RNA and an oligo(dT) celluose column and purification kit (Amersham Pharmacia Biotech) and yielded 10 μg of poly(A)-rich RNA from 1.45 mg of G. fruticosa total RNA. For Northern blot analysis, the mRNA was purified using an Oligotex spin column (Oligotex mRNA midi kit, QIAGEN Inc.). Approximately 20 μg of mRNA was recovered from 330 μg of total RNA using this protocol.

DNA Sequencing and Sequence Analyses

Plasmid DNA was isolated using the QIAGEN plasmid purification system. The clones were sequenced using an ABI Prism dye terminator cycle sequencing kit (PerkinElmer Life Sciences) and an ABI Prism 310 genetic analyzer. The amino acid sequence data were compared with entries in the GenBank®EBI Data Bank using BLAST (25). The signal peptide cleavage site was predicted using SignalP Version 1.1 (26). Multiple sequence alignments were obtained with the Clustal method using the Lasergene program (DNASTAR, Inc.).

Northern Blot Analysis

Poly(A)-rich RNA (1 and 10 μg) from G. fruticosa was fractionated on a 1.0% denatured formaldehyde-agarose gel and immobilized on a Hybond-N° nylon membrane (Amersham Pharmacia Biotech RPN119B). Immobilized RNA was hybridized with radiolabeled DNA probes using the nylon membranes at 65 °C for 2 h with the initial 566-bp PCR product labeled with [35S]dCTP using ExpressHyb hybridization solution (CLONTECH 8015-2). The membranes were then washed as follows: 2 × 25 min with 2× SSC + 0.1% SDS at room temperature; 2 × 15 min with 2× SSC + 0.1% SDS at 65 °C; and 1 × 15 min with 1× SSC + 0.1% SDS at 65 °C. Hybridization was visualized by autoradiography after overnight exposure at −70 °C using Kodak x-ray film.

Plasmids

The ORF of the coral COX cDNA was subcloned into the eukaryotic expression vector pcR3.1 (Invitrogen) for expression in the vaccinia/HeLa expression system and into pcR3.1, pcDNA3 (Invitrogen), and pcCG-E2Tag (27) vectors for transient expression in COS-7 cells. The 1.8-kilobase BamHI/EcoRV fragment was isolated from the coral COX-gEM-T Easy construct and cloned into a suitable expression vector.
This fragment contains the translational start and stop codons, and the cohesive ends allow the fragment to be ligated into expression vectors in the proper orientation. The XbaI/BamHI digestion removed the E2Tag from the N terminus of the coral COX sequence in the pCZ-E2Tag construct (see Fig. 4A). For insertion of the E2Tag epitope in the middle of the protein, the EcoRII/NotI site, 23 amino acids from the C terminus, was used (see Fig. 4A). The pCDNA3.1 expression vector containing rabbit COX-2 cDNA was a kind gift from Dr. Matthew Breyer (Vanderbilt University).

**Cells and Transfections**

The COS-7 cells were maintained in Iscove's modified Dulbecco's medium with 10% fetal bovine serum. For transfection, COS-7 cells were trypsinized, harvested by centrifugation, and resuspended in Iscove's modified Dulbecco's medium containing 10% fetal calf serum at a density of 1 × 10⁶ cells/ml. 250 μl of cell suspension was mixed with 800 ng of plasmid DNA and 50 μg of salmon sperm DNA in a disposable electroporation cuvette and was subjected to an electric discharge of 180 V using a Bio-Rad Gene Pulser at 970-microfarad capacitance. After the discharge, the cell/DNA mixture was left at room temperature for 15 min, and then cells were washed and plated. Cells were grown either at 37 °C for 48 h or at 28 °C for 72 h. Cells were collected using a rubber policeman, washed with ice-cold PBS, and collected by centrifugation at 1000 × g for 10 min. Transfected cells from 2–12 tissue culture plates (100 mm, 2–2.5 × 10⁶ cells/plate) were resuspended in 1 ml of ice-cold 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol and disrupted by sonication. The membranes were resuspended by homogenization in a volume of 50 μl Tris-HCl (pH 8.0) convenient for cyclooxygenase assay.

**Western Blotting**

COS-7 cells transfected with expression plasmid in 100-mm diameter dishes were lysed 48 h after electroporation in 200 μl of Laemmli sample buffer (28). Proteins were separated by SDS-8% polyacrylamide gel electrophoresis. After transfer of proteins by a semidry blotting method, a polyvinylidene difluoride membrane (Millipore Corp.) was incubated with anti-E2Tag mouse monoclonal antibody (final concentration of 0.1 μg/ml) (30), ovine COX-1-specific polyclonal or monoclonal antibodies (Cayman Chemical Co., Inc.), or rat COX-2-specific mouse monoclonal antibody (Pharmingen) and with a secondary horseradish peroxidase-conjugated antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (LabAs Ltd., Tartu, Estonia) according to the manufacturer’s recommendations. Detection was performed using an ECL detection kit (Amersham Pharmacia Biotech) following the recommendations of the manufacturer’s manual.

**Immunofluorescence Analysis**

For immunofluorescence analysis, COS-7 cells transfected with the appropriate expression vector were grown on coverslips for 48 h and fixed in cold (−20 °C) acetone/methanol (1:1) for 15 min. Coverslips were washed with PBS and blocked with bovine serum albumin (1 mg/ml in PBS) for 30 min. Anti-E2Tag monoclonal antibody was added at a concentration of 5 μg/ml (in bovine serum albumin/PBS) and anti-COX-2 monoclonal antibody in a dilution of 1:100, and both were incubated for 1 h at room temperature and washed with PBS. As a secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (LabAs Ltd.) was used at the concentration recommended by the manufacturer. Cells were examined on an Olympus Vanox-S AH2 microscope.

**Enzyme Assay**

Incubations of the microsomal fraction of transfected cells with [14C]arachidonic acid (final concentration of 50 μM) were performed in 1 ml of 50 mM Tris-HCl (pH 8.0) containing 1 mM adenine and 1 μM hemin for 10 min at room temperature. The reactions were terminated by addition of 100 μl of 100 mM SnCl₂ as an aqueous suspension. After acidification to pH 3, the products were recovered by extraction with ethyl acetate and subjected to TLC analysis with unlabeled authentic standards (a generous gift from Kevil Ltd., Tallinn, Estonia) (120).

For inhibition studies, the enzyme (microsomal fraction) was reconstituted with hemin (1 μM) in 50 mM Tris-HCl (pH 8.0) containing 1 mM adenine and preincubated at room temperature for 5 min with various amounts of inhibitors added in a few microliters of ethanol. An equal amount of ethanol was added to the control. The reaction was initiated with [14C]arachidonic acid (final concentration of 50 μM). The incubation was carried out for 10 min, followed by treatment with SnCl₂. The products were extracted and analyzed as described above.

**RESULTS**

**Cloning of COX by Degenerate Oligonucleotide-primed PCR**—Vertebrate cyclooxygenases have highly conserved residues in their functionally important regions of the catalytic domain. In an effort to clone the gene responsible for prostaglandin synthesis in the soft coral G. fruticosa, a homology-based reverse-transcription-PCR cloning strategy and degenerate primers were used to amplify COX sequences from coral cDNA (Fig. 1). Upstream degenerate primers were based on the conserved amino acid sequence of mammalian COX-1 and COX-2, FAFPAQHHTHFQFKT (primers F1 and F2). Downstream primers were based on the conserved sequence TF GG DVG (primer R1) and, for the second round of nested PCR, on the conserved sequence in the region of the active-site tyrosine, WH PLLPD (primer R2). First round PCR experiments gave no visible bands on an ethidium bromide-stained agarose gel. Second round half-nested or nested PCR with primers F2 and R2 gave a product of the expected size (566 bp) that, when cloned and sequenced, was found to have high homology to mammalian COX genes. Extension of the 5'- and 3'-ends was achieved using RACE-PCR methodology. Overall, the nucleotide sequence obtained from the three overlapping products translated in one reading frame to give the full-length cDNA sequence. The resulting clone contained, in addition to 1767 bp of coral COX ORF, also 161 bp of 5'-untranslated region and 197 bp of 3'-untranslated region, including the polyadenylation signal AATAAA. Northern blot analysis of G. fruticosa poly(A)-rich RNA with the 32P-labeled initial PCR product showed that the coral COX mRNA is ~2.1 kilobases in size, substantially smaller than the mRNAs for mammalian COX gene products (Fig. 2). The mRNAs of the mammalian constitutive and inducible enzymes are ~2.8 and 4.5 kilobases, respectively (8). Subsequently, the ORF of the full-length clone was amplified from the first strand cDNA by PCR using gene-specific primers and a proofreading polymerase. Five full-length clones were sequenced.

**Sequence Analysis**—The coral cDNA (GenBank™/EMBL accession number AF004222) contains an ORF encoding a protein of 589 amino acid residues with a calculated molecular mass of 67,934 Da. Multiple alignment of the predicted sequence of the coral enzyme with other known COX sequences revealed a high level of conservation within these molecules (Fig. 3). The amino acid sequence identity to vertebrate COX-1 and COX-2 was approximately equal, 45–49% to each. There are 21 amino acids in the coral sequence that can be considered COX-1-specific (i.e. conserved in all COX-1 sequences and not in COX-2, e.g., Ile623), and 23 coral residues are COX-2-specific (e.g., Leu698). (The numbering of residues used here corresponds to the amino acids in sheep COX-1.) The key residues determined to be essential for substrate binding, catalysis, and inhibition are well conserved in the coral enzyme. Also, five pairs of cysteines that form disulfide bonds (Cys36−Cys47, Cys37−Cys159, Cys41−Cys87, Cys56−Cys69, and Cys569−Cys575) are conserved in coral COX (10).
Expression of Coral COX cDNA—The ORF of the coral COX cDNA was subcloned into the eukaryotic expression vector pCR3.1 for transient expression in COS-7 cells and vaccinia/HeLa cell expression systems. For deletion of the 5′- and 3′-untranslated regions, it was necessary to synthesize two oligonucleotide linkers to rebuild the initiator methionine with a Kozak translational start sequence and a termination codon at the 3′-end of the ORF. These linkers contained a BamHI restriction site for the 5′-end and an EcoRV site for the 3′-end of the ORF. This initial construct lacked detectable enzyme activity in transfection experiments at 37 °C in COS-7 cells as well as in vaccinia/HeLa cell expression systems. We were also unable to detect the expression level of the protein at this stage due to the lack of a suitable antibody.

To follow the expression of the protein, the ORF of the coral COX cDNA was subcloned into the epitope-tagged eukaryotic expression vector pCG-E2Tag (27, 29). In this way, we fused the bovine papilloma virus type 1 E2 protein-derived epitope (E2Tag, GVSSTSSDFRDR) (30) in frame into the N terminus of coral COX. As both extreme ends of cyclooxygenases have been shown to be important for processing the enzyme within a mammalian cell (6), we also inserted the tag into the C-terminal part of the protein, 23 amino acids from the C terminus (Fig. 4A). The resulting plasmids, pCG-E2Tag-COX and pCG-COX/E2Tag, were expressed in COS-7 cells; and 48 h after transfection, the expression of the coral COX protein was analyzed by immunoblot analysis. At the same time, we found that a mouse monoclonal antibody raised against a 236-amino acid C-terminal part of the rat COX-2 peptide, but not anti-ovine COX-1 polyclonal or monoclonal antibodies (data not shown), reacted specifically with both native (Fig. 4B, lane 8) and recombinant (lanes 6 and 7) coral enzymes upon Western blotting. Rabbit COX-2 in pcDNA3.1 was used as a positive control (lane 5). As shown in Fig. 4B, anti-E2Tag monoclonal antibody recognized the COX/E2Tag protein (lane 3), where the tag was fused in the middle of the enzyme, but was not able to recognize N-terminally fused E2Tag (lane 2). As the protein E2Tag-COX was readily detectable by COX-specific antibody (lane 7), we can conclude that coral COX, similar to mammalian COX enzymes, contains a signal peptide in its N terminus and that this signal peptide is cleaved during the processing of the protein within the cell. In conclusion, Western blotting of recombinant coral COX revealed that (i) recombinant coral COX expressed in a mammalian cell expression system gives one distinct band of ~72 kDa, similar to that of the native coral enzyme; and (ii) coral COX contains an N-terminal signal peptide, which is cleaved to yield the mature protein.

Expression at 37 °C in either COS-7 cells or vaccinia-infected HeLa cells produced COX protein as determined by Western analysis, but there was no detectable conversion of [14C]arachidonic acid with the recombinant enzyme. The soft coral G. fruticosa grows in the Arctic White Sea at depths below 20 m, where temperature and light conditions do not change significantly during the year. Even in the summer months, the water temperature is ~5 °C. To survive under such extreme conditions, enzymes must catalyze efficiently at low temperatures. The molecular basis of cold adaptation of psychrophilic enzymes is relatively poorly understood (31, 32). Some recombinant psychrophilic enzymes that have been used to measure specific activity may not fold properly in mesophilic hosts or may be partially inactivated as a result of the high temperature (>30 °C) used for their expression (32).

Based on these considerations, we decided to grow the transfected COS-7 cells expressing coral COX at 28 °C. To follow the expression level of the COX enzyme under such extreme conditions, the pCG-COX(E2Tag) plasmid was transfected into the COS-7 cells; the cells growing at two different temperatures were harvested at different time points; and the expressed protein level was determined by Western blot analysis. As shown in Fig. 4C, COS-7 cells grown at 28 °C did express the coral COX protein, although at a reduced level compared with the normal temperature (37 °C). To obtain sufficient recombinant enzyme for cyclooxygenase assay, the COS-7 cells transfected with pCG-E2Tag-COX were grown at 28 °C for 72 h. The microsomal fraction was prepared and incubated with [14C]arachidonic acid. The products were separated by TLC, followed by monitoring of the radioactivity. Four well-separated polar bands comigrated with natural mammalian prostaglandin standards and gave color reactions with anisaldehyde spray reagent characteristic of the corresponding authentic prostaglandins (Fig. 5). The prostaglandins PGD2, PGE2, PGF2α, and 15-keto-PGF2α, formed from the exogenous arachidonic acid with the G. fruticosa acetone powder were characterized earlier by high pressure liquid chromatography, gas chromatography–mass spectrometry, 13C NMR, and optical rotation measurements in comparison with authentic standards (18, 20). So, the active recombinant COX enzyme of the Arctic coral G. fruticosa was expressed at 28 °C, whereas the protein expressed at 37 °C was not functional and was unable to catalyze the conversion of arachidonate to prostaglandins (Table I).

The effect of typical mammalian cyclooxygenase inhibitors (indomethacin and nimesulide) was tested on product formation by recombinant coral COX. As shown in Table I, indomethacin inhibited the COX activity in microsomal fractions of COS-7 cells transfected with coral COX cDNA. The selective COX-2 inhibitor nimesulide was found to be ineffective up to concentrations of 40 μM.

Intracellular Localization of Coral COX—To determine the subcellular localization of recombinant coral COX, pCG-E2Tag-COX, pcCG-COX/E2Tag, and pcDNA3.1 rabbit COX-2 were transfected into COS-7 cells. The cells were grown and fixed on coverslips, and immunofluorescence staining was performed. As shown in Fig. 6 (B and C), coral COX and coral COX/E2Tag both exhibited the signal at the endoplasmic reticulum and the nuclear envelope, similar to the location of the rabbit COX-2 protein (Fig. 6A). No specific immunofluorescence signal was detected in control COS-7 cells transfected with the carrier salmon sperm DNA (Fig. 6D).

DISCUSSION

G. fruticosa cyclooxygenase cDNA was cloned by PCR using degenerate primers based on conserved sequences of known vertebrate COX enzymes. The primary structure of coral COX has ~50% amino acid identity to both mammalian COX-1 and COX-2. The residues shown to be catalytically important for
both peroxidase and cyclooxygenase activities in mammalian COX isozymes are present in coral COX. However, significant structural differences can be found around the catalytic sites as well as in the pattern of glycosylation.

The Peroxidase Active Site—The hydroperoxide-reducing site of mammalian COX-1 and COX-2 lies on the surface of the catalytic domain on the distal side of the liganded heme prosthetic group (10). Amino acids proposed to be important for heme binding and catalytic activity (proximal heme ligand His388 and distal heme His207 and Gln203 (6)) are conserved in the coral enzyme (Fig. 3). However, there are significant differences in the amino acid substitutions at residues 289–295 that are proposed to form a small shield above His207 and Gln203. The occurrence of the sequence HPFYSML in G. fruticosa instead of QEVFGLL in COX-1 may reduce the opening to the hydroperoxide-reducing site and thus sterically restrict access of bulky fatty acid hydroperoxides to the heme iron. Also, essential sequence differences between the coral and mammalian COX enzymes can be found in the loop of residues at positions 211–220 that are supposed to form binding sites for PGG2 and the reducing substrates (10). Our earlier studies showed a very low hydroperoxide-reducing activity of native G. fruticosa preparations as evidenced by (i) significant amounts of 15-keto-PGs among the endogenous prostaglandins, (ii) formation of 15-keto-PGs in incubations with exogenous arachidonic acid, and (iii) accumulation of PGG2 instead of PGH2 in short incubations even in the presence of different electron donors (18–20). Structural changes around the peroxidase catalytic site of the coral enzyme that may affect the hydroperoxide-reducing ability remain to be established by mutagenesis studies.

The Cyclooxygenase Active Site—The positioning of Arg120, Tyr355 (important for fatty acid substrate binding), catalytic Tyr385, and Ser530 (the residue that is acetylated by aspirin and that is essential for its inhibitory activity (5, 6, 9, 33, 34)) is well conserved between mammalian and coral COX proteins (Fig. 3). The volume of the arachidonate-binding channel of mammalian COX isozymes is determined by the Ile-to-Val substitution at position 523. Indeed, the V523I replacement in human COX-2 opens access to a side pocket in the arachidonate-binding channel for specific COX-2 inhibitors (12, 35). The presence of Ile523 in coral COX resembles the COX-1 active site. However, substitution at another crucial position (position 503) is different; in G. fruticosa, similar to COX-2 (36), there is a leucine at position 503. Moreover, substitutions with Leu513 (His in COX-1 and Arg in COX-2), Ala524 (Glu in COX-1 and COX-2), and Met434 (Ile and Val in COX-1 and COX-2, respectively) in coral COX indicate additional structural differences in the hydrophobic tunnel that forms the cyclooxygenase active site (11). These may reflect different catalytic and inhibition properties of the coral and mammalian enzymes. Our inhibition studies with native and recombinant G. fruticosa COX enzymes showed that both preparations are inhibited by the nonselective COX inhibitor indomethacin (Ref. 20 and Table I). The relatively high IC50 compared with mammalian COX isozymes appears to indicate that coral COX is less sensitive to indomethacin. Also, the selective COX-2 inhibitor nimesulide had no effect on coral COX at concentrations up to 40 μM, indicating that the coral enzyme is even less susceptible to this inhibitor than is mammalian COX-1 (4).

Consensus N-Glycosylation Sequences—Mutagenesis studies with vertebrate COX proteins transiently expressed in COS-1
cells indicate that COX-1 is N-glycosylated at three sites, Asn68, Asn144, and Asn410 (37). COX-2 contains one (trout) or two (chicken and mammals) additional potential N-glycosylation sites (38); in mammals, only one of them, Asn580, is occupied in 50% of molecules (37). The *G. fruticosa* COX enzyme has three potential N-glycosylation sites: one is in a conserved position at Asn144, whereas the first and third are shifted to Asn73 and Asn396 (Fig. 3).

**Signal Sequences**—The major differences in primary structure between mammalian COX isozymes are a shorter signal peptide in the N terminus and an 18-amino acid C-terminal insertion in COX-2 (6, 8). The C terminus of coral COX is similar to that of COX-1, but the N terminus differs from those of both mammalian isozymes. The putative N-terminal signal peptide of the coral enzyme is cleaved between Ala22 and Val23, at a position corresponding to the cleavage site of all COX-2 proteins (Fig. 3) (26). However, based on the size of the cleaved

**TABLE I**

| **Cyclooxygenase activity of coral COX and rabbit COX-2 expressed in COS-7 cells** |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **COS-7 cells** | **Indomethacin** | **Nimesulide** | **pCG-E2Tag-COX** | **pCG-coral COX (E2Tag)** | **pcDNA3.1 rabbit COX-2** | |
| 37 °C | µg | 37 °C | µg | 37 °C | µg | 37 °C | µg | 37 °C | µg | 37 °C | µg |
| Sham-transfected | <0.01 | <0.01 | 0.91 | 0.46 | | |
| pCG-E2Tag-coral COX | 0.01 | 0.01 | 0.01 | 0.01 | 0.61 | 0.61 | |
| Indomethacin | 10 µM | ND | 40 µM | ND | 0.42 (36% inhibition) | 0.07 (89% inhibition) | |
| Nimesulide | 10 µM | ND | 40 µM | ND | 0.69 (0% inhibition) | 0.69 (0% inhibition) | |
| pCG-coral COX (E2Tag) | <0.01 | <0.01 | | | |
| pcDNA3.1 rabbit COX-2 | 0.91 | 0.46 | | | |

*Sum of PGD<sub>2</sub>, 15-keto-PGF<sub>2α</sub>, PGE<sub>2</sub>, and PGF<sub>2α</sub>.*
signal peptide, coral COX is more similar to COX-1 (23 cleaved residues) than to COX-2 (17 cleaved residues) (6).

In subcellular localization, the mammalian cyclooxygenases are associated with the endoplasmic reticulum and nuclear membranes (39, 40). The coral COX primary structure ends with the sequence RDEL (Fig. 3), which closely resembles the classic endoplasmic reticulum targeting signal for soluble proteins, KDEL. A similar sequence, P/S/TEL, found on the carboxyl terminus of all mammalian cyclooxygenases, has been reported to be necessary for retention of these enzymes within the endoplasmic reticulum (41). Some other studies indicate that the extreme C-terminal region is not an essential part of the intracellular targeting mechanism of COX-1 and COX-2 (42, 43). In our immunofluorescence studies, the COX protein of the Arctic soft coral exhibited subcellular localization similar to that of the mammalian cyclooxygenases, on the endoplasmic reticulum and nuclear envelope. The localization was not affected by the inserted tag within the protein, 23 amino acids from the C terminus. However, when the pCG-corall COX(E2Tag) plasmid expressing the protein containing E2Tag was used for transfection, no cyclooxygenase activity was detected (Table I).

The relationship between the coral COX amino acid sequence and other known COX sequences is shown in the phylogenetic tree in Fig. 7. Coral sequences form a separate arm on the phylogenetic tree, indicating that invertebrate COX might be a common ancestor of vertebrate COX isozymes and that divergence of COX-1 and COX-2 genes occurred later in evolution, after divergence of the animal kingdom to vertebrates and invertebrates.

In summary, the results of this study demonstrate the existence of a COX isozyme in marine invertebrates. This is the first study to confirm experimentally the presence of a cyclooxygenase in non-vertebrates. Our results establish that the COX enzyme is conserved from lower animals to human beings. They also lay to rest the long-standing issue of the origin of prostaglandins in coral, showing clearly that prostaglandins in coral are synthesized from arachidonic acid via an endoperoxide intermediate and that the conversion is catalyzed by an enzyme highly homologous to vertebrate COX isozymes. The expressed coral enzyme has some unusual catalytic activities that will make it a striking research object from both the evolutionary and mechanistic standpoints. The interrelationship of the peroxidase and oxygenase activities in initiating and maintaining PG endoperoxide synthesis remains a subject of intense investigation. The different structure and coupling of the peroxidase in coral COX present an opportunity to gain additional insights.

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