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Matrix Gla protein in turbot (*Scophthalmus maximus*): Gene expression analysis and identification of sites of protein accumulation

Vânia P. Roberto [a], Sofia Cavaco [a], Carla S.B. Viegas [a], Dina C. Simes [a], Juan-Bosco Ortiz-Delgado [a,b], M. Carmen Sarasquete [b], Paulo J. Gavaia [a], M. Leonor Cancela [a,*]

[a] CCMAR, Universidade do Algarve, 8005-139 Faro, Portugal
[b] Institute of Marine Sciences of Andalucía, CSIC, Polígono Río San Pedro Apdo Oficial, 11510 Puerto Real, Cádiz, Spain

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ABSTRACT

Matrix Gla protein (Mgp) is a secreted vitamin K-dependent extracellular matrix protein and a physiological inhibitor of calcification whose gene structure, amino acid sequence and tissue distribution have been conserved throughout evolution. In the present work, the turbot (*Scophthalmus maximus*) *mgp* cDNA was cloned and the sequence of the deduced protein compared to that of other vertebrates. As expected, it was closer to teleosts than to other vertebrate groups but there was a strict conservation of amino-acids thought to be important for protein function. Analysis of *mgp* gene expression indicated branchial arches as the site with higher levels of expression, followed by heart, vertebra and kidney. These results were confirmed by *in situ* hybridization with a strong *mgp* expression in branchial arch chondrocytes.

Mgp was found to accumulate in gills where it appeared to be restricted to chondrocytes from branchial filaments, while in vertebrae it was localized in vertebral end plates, in growth zones, in vertebral arches and spines and in notochord cells. In the soft tissues analysed, Mgp was mainly detected in kidney and heart, consistent with previous data and providing further evidence for a role of Mgp as a calcification inhibitor and a modulator of the mineralization process. Our studies provide evidence that turbot, an important new species for aquaculture, is also a useful model to study function and expression of Mgp.

1. Introduction

Matrix γ-carboxyglutamic acid (Gla) protein (Mgp) is a vitamin K-dependent protein known for its capability of binding mineral ions through its gamma-carboxylated glutamic acid residues. Mgp is normally found associated with the organic matrix of cartilage and bone *in vivo*, and it is expressed in various vertebrate tissues such as cartilage, kidney, lung, aorta, bone and tooth (Fraser and Price, 1988; Hao et al., 1995; Hashimoto et al., 2001; Hale et al., 1988). Although a wide range of cell types express Mgp, only chondrocytes, endothelial cells and VSMC (vascular smooth muscle cells) synthesize significant levels of Mgp *in vivo* (Conceição, et al., 2002; Luo et al., 1997; Ortiz-Delgado et al., 2005, b; Simes et al., 2003). After the discovery of Mgp in various soft tissues, it was proposed that this protein could act as a local inhibitor of mineralization (Fraser and Price, 1988). Accordingly, a number of later studies using various genetic and biochemistry approaches established Mgp as the first *in vivo* calcification inhibitor, although its molecular mechanism of action remains incompletely understood (Braam et al., 2000; Luo et al., 1997; Meier et al., 2001; Proudfoot et al., 1998; Speer et al., 2002; Wallin et al., 2001). Mgp plays also a role in chondrocyte differentiation and maturation and is a key factor for normal endochondral and intramembranous ossification (Luo et al., 1997; Newman et al., 2001; Yagami et al., 1999).

The skeleton is highly diversified throughout fish species and different aquatic habitats and is responsible for body shape, movement and muscle attachment, as well as protection of internal organs. It also plays an important role in physiological functions such as feeding and reproduction, and is the major calcium and/or phosphate reservoir for many species (Du et al., 2001; Lagler et al., 1987; Marshal and Hughes, 1985; Walker and Liem, 1994). For this study, we have selected a marine teleost species which undergoes major alterations in its skeletal morphology during development, the pleuronectiform *Scophthalmus maximus*, commonly known as turbot. This species is becoming progressively more important for aquaculture given its high commercial value, and at present its rate of production is increasing worldwide. However, there is a scarce knowledge on the mechanisms involved in turbot skeletal development, remodelling during metamorphosis and subsequent growth maintenance. Therefore, the main objective of this work was to characterize the structure of the cartilaginous tissues of this species through an extensive histomorphological analysis. Furthermore, and given the possible involvement of Mgp in these processes, we have also identified the major sites of turbot *mgp* gene expression and protein accumulation and compared the results with those previously obtained for other fish.

* Corresponding author. Tel.: +34 351 289 800 971; fax: +34 351 289 818 353.
E-mail addresses: vroberto@ualg.pt (V.P. Roberto), dsimes@ualg.pt (D.C. Simes), pgavaia@ualg.pt (P.J. Gavaia), lcancela@ualg.pt (M.L. Cancela).

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2. Materials and methods

2.1. Specimen collection and processing

Turbot juveniles (~6 g) were collected from an aquaculture company (STOLT seafarm, Galicia-Spain) in collaboration with the Institute of Marine Sciences of Andaluzia (ICMAN-CSIC, Cadiz). Juveniles were anaesthetized in 2-phenoxyethanol (Sigma, St Louis, MO) and sacrificed by post-cranial sectioning of the spinal chord. Samples of various organs were either collected in TRIZOL Reagent (Gibco-Invitrogen) and preserved at −80 °C for subsequent RNA purification, or fixed in buffered 4% paraformaldehyde (pH 7.4 in PBS) for 24 h at 4 °C. After fixation, samples were either immediately preserved in methanol at −20 °C or decalcified in ethylenediamine-tetraacetic acid solution (EDTA 10%/formaldehyde 2%) at 4 °C and then preserved in methanol at −20 °C.

2.2. RNA purification

Total RNA was purified from turbot tissues (vertebrae, liver, kidney, heart and branchial arches) using the TRIZOL (Gibco) method, according to supplier instructions. Integrity of purified RNA was evaluated by gel electrophoresis.

2.3. Molecular cloning of mgp cDNA

The turbot *mgp* sequence was obtained using 1 μg of total RNA derived from heart, that was reverse-transcribed at 37 °C for 1 h, using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT, Invitrogen) and a reverse oligo(dT)-adapter (5′-ACGCGTCGACCTCGA GATCGATG$(T)_{18}$-3′). Amplification of the 3′ end of *mgp* cDNA was performed by the polymerase chain reaction (PCR) using one forward oligonucleotide designed according to *Sparus aurata mgp* cDNA (Pinto et al., 2003) (spMGPcF2: 5′-GAGGACTACTCGCCCTGCCGCTTCT-3′) and the universal dT adapter (5′-ACGCGTCGACCTCGAGATCGATG-3′). The corresponding PCR reactions were conducted for 30 cycles (1 cycle: 95 °C: 30 s, 60 °C: 45 s, and 72 °C: 60 s), preceded by 5 min of an initial denaturing step at 95 °C, and followed by a 10 min final extension at 72 °C, with Taq DNA polymerase (Sigma).

Poly-A^+ RNA from turbot was purified from 500 μg of total RNA (a mixture of heart, kidney, branchial arches, vertebrae and liver in equal amounts) with the Oligotex mRNA Midi Kit (Qiagen). 1 μg of this mRNA was used to construct one Marathon cDNA library (BD Bioscience Clontech) and the 5′ end of *mgp* cDNA was cloned following 5′ rapid amplification of cDNA ends (RACE)-PCR with the Marathon cDNA Amplification Kit (BD Bioscience Clontech), using Advantage cDNA polymerase, the AP1 primer and the *mgp* specific primer RvMGP1R (5′-TGGATAGAGAACGAAACACTTAATTCAAGCGTA-3′), designed within the partial sequences previously obtained. Amplification conditions were those suggested by the supplier.

All PCR products obtained were fractionated by agarose gel electrophoresis, purified from the gel and cloned into pCR^{II}TOPO (Invitrogen). Final identification was achieved by DNA sequence analysis using SP6 vector specific primer at Macrogen Inc. (South Korea).

2.4. Molecular cloning of partial 18S ribosomal RNA

Turbot 18S ribosomal RNA was cloned using 1 μg of total RNA derived from liver and RT-PCR was conducted as described above, using primers Cod18SRNA01F (5′-CACATCCAAGGAAGGCAGCAGGCG-3′) and Cod18SRNA01R (5′-(AG)GT(CT)GGCATCGTTTA(CT)GGTCGCAACTA-3′), designed based on the conserved sequences derived from the nucleotide alignment of 18S ribosomal RNA sequences available for different species. The PCR products were analysed as described above and partial 18S rRNA sequence was obtained (GenBank accession no. DQ302409).

2.5. Analysis of gene expression by real-time quantitative PCR (qPCR)

Real-time qPCR assays were performed using iCycler PCR system and software to quantify nucleic acids (Bio-Rad, Richmond, USA). Total RNA (1 μg) was reverse-transcribed as described above. Reaction mixture containing 1× iQ SYBR Green I mix (Bio-Rad), 0.2 μM of forward and reverse primers and 5 ng of reverse-transcribed RNA was submitted to the following PCR conditions: 13 min at 95 °C, 50 cycles (each cycle is 30 s at 95 °C, 15 s at 68 °C). 18S ribosomal relative gene expression was used to normalize *mgp* gene expression levels and the reference sample was the liver. Fragments of 144-bp for *mgp* cDNA and 194-bp for 18S ribosomal cDNA were amplified using the primer sets SmMGP02F (5′-CCCCAG GATGAGGAGCCTTCTTCG-3′)/SmMGP02R (5′-TGTGGCGTGAAG GAGTTGGCT-3′) and Sm18S02F (5′-GCCACCGTCTCCCAGCCCCT-3′)/ Sm18S02R (5′-CCCAGTTCAGAGAGAGAAAACCCACA-3′), respectively.

2.6. Histological procedures

Turbot liver, spleen, heart, kidney, gut, vertebrae and branchial arches were included in paraffin blocks, sectioned (5–7 μm) and collected in TESPA (3-aminopropyltriethoxysilane, Sigma)-coated slides. Staining was carried out with alcian blue 8GX (pH 1.0) (CI 74240; Sigma), for cartilaginous tissue detection, and with Harris haematoxylin–eosin (HE) (CI 75290, Sigma and CI 1143463, Merck), for identification of structures and morphological features. After staining, sections were dehydrated and mounted with EUKIT (Merck).

2.7. Purification of Mgp from vertebra and heart tissues

Vertebra from adult *S. maximus* were crushed in liquid nitrogen, acid extracted and a crude precipitate was obtained as described in Simes et al., 2003. Following dialysis against 50 mM HCl, 30–50 μg of total protein from the resulting Mgp-containing extract were loaded into parallel lanes of a 4–12% SDS-PAGE gel (Nupage, Invitrogen) and electrophoresed. The gel was then cut into two halves and stained with either CBB for total protein detection or DBS for specific Gla residue detection, as described (Simes et al., 2003).

The hearts from four *S. maximus* adult specimens were cleaned from blood by extensive washing with milli-Q water then frozen in liquid nitrogen and ground with a mortar and pestle. The resulting tissue powder was extracted using a 10-fold excess (w/v) of 6 M guanidine-HCl solution with vigorous stirring at room temperature for 24 h followed by a 10 min centrifugation at 10,000 rpm, at room temperature. The tissue pellet was subjected to a similar second extraction procedure during 24 h. Supernatants from both extractions were mixed, dialyzed (SpectraPor 3; Spectrum, Gardena, CA, USA) against 50 mM HCl at 4 °C, cleared by centrifugation at (10,000 rpm, 10 min, 4 °C) and total supernatant protein content was as previously described (Simes et al., 2004). The presence of Mgp in both dialyzed guanidine extracts was analysed by western blot. In brief, a total of 200 μg of protein from each heart extract (samples 1 and 2) were dissolved in SDS reducing sample buffer containing reducing agent (NuPage, Invitrogen, La Jolla, CA,USA), applied to a 4–12% gradient polyacrylamide precast gel containing 0.1% SDS (NuPage, Invitrogen) and fractionated at constant 140 V.

Western-blot analysis for both validation of ArMgp antibody (developed against *Argyrosomus regius*, meagre, Mgp) and detection of Mgp in heart samples was performed as described (Simes et al., 2004) using 1:250 dilution of ArMgp primary polyclonal antibody. Immunoreactive protein bands were detected using alkaline phosphatase-labelled goat anti-rabbit IgG antibody (Gibco-BRL, Paisley, UK) diluted 1:20,000 in TBST and visualized using NBT/BCIP substrate solution (Sigma).

2.8. Immunohistochemistry

Immunohistochemistry was performed with ArMgp primary antibodies validated for turbot Mgp by western blotting

(supplementary Fig. 5), using as secondary antibodies peroxidase-conjugated or FITC-conjugated goat anti-rabbit IgG, as described for other species (Simes et al., 2003). .Histological sections were incubated overnight with rabbit anti-Mgp polyclonal antibody, washed and incubated for another 2 h with goat anti-rabbit IgG peroxidase-conjugated (Sigma), then stained with DAB (3,3′-Diamino Benzidine, Sigma) as described (Simes et al., 2003). The endogenous peroxidase activity was blocked with 3% H_2O_2 before immunostaining. Results were visualized with a BX-41 Olympus light microscope equipped with a C3030 Olympus digital camera.

Immunofluorescent Mgp detection procedure was similar to that described above but incubation with goat anti-rabbit IgG FITC-conjugated (Sigma) was performed in a humidified dark chamber. Sections were washed and mounted with glycerol immediately before observation. Results were visualized with a Leica DMLB fluorescence microscope equipped with a Leica DC 300 FX camera.

Specificity of the immunoreactions was confirmed by negative controls where (1) primary antibody was replaced with BSA, (2) secondary antibody was replaced with Coon's buffer or (3) either primary or secondary antibodies were omitted.

2.9. In situ hybridization

Sense and anti-sense RNA probes were synthesised from a 575-bp partial sequence of turbot *mgp* cDNA (spanning from nucleotide 247 to the 3′ end of the cDNA), linearized with *Apa*I and transcribed with SP6 RNA polymerase to generate a sense probe, or linearized with *Spe*I and transcribed with T7 RNA polymerase to generate an anti-sense probe. Quality of synthesised probes was verified by agarose gel electrophoresis. Both probes were then labelled with digoxigenin using a RNA labelling kit (Roche, Mannheim, Germany) according to manufacturer's instructions.

Treatment of slides and hybridization conditions were performed as previously described (Viegas et al., 2008). Hybridization was performed at 65 °C overnight in a humidified chamber. After hybridization, the sections were washed two times, each of them for 30 min at 65 °C, in 50% formamide/50% 2× SSC, then a third time under similar conditions but using 50% formamide/50% 0.2× SSC. Hybridized probe was detected with the alkaline phosphatase-coupled anti-digoxigenin-AP antibody (Boehringer, Mannheim, Germany) and NBT/BCIP substrate solution (Sigma) as described (Simes et al., 2003). The controls included hybridization with the sense probe, RNase treatment before hybridization and absence of either anti-sense RNA probe or anti-digoxigenin antibody. All three control experiments showed no detectable signals (results not shown).

2.10. Statistical analysis

Statistical analysis of expression levels was first performed using ANOVA with a p-value of <0.001, followed by the Holm–Sidak method, with a p-value of 0.05 using SigmaStat® version 3.5.

3. Results

3.1. Identification and molecular cloning of turbot mgp

The complete nucleotide sequence of turbot *mgp* cDNA (GenBank accession no. DQ304476) was obtained by a combination of RT- and 5′ RACE-PCR. The longest *mgp* cDNA obtained spans 864 base pairs (bp) and comprises an open reading frame (ORF) of 354 bp (Fig. 1)

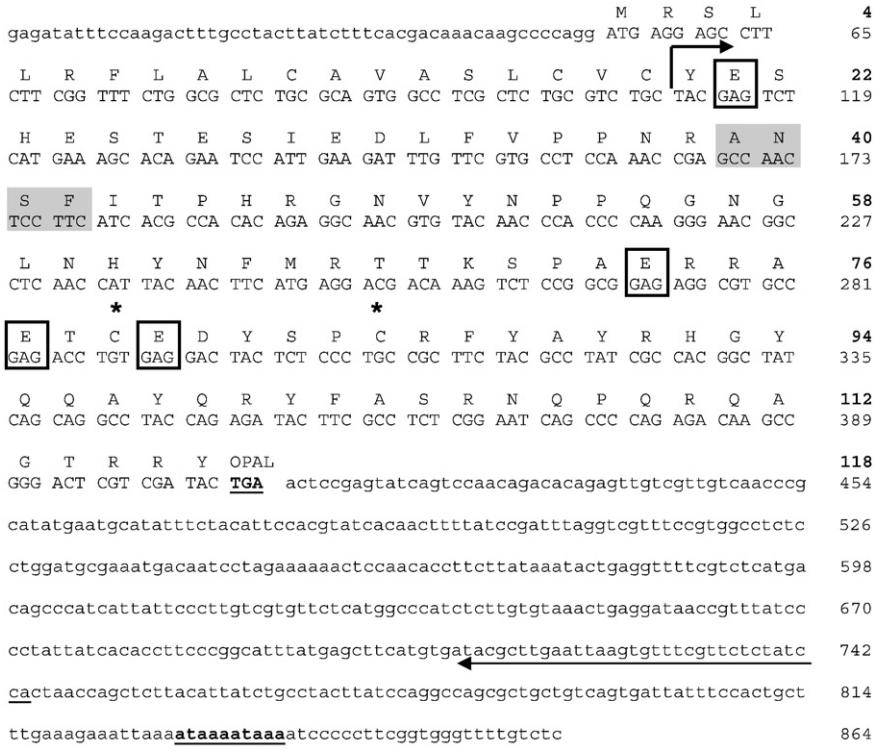

Fig. 1. Nucleotide sequence of the cDNA encoding turbot *mgp*. The cDNA was obtained by a combination of RT-PCR and RACE amplification. Numbering on the lower right end side is according to the first nucleotide from the longest 5′ RACE extension product obtained. Amino acid residues are numbered according to the first residue of the mature protein and are shown in bold above nucleotide numbering. Beginning of the mature protein is marked with a curved arrow. ANxF domain is shown in a grey box. Conserved cysteine residues required for the disulfide bridge are marked with an asterisk above. Gla residues predicted from comparative analysis are boxed (based on homology with other *mgp* sequences). Stop codon (opal) and overlapping polyadenylation signals are shown in bold and underlined. The sequence used for construction of the reverse primer used for *mgp* cDNA 5′ end amplification is denoted by a horizontal arrow.

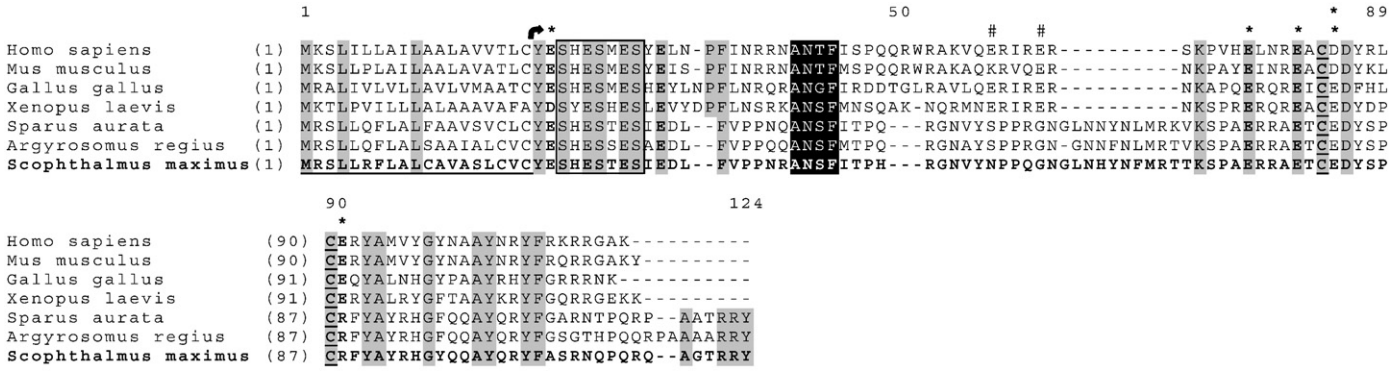

Fig. 2. Amino acid sequence alignment of *mgp* derived from mammalian, avian, amphibian and fish species. Turbot shown in bold. Sequences were aligned and conserved residues highlighted. Dashes indicate gaps in sequence, introduced to increase homology. The signal peptide is indicated by a continuous dark line below the corresponding sequence. First aminoacid of mature protein is signalled by a curved arrow. Phosphorylation domain is boxed, and residues within the ANxF domain are shown as white letters in a black background. Asterisks signal the position of predicted γ-carboxylated glutamic acid residues (a single asterisk indicates a conserved Gla in all *taxa*, a double asterisk corresponds to Gla residues conserved in non-mammalian species, a cardinal indicates Gla residues conserved in non-fish species). Cysteine residues forming the disulphide bridge are in bold and underlined. Accession numbers for these sequences are: *Homo sapiens* CR450358.1; *M. musculus* NM_008597.3; *Gallus gallus* NM_205044.1; *Xenopus laevis* AF055588.2; *S. aurata* AY065652.1; *A. regius* AF334473.1; *S. maximus* DQ304476.

encoding a polypeptide of 117 amino acid residues (aa), and 5′ and 3′ UTRs of 53 and 457 bp, respectively. The site of insertion of the poly-A tail is located 25 bp after two consensus consecutive polyadenylation signals (aataaa). By comparison with the protein sequences deduced from the complete cDNAs identified from other vertebrates, we hypothesize that the turbot *mgp* contains a 19 aa signal peptide, followed by a mature protein of 98 residues. Amino acid sequence alignments (Fig. 2) allowed identification of residues and domains conserved between turbot and other Mgp sequences, including: (1) a transmembrane signal peptide to control protein entry into the secretory pathway; (2) a phosphorylation domain; (3) a γ-glutamyl carboxylation recognition site; (4) an ANxF proteolytic cleavage site thought to be involved in post-translational processing; (5) two invariable cysteine residues required for establishing an intramolecular disulphide bridge; and (6) a C-terminal Gla domain where the majority of the Glu residues are expected to be γ-carboxylated, and thus responsible for the high affinity binding of calcium ions.

3.2. Tissue distribution of mgp gene expression

Distribution and levels of turbot *mgp* gene expression in the different tissues were determined by quantitative real-time PCR and *in situ* hybridization to attempt to correlate the relative levels of gene expression with the sites where expression occurred within the tissues analysed.

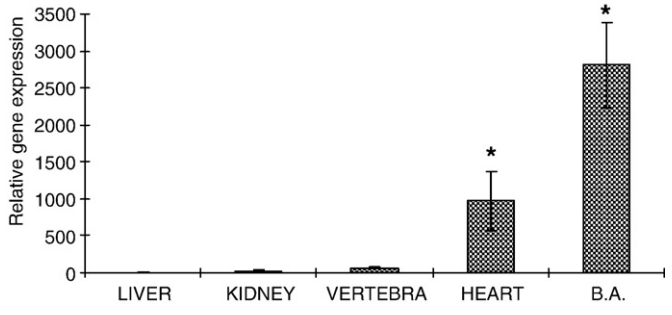

Fig. 3. Relative turbot *mgp* gene expression in tissues. Liver was selected as reference sample and the corresponding *mgp* gene expression levels were arbitrarily set to 1. Values are the mean of 3 independent real-time qPCR experiments. Turbot 18S (accession number DQ302409) was used to normalize *mgp* gene expression to the total amount of RNA in the sample. Asterisk indicates that expression levels are significantly different from those detected in all others samples in the figure. (B.A. — branchial arches.)

3.2.1. Quantitative real-time PCR

From all tissues analysed, liver had the lowest levels of *mgp* expression and thus was selected as reference sample. No significant differences were found between levels detected in liver, kidney and vertebrae for *mgp* mRNA. Branchial arches had the highest levels of *mgp* gene expression (over 2500 fold higher than the reference sample) while heart was found to exhibit close to 1000 fold increase over reference (Fig. 3).

3.2.2. In situ hybridization

Confirming the qPCR data, *mgp* was found to be strongly expressed by chondrocytes in branchial arches. A strong signal was observed in all cartilaginous structures including in hyaline cartilage of ceratobranchial and Zellknorpel cartilages of branchial filaments (Fig. 4.1 and 4.2). No *mgp* expression was detected in adjacent bony tissues (Fig. 4.1). In vertebrae, we detected expression of *mgp* only in chordoblasts (Fig. 4.3) and in cell population of the growth zone, known to contain chondroblast and osteoblast precursors (Fig. 4.4). Cartilaginous islets at the base of vertebral arches also showed expression of *mgp* in some chondrocytes (not shown), although at much lower levels than observed in branchial arch cartilages. From all soft tissues analysed, only kidney and heart showed significant levels of expression of *mgp*. In heart, *mgp* mRNA was associated to cardiomyocytes throughout the cardiac muscle (Fig. 4.5) while in kidney (Fig. 4.6), *mgp* mRNA was localized in cells from endothelium of urinary tubules and in some glomeruli.

3.3. Immunolocalization of Mgp and tissue characterization

The antibodies developed against *A. regius* Mgp were validated for the turbot protein using Mgp extracted and purified from *S. maximus* vertebra. DBS staining permitted the identification of a Gla containing protein in sample extracts (Fig. 5.1) corresponding to a single immunoreactive band observed in western blotting with anti-ArMgp with a migration behaviour (14–18 kDa) similar to that previously observed for other fish Mgp entities (Simes et al., 2004). Similarly, SDS-PAGE electrophoresis of guanidine-HCL extracts obtained from heart, followed by blotting onto nitrocellulose membranes and incubation with anti-ArMgp polyclonal antiserum, revealed the

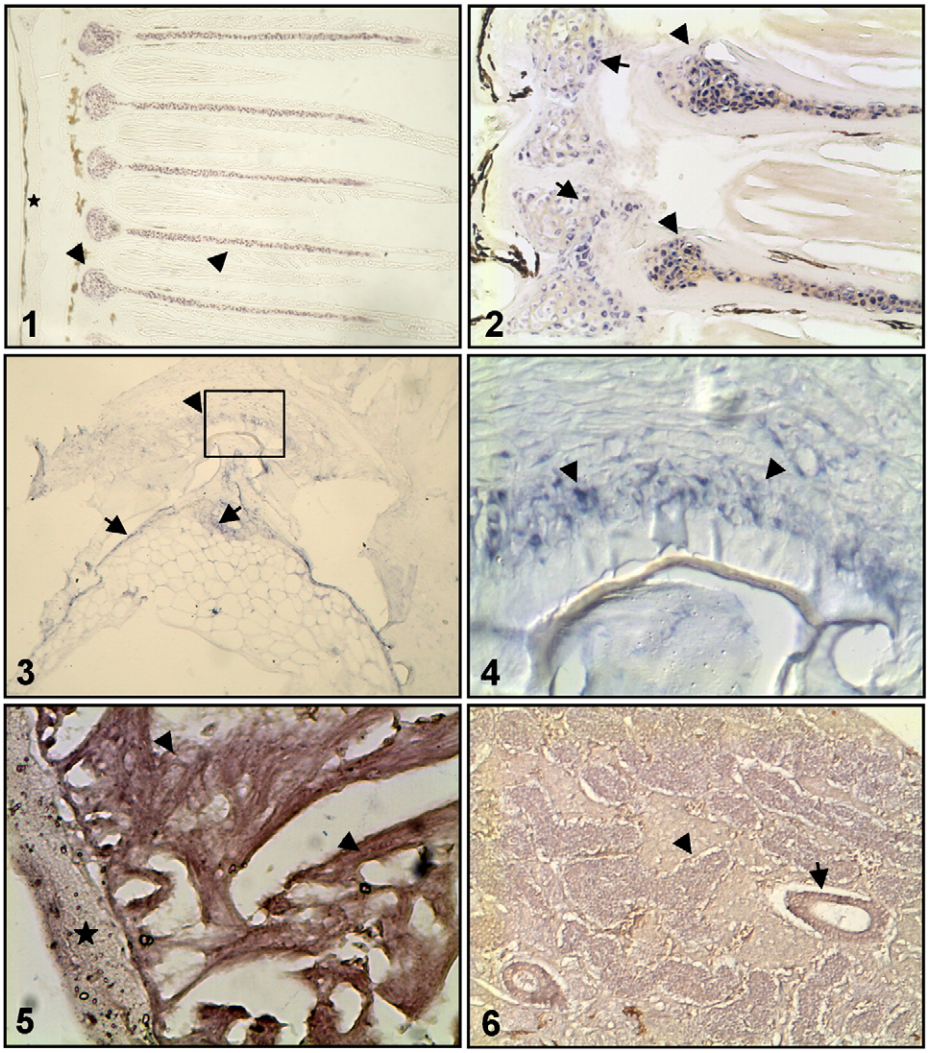

Fig. 4. Sites of expression of *mgp* were detected by *in situ* hybridization. 1) Branchial arches: *mgp* expressed by chondrocytes (arrowheads) in the Zellknorpel cartilage of branchial filaments, but not in the calcified tissue of the ceratobranchial (asterisk); 2) branchial arches: *mgp* expressed in the hyaline cartilage of the ceratobranchial (arrows) and at the base of branchial filaments (arrowheads); 3) vertebra: *mgp* expression detected in chordoblasts (arrows) and in chondroblast/osteoblast precursors of vertebral growth zones (arrowhead); 4) vertebra: magnification of box in 4.3 showing *mgp* expression on proliferating precursors (arrowheads); 5) heart: *mgp* revealed a widespread expression by cardiomyocytes (arrowheads) and absence of expression in adjacent mesenchymal tissue (star); 6) kidney: *mgp* expression was associated to the endothelium of urinary tubules (arrow) and *to* glomeruli (arrowhead).

presence, in heart extract, of an immunoreactive band corresponding to Mgp (Fig. 5.2).

3.3.1. The gills of turbot

In turbot, the gills consist of four to five branchial arches on each side of the esophagus that attach 2 sets of branchial filaments composed by the primary and secondary lamellas. Both branchial arches and primary lamella have a central core of cartilage (nomenclature used for cartilage follows that of Benjamin et al. (1992)), but while Zellknorpel cartilage, in branchial filaments, present proliferating chondrocytes, those of ceratobranchial contain mature chondrocytes immersed in a homogeneous (hyaline) cartilage matrix. ECM from both cartilages was stained with alcian blue, as seen in the basal part of branchial filaments (Fig. 6.1 and 6.5). With HE, bone matrix from ceratobranchial and acidophilic structures stained with eosin while nuclei of chondrocytes from branchial filaments stained with haematoxylin (Fig. 6.2 and 6.3). Using immunofluorescence and peroxidase-conjugated immunodetection, Mgp was found to accumulate mainly within proliferating chondrocytes from branchial filaments, and it was not detected in the extracellular matrix of cartilage (Fig. 6.4 and 6.6).

3.3.2. The vertebrae of turbot

The turbot vertebra is composed by the vertebral *centrum* (spongiosa) and 2 vertebral end plates. Bone matrix at vertebral end plates and *centrum* was stained with eosin, indicating a basic nature (Fig. 7.1). Mgp was immunodetected in the bone matrix of vertebral end plates and in vertebrae growth zones, within the contact area of opposing vertebral body end plates (Fig. 7.2 and 7.3). The bone matrix was clearly stained by alcian blue, although no staining was observed in the growth zones with this technique (Fig. 7.4).

In the centre of two adjacent vertebrae, and filling the intervertebral space, is the notochord (Fig. 7.1 and 7.2). This tissue is composed by an acellular, fibrous elastic sheath, covering a glycosaminoglycan-rich collagenous layer (Grotmol et al., 2005; Sanatamaría et al., 2005), that encloses and constrains the net of chordocytes in the inner core, surrounded by an outer layer of chordoblasts. Using HE, the chordoblast nucleus is stained by haematoxylin while the notochord is clearly stained with eosin, (Fig. 7.1). Mgp was found to accumulate in the notochord cells, both in chordoblasts and chordocytes, as detected by both immunocytochemistry and immunofluorescence (Fig. 7.2 and 7.3). In contrast, no immunostaining was observed in the notochordal

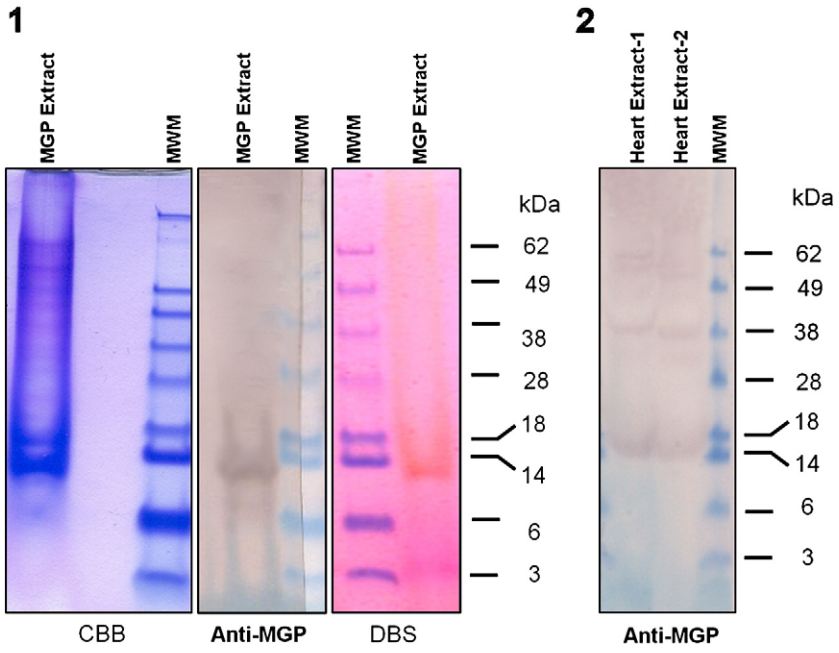

Fig. 5. Validation of Mgp antibodies by Western blot. 1 — SDS-PAGE and Western-blot analysis of crude precipitate after acid extraction of turbot vertebra. Electrophoresis analysis of dialyzed acid vertebra extract containing Mgp (Mgp extract) was performed using 4–12% SDS-PAGE gels (Nupage, Invitrogen) and gel was stained either with CBB, DBS gla-specific staining method or transferred for western-blot analysis using the anti-ArMgp polyclonal antiserum (Anti-Mgp) as described in the Material and Methods section. MWM, SeeBlue pre-stained molecular weight markers (Invitrogen); 2 — western-blot analysis of dialyzed turbot heart guanidine-HCl 6 M extract. Validated anti-ArMgp polyclonal antiserum (Simes et al., 2004) was used to detect the presence of Mgp in the first (heart extract-1) and second (heart extract-2) dialyzed guanidine extraction solution by western blot as described in the Materials and Methods. MWM, SeeBlue pre-stained molecular weight markers (Invitrogen).

sheath (Fig. 7.4, stained by alcian blue) confirming the cellular location of Mgp.

Each trunk vertebrae comprises one haemal and one neural process. Together, the haemal arches form the haemal canal which carries the primary blood vessels, while the neural arches accommodate the spinal cord, and together form the neural canal. From each neural and haemal arch, a spine extends providing anchorage for muscle attachment. Mgp was immunodetected in vertebral arches and spines, by both techniques used (Fig. 7.5 and 7.6), and appeared to be restricted to the corresponding mineralizing fronts.

3.3.3. Soft tissues of turbot

From all turbot soft tissues analysed (heart, gut, kidney, liver and spleen), accumulation of Mgp was immunodetected only in the endothelium of urinary tubules of kidney (Fig. 8.1 and 8.2) and in heart cardiomyocytes (Fig. 8.3) suggesting a specific function in these two organs.

4. Discussion

4.1. There is a high degree of evolutionary conservation among fish Mgp sequences

Comparison of turbot Mgp with sequences from other organisms indicated a higher degree of identity with homologous sequences from fish species (ranging from 80% to 60% amino acid conservation) than with other groups of vertebrates (ranging from 40% to 30% amino acid conservation). However, the evolutionary conservation of many invariant residues identified from the alignment of all vertebrate Mgp sequences, suggests that they are required for maintenance of a correct protein structure and/or to preserve a critical function. Among the unique characteristics that distinguish Mgp from other vitamin K-dependent proteins is the lack of a pro-peptide, with the gamma-carboxylase recognition sequence encoded inside the mature protein (Cancela et al., 2001; Price et al., 1987), demonstrating that gamma-carboxylation and secretion of this VKD protein are not directly related to the proteolytic cleavage of a pro-peptide domain (Price et al., 1987). An ANxF putative proteolytic cleavage site (reviewed in Laizé et al., 2005) and three highly conserved serine phosphorylation sites near the N-terminus of all known Mgps (Laizé et al., 2005; Price et al., 1994; Simes et al., 2003) are some of the potential targets for post-translational events that may alter the activity and/or properties of Mgp.

4.2. The highest levels of turbot mgp gene expression are found in branchial arches and heart

Previous findings showed that tissue distribution of *mgp* in fish (Pinto et al., 2003; Simes et al., 2003) essentially parallels that seen in mammals (Fraser and Price, 1988; Luo et al., 1997) and amphibians (Cancela et al., 2001). In this work, high levels of *mgp* gene expression were detected in branchial arches by qPCR. Since branchial arches contain cartilage with chondrocytes, which secrete *mgp*, this justifies the high levels detected. Results obtained by *in situ* hybridization confirmed the previous statement by revealing a strong *mgp* expression associated with chondrocytes in branchial arches and branchial filaments (Fig. 4). This could be related to Mgp proposed role in maintenance of a non calcified cartilage matrix, but also as a possible mediator of calcium metabolism occurring at the gill level. Accordingly, a strong *mgp* expression associated with these structures was previously observed in other fish species such as gilthead seabream (*S. aurata*), zebrafish (*Danio rerio*) and Senegalese sole (*Solea senegalensis*) (Gavaia et al., 2006; Pinto et al., 2003). Furthermore, the purification of sizeable amounts of Mgp from fish was only possible using calcified branchial arches (Simes et al., 2003) confirming the high content of Mgp in these structures, probably due to its high affinity for calcified matrices.

After branchial arches, heart was the other tissue where significant levels of *mgp* expression were detected by qPCR. Simes et al. (2003) reported heart as the major site for *mgp* expression in adult meagre,

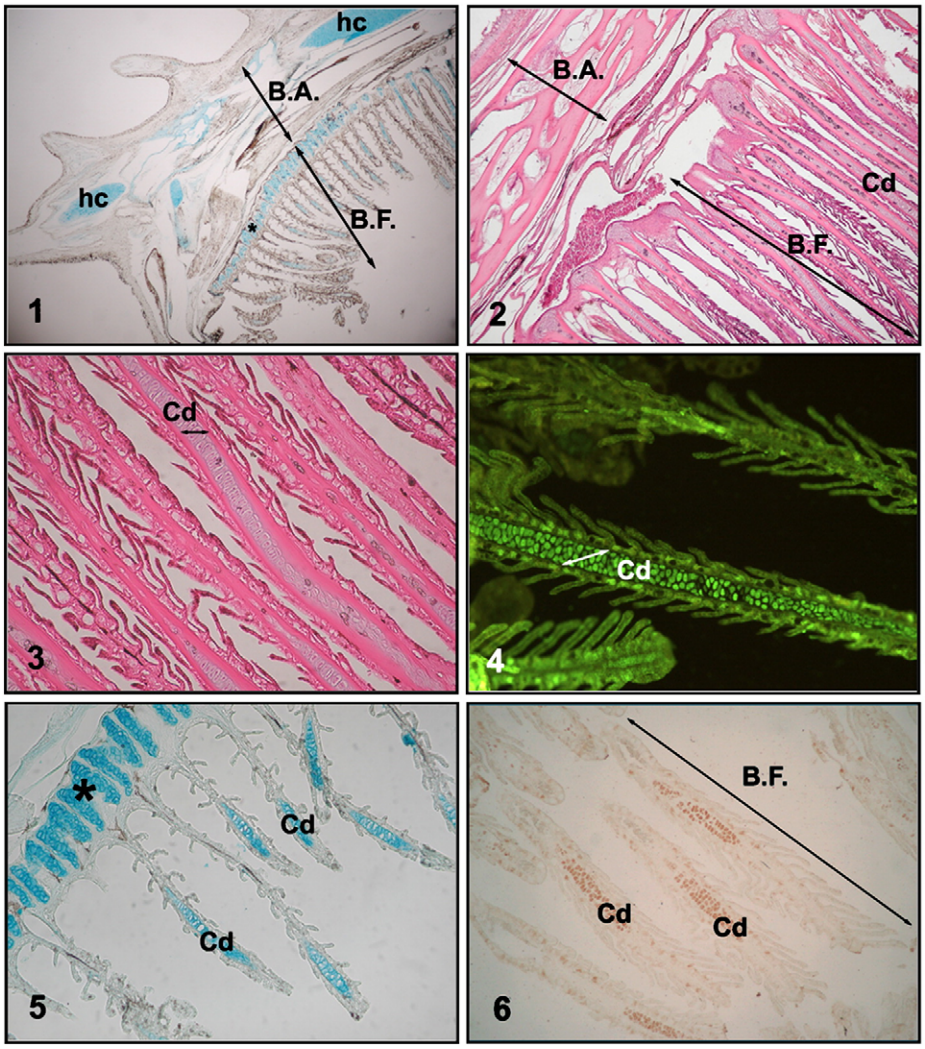

Fig. 6. Histological analysis of the gills of turbot and identification of sites of Mgp accumulation. 1— Alcian blue stained the hyaline cartilage from ceratobranchial bone (hc) in the branchial arch (B.A.), the proliferating chondrocytes from the branchial filaments (B.F.), and the cartilage from the base of branchial filaments (star) (50×); 2 — HE staining: eosin stained the bone matrix in branchial arches (B.A.); nucleus of chondrocytes (Cd) from branchial filaments (B.F.) stained with haematoxylin (100x); 3 — magnification of proliferating chondrocytes (Cd) from the Zellknorpel cartilage stained with HE (200×); 4 — immunofluorescent detection of Mgp in proliferating chondrocytes from Zellknorpel cartilage (arrow, Cd) (200×); 5 — alcian blue staining, magnification of 1, showing the chondrocytes piled up in the cartilage of gill primary lamella (Cd) and hyaline cartilage from the ceratobranchial (asterisk) (200×); 6 — confirmation of the site of Mgp accumulation in the branchial filaments (B.F.), as shown in 4, by peroxidase-conjugate immunodetection (Cd) (100×).

followed by branchial arches, and Pinto et al. (2003) reported levels of *mgp* expression in heart almost equivalent to those observed in branchial arches for juvenile gilthead seabream. The high expression levels of *mgp* observed in heart are in agreement with those previously observed by others for fish (Simes et al., 2003), amphibians (Cancela et al., 2001) and mammals (Fraser and Price, 1988). Our results by *in situ* hybridization confirmed that cardiomyocytes were expressing *mgp*. Furthermore, localization of *mgp* mRNA in cardiac structures by *in situ* hybridization was also previously shown to occur in fish by our group (Gavaia et al., 2006; Ortiz-Delgado et al., 2006; Pinto et al., 2003). The presence of high levels of *mgp* in heart tissue is indicative of its function as a calcification inhibitor since this tissue has been shown to be a target of ectopic calcifications during pathological conditions, where expression and/or function of *mgp* is altered (Jono et al., 2006; Luo et al., 1997; Proudfoot and Shanahan, 2006; Schurgers et al., 2005). Mgp is reported to act in the cardiovascular system as a regulator of calcium deposition by binding calcium ions and crystals, by modulating bone morphogenetic protein-2, by binding to extracellular matrix (ECM) and as a regulator of apoptosis (Braam et al., 2000; Hruska et al., 2005; Proudfoot and Shanahan, 2006).

Vertebrae and kidney, in contrast with heart and branchial arches, showed very low levels of *mgp* mRNA by qPCR although expression in these tissues was confirmed by *in situ* hybridization. In vertebrae, *mgp* expression was associated mainly to the proliferating and differentiated chondrocytes from the intervertebral cartilage, in agreement with previous reports (Gavaia et al., 2006; Pinto et al., 2003), and to the notochord, in particular the chordoblasts.

In the kidney, *mgp* expression was detected in some renal tubules and glumeruli which can be related to a need for inhibition of mineralization in this organ, a process which could be mediated by Mgp. It is known that nephrocalcinosis is a common disease affecting aquaculture produced fish (Gillespie and Evans, 1979; Golomazou et al., 2006; Srinivasa and Lakshmi, 1986), and Mgp presence in this organ can be explained by the need of preventing passive accumulation of calcium Expression of *mgp* in kidney assessed by Northern blotting or qPCR was reported in various studies in fish and mammals (Fraser and Price, 1998; Pinto et al., 2003; Simes et al., 2003; Zhao and Nishimoto, 1996), although only few studies referred to *in situ* localization of *mgp* expression on this organ, namely in rat (*Mus musculus*) (Zhao and Nishimoto, 1996) and in blue shark (Ortiz-Delgado et al., 2006).

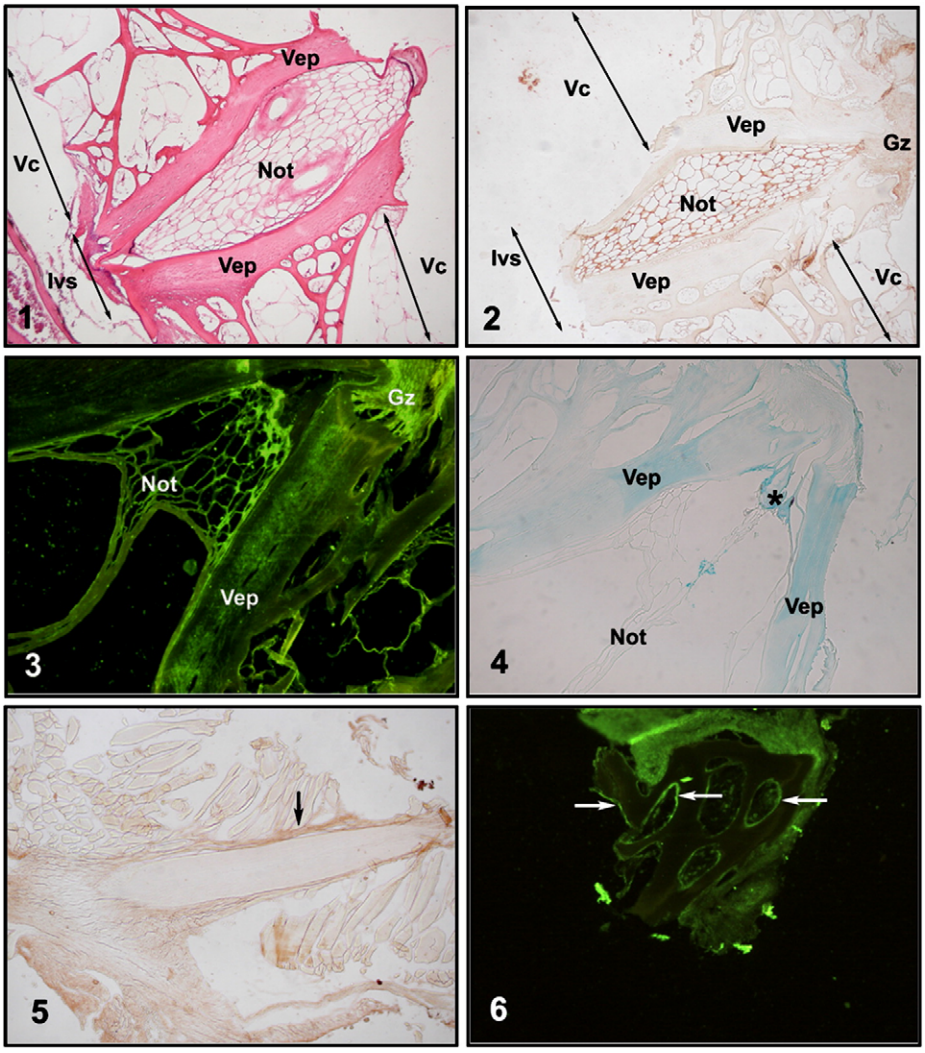

Fig. 7. Histological analysis of the vertebra of turbot and identification of sites of Mgp accumulation. 1 — Staining of vertebrae with HE. Bone matrix of vertebral *centrum* (Vc) was stained with eosin, as well as the vertebral end plates (Vep). The notochord (Not) is visible in the intervertebral space (Ivs) (100×); 2 — immunodetection of Mgp with peroxidase-conjugated antibodies in the notochord (Not) and growth zone of vertebra (Gz) (200×). 3 — immunofluorescent detection of Mgp in the notochord (Not), bone matrix of vertebral end plates (Vep) and growth zone of vertebra (Gz) (400×); 4 — strong staining of vertebral end plates (Vep) and notochordal sheath (star) with alcian blue (200×); 5 — immunodetection of Mgp in the periphery of haemal spine (arrow) (200×); 6 — immunofluorescent detection of Mgp in cavities and periphery of the neural arch (arrows) (200×).

4.3. Mgp accumulates in proliferating chondrocytes, in growth fronts during bone mineralization and in notochord cells

Immunostaining showed Mgp accumulation in the gills only at sites of proliferating chondrocytes from Zellknorpel cartilage of branchial filaments (Fig. 6). A similar pattern of Mgp accumulation was previously observed in branchial filaments of meagre (Simes et al., 2003).

In turbot vertebrae, Mgp accumulation was observed at four different sites (Fig. 7). The immunodetection of Mgp in the mineralizing fronts of vertebral arches and spines, and in vertebral

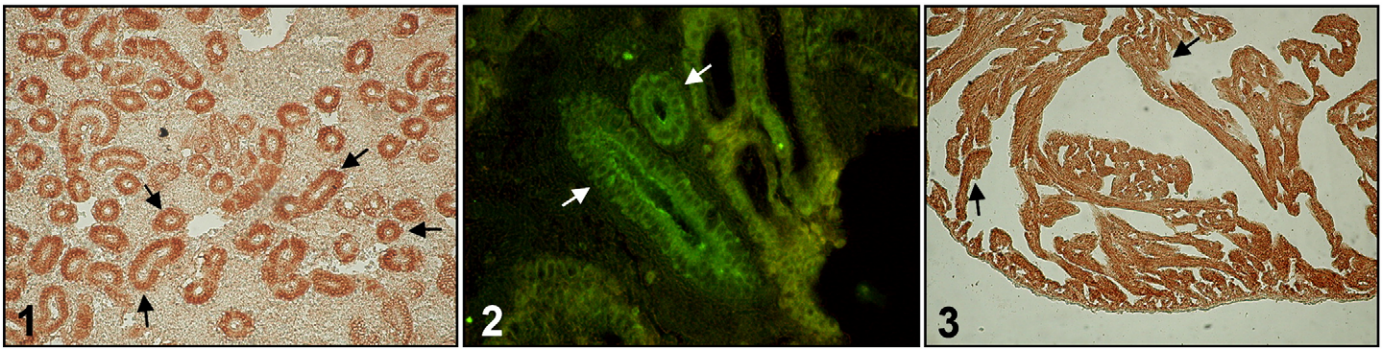

Fig. 8. Identification of sites of Mgp accumulation in kidney and in heart of turbot. 1— Localization of Mgp accumulation in the urinary tubules, by immunoperoxidase (arrows); 2 — immunofluorescence showed a more precise location of Mgp accumulation in the endothelium of urinary tubules (arrows) (400×); 3 — Detection of Mgp associated to cardiomyocytes in a transverse section of a juvenile specimen heart (400×).

growth zones, corroborates previous findings in vertebrates which indicated that Mgp appears first in sites that will be later calcified, functioning early in the bone formation process (Otawara and Price, 1986). The presence of Mgp in these sites may result from the fact that in fish, bone growth occurs (i) in the contact area of opposing vertebral body end plates (Witten et al., 2005), and (ii) at the periphery of arches and spines. Our work is in agreement with other studies which have also reported the presence of Mgp in the mineralization front in fish (Pinto et al. 2003 for gilthead seabream; Simes et al. 2003 for meagre; among other authors).

Furthermore, Mgp accumulation was evident in the matrix of vertebral end plates from turbot, a site near vertebrae growth zone. The positive staining with alcian blue in a contiguous slide seems to indicate that this matrix is still undergoing calcification. This result is in contrast with that found in meagre (Simes et al., 2003) where Mgp was restricted to cartilage but is in agreement with the results of Gavaia et al. (2006) for zebrafish and Senegalese sole.

Immunofluorescence and immunohistochemistry revealed accumulation of Mgp in the notochord cells, both chordoblasts and chordocytes. Since no reports of Mgp accumulation and/or expression in the notochord structures were found in the literature, this is the first report of Mgp accumulation in this structure. The presence of Mgp in this site could be related to a possible role of Mgp in teleost osteogenesis while also preventing local calcification, in agreement with its proposed function in fish and mammals.

4.4. In soft tissues, Mgp accumulates essentially in heart and kidney

Immunodetection of Mgp in soft tissues was positive in heart and kidney (Fig. 8), a finding that could be related to its alleged protection role against ectopic calcification of soft tissues. Alternatively, and since Mgp is present in the vascular system, it could be argued that the levels observed were due to the high degree of vascularization of these two organs. However, this explanation is unlikely since liver is also highly vascularized but no accumulation of Mgp was detected in this organ.

In kidney, accumulation of Mgp was immunodetected in the renal tubules corroborating the results obtained for the expression of this protein. Our results confirm previous reports of Mgp accumulation in kidney by Ortiz-Delgado et al. (2006) for blue shark and Zhao and Nishimoto (1996) for rat. The fact that only few articles report this accumulation might be the time scale of Mgp expression/accumulation in kidney, as demonstrated by Zhao and Nishimoto, 1996, and/or the possibility that the need for this protein in kidney may vary among species.

In this report, in addition to the presence of *mgp* mRNA detected in heart tissue, we were also able to detect Mgp accumulation in heart cells and ECM by immunostaining, suggesting that this accumulation could be related to its role as a calcification inhibitor. The only reference reporting a similar accumulation for Mgp in mammalian was in the work from Fraser and Price (1988), who were able to quantify Mgp in rat heart by RIA, detecting minute amounts compared to calcified tissues. These authors attributed its location to cardiomyocytes by immunofluorescence. The presence of Mgp in arterial walls, expressed by vascular smooth muscle cells, is also well documented, suggesting its involvement in the prevention of vascular calcification and atherosclerotic plate formation (Dhore et al., 2001; Engelese et al., 2001; Proudfoot and Shanahan, 2006). Thus, a similar effect in heart tissue is a likely possibility, since heart valves are also a site of calcification in pathological conditions (Price et al., 1998; Proudfoot and Shanahan, 2006). Mgp accumulation in heart structures of other fish (aorta and cardiac arterial bulbus) was previously described in Senegalese sole (Gavaia et al., 2006) and in blue shark (Ortiz-Delgado et al., 2006), confirming that a conservation of specific cardiac accumulation of Mgp is widespread among very different taxa which reinforces the hypothesis of a conserved function crucial for all studied vertebrates.

In conclusion, the results presented in this report strongly suggest that in turbot, Mgp expression and function is associated with regulation of mineralization, and provides further evidence confirming that bony fish are indeed useful models to study function, regulation and expression of Mgp. The identification of Mgp in the notochord cells is a novel finding and opens new perspectives for Mgp function in this tissue. In addition we report the intracellular localization of *mgp* in fish heart, in agreement with an older report for rat (Fraser and Price, 1988) which provides additional evidence for a conserved role for Mgp in heart tissue.

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