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Diverse cell-specific expression of myoglobin isoforms in brain, kidney, gill and liver of the hypoxia-tolerant carp and zebrafish

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SUMMARY

Myoglobin (Mb) is famous as a muscle-specific protein – yet the common carp expresses the gene (*cMb1*) encoding this protein in a range of non-muscle tissues and also expresses a novel isoform (*cMb2*) in the brain. Using a homologous antibody and riboprobes, we have established the relative amounts and cellular sites of non-muscle Mb expression in different tissues. The amounts of carp myoglobin (cMb) in supernatants of different tissues were just 0.4–0.7% relative to that of heart supernatants and were upregulated by two-to-four fold in liver, gill and brain following 5 days of hypoxic treatment. Brain exhibited both cMb proteins in western analysis, whereas all other tissues had only cMb1. We have also identified cells expressing cMb protein and *cMb* mRNA using immunohistology and RNA *in situ* hybridisation (RNA-ISH), respectively. Mb was strongly expressed throughout all cardiac myocytes and a subset of skeletal muscle fibres, whereas it was restricted to a small range of specific cell types in each of the non-muscle tissues. These include pillar and epithelial cells in secondary gill lamellae, hepatocytes, some neurones, and tubular epithelial cells in the kidney. Capillaries and small blood vessels in all tissues exhibited Mb expression within vascular endothelial cells. The *cMb2* riboprobe located expression to a subset of neurones but not to endothelial cells. In zebrafish, which possesses only one *Mb* gene, a similar expression pattern of Mb protein and mRNA was observed. This establishes a surprisingly cell-specific distribution of Mb within non-muscle tissues in both carp and zebrafish, where it probably plays an important role in the regulation of microvascular, renal and brain function.

Key words: immunohistology, *in situ* hybridisation, endothelial cells.

INTRODUCTION

Myoglobin (Mb) occupies a key place in the modern history of biology because it was the first protein with a resolved molecular structure (Kendrew et al., 1960) but also because it was ascribed an apparently straightforward function, namely the binding of oxygen. It is distributed widely throughout the vertebrates but is generally regarded as being expressed only in cardiac and oxidative skeletal myocytes (Ordway and Garry, 2004), where it binds to oxygen and delivers it during times of oxygen scarcity (Hochachka and Somero, 2002; Wittenberg and Wittenberg, 2003). Mb protein is particularly strongly expressed in diving mammals such as whales and seals, where it provides a reserve capacity for oxygen to sustain oxidative energy production when under water (Kooyman and Ponganis, 1998).

This picture has recently changed in two important respects. First, Mb is now recognised to display alternative functions, including the metabolism and scavenging of other diatomic gases, notably including nitric oxide (Brunori, 2001). During ischaemic episodes, it also acts as a nitrite reductase to generate nitric oxide, which in turn offers cytoprotection during ischaemia (Hendgen-Cotta et al., 2008; Rassaf et al., 2007). Indeed, knocking-out this function appears to be phenotypically more important than oxygen binding (Cossins and Berenbrink, 2008). Second, myoglobin transcript (*Mb*) expression has recently been demonstrated in several non-muscle tissues of the hypoxia-tolerant common carp, *Cyprinus carpio* (Fraser et al., 2006). Thus brain-, liver-, kidney- and gill-expressed *Mb* transcripts (*cMb*) and protein (cMb) expression was verified in liver using mass spectrometry. In that study, transcript amounts were increased several fold in these tissues following chronic hypoxia treatment, and liver Mb amounts were upregulated approximately three fold. Uniquely, the carp also possesses a second *mb* isoform (*cMb2*), whose transcript was expressed exclusively in the brain alongside the cardiac isoform (*cMb1*) (Fraser et al., 2006). The closely related goldfish also expresses a second *mb* isoform (Roesner et al., 2006).

This discovery of non-muscle expression of *mb* occurs against the backdrop of an expansion of the globin family, notably by the recent discovery of cytoglobin, neuroglobin and globins X and Y (Burmester et al., 2002a). These proteins are also distributed widely in non-muscle tissues (Hankeln et al., 2004), but their molecular functions, and even their wider physiological significance, are not well understood. Based on its focal distribution in retinal nerve cells, neuroglobin has been linked to the intense metabolic activity of neurones (Fuchs et al., 2004), whereas cytoglobin, with a wider tissue distribution, has been linked to the production of extracellular matrix proteins (Hankeln et al., 2004). Even haemoglobin, famous for its restriction to erythroid cells, has now been localised to other cell types (Bhaskaran et al., 2005; Liu et al., 1999; Newton et al., 2006). The expression of different globin genes across different tissues and the functional relationship of these to each other are thus major contemporary issues, and early ideas of myoglobin distribution need to be re-evaluated.

Here, we report the use of an anti-peptide cMb antibody and carp *cMb* riboprobes to quantify cMb protein amounts in different carp and zebrafish tissues and to identify cells that express cMb. We show that, although cMb is expressed in many different non-muscle

cell types across different tissues, it comprises just ~0.5% of that in cardiac tissue. We identify a particularly consistent expression across tissues in vascular endothelial cells of capillaries, which is consistent with a role in the regulation of vascular function, but we also reveal a range of other specific Mb-expressing cell types in other tissues. All tissues tested apart from the heart display increased Mb protein expression following hypoxia.

MATERIALS AND METHODS

Fish and hypoxia treatment

Carp (*Cyprinus carpio* L.) were obtained from a commercial fish farm. They were maintained in tanks of volume 2000 l at 30°C±0.1°C and a 16 h:8 h light:dark photoperiod, and each was provided with a recirculating water treatment system. The fish were fed daily at ~1% w/w body mass with commercial carp pellets. One group of carp was exposed to hypoxia at 10% saturation (0.8 mg O_2 l^{-1}) for 5 days, as described previously (Fraser et al., 2006). Zebrafish (*Danio rerio* Hamilton) were maintained in Aquatic Habitat (Apopka, FL, USA) polycarbonate tanks under a recirculating flow of water at 26±1°C and 14 h:8 h light:dark photoperiod with artificial dawn and dusk. They were fed twice daily on crushed Tetramin fish flakes.

Tissue preparation

For western analysis, small pieces of tissue from carp liver, gill, brain and heart were excised, rapidly frozen and stored at –20°C. For immunohistology (IH) and RNA *in situ* hybridisation (RNA-ISH), tissue samples of carp (liver, gill, brain, heart, intestine, kidney and epaxial skeletal muscle) and zebrafish (liver, gill, brain, heart and epaxial skeletal muscle) were excised and fixed in 4% paraformaldehyde (PFA; pH 7.4) for 24–48 h, followed by embedding into paraffin wax. Sections of thickness 3–5 μm were cut and mounted on poly-L-lysine-coated slides.

Production of antibody against myoglobin

The C-terminal segment of cMb2 (RDIDRYYKEIGFAG) was submitted for peptide synthesis, antibody production in rabbits (Covalab, Cambridge, Cambridgeshire, UK) and subsequent affinity purification using the peptide. This antibody was also found to recognise the near-identical C-terminal peptide region of carp cMb1, namely GDIDTYYKEIGFAG.

Western analysis

Protein extracts were prepared by homogenising tissue samples in 10 mmol l^{-1} Tris buffer (pH 8.0) containing Complete Mini Protease Inhibitor Cocktail Tablets (1 tablet/20 ml buffer; Roche Diagnostics, Burgess Hill, UK), followed by centrifugation at 10,000 ***g*** for 10 min at 4°C. Supernatants were decanted and stored frozen at –20°C. Protein concentration was determined (Bradford, 1976) using bovine serum albumin as a standard.

For all tissues other than heart, a sample of 25 μg of supernatant protein was electrophoresed on polyacrylamide gels. Heart samples gave very strong signals, and 0.1 μg supernatant protein was sufficient to give signals comparable to those of the other tissues. All supernatants were heated at 70°C for 10 min in 1× Laemmli buffer (Sigma, Poole, UK) before electrophoresis alongside PageRuler™ Prestained Protein Ladder (Fermentas, York, UK) used as a size marker. Electrophoresis and blotting were performed using precast NuPAGE® MES Gels employing the XCell *SureLock*™ Mini-Cell apparatus (Invitrogen, Paisley, UK), following the manufacturer's instructions. The corresponding blotting module for this unit was also used to transfer the protein onto the nitrocellulose membrane. The membrane was blocked overnight in 5% (w/v) non-fat milk (NFM) in Tris-buffered saline (TBS, pH 7.4). The antibody against cMb was incubated at 1:2000 dilution in 0.5% (w/v) NFM in TBS for 2 h and, after 5×5 min washes in TBS, the secondary antibody sheep anti-rabbit horseradish peroxidase (GE Healthcare, Amersham, UK) was incubated at 1:2000 dilution in 2% (w/v) NFM in 1× TBS for 2 h. The membrane was washed for 5×5 min in 1× TBS before application of ECL reagent (GE Healthcare) and exposure of the membrane to autoradiography film.

Actin was used as the loading control for brain and gill. A 1:500 dilution of an antibody against actin (actin H-15; AbCam, Cambridge, UK) in TBS was used for gill, overnight at 4°C, whereas a 1:1000 dilution in 0.5% NFM in TBS for 2 h at room temperature was used for brain. The secondary antibody was sheep anti-mouse horseradish peroxidase (GE Healthcare) and was used as described for the anti-rabbit antibody. Although several commercial loading control antibodies with apparent fish cross-reactivity, including tubulin, actin and β-actin, were examined, we were unable to achieve a consistent band signal for carp liver extracts. We excluded the commonly used GAPDH loading control from consideration because it is known to be induced by hypoxia (Yamaji et al., 2003). For liver, we therefore confirmed equivalent loading by staining with 0.1% (w/v) Ponceau S in 5% acetic acid. Heart samples were loaded at 0.1 μg per well, based on Bradford assay results, but variations in loading could not be determined owing to the very low amounts of protein used.

For the quantitative comparison of Mb amounts in multiple tissues, we used a Bio Dot SF slot-blotter (Bio-Rad, Hemel Hempstead, Herts) with 10 μg of protein for brain, gill and liver samples and 0.1 μg for heart samples. Protein supernatants were transferred by vacuum onto a nitrocellulose membrane together with three sheets of Whatman paper pre-soaked in TBS. The membrane was then blocked and incubated with the antibody against Mb, as described for the western transfer. Bands on autoradiography film were quantified densitometrically using a ChemiDoc XRS system (Bio-Rad) using Quantity One image-analysis software (Bio-Rad).

Immunohistology for Mb

The antibody against cMb and the peroxidase anti-peroxidase (PAP) method was employed, as described previously (Kipar et al., 1998), on tissue sections from carp and zebrafish. Briefly, following removal of paraffin by xylene and rehydration through graded alcohols, sections were incubated in methanol with 0.5% v/v H_2O_2 (Perhydrol 30%, Fisher Scientific, Loughborough, UK) for 30 min to inactivate endogenous peroxidase. Slides were pre-treated with protease for antigen retrieval [5 min wash with phosphate-buffered saline (PBS, pH 7.2) at 37°C, followed by 5 min incubation in 0.05% w/v protease (bacterial protease type XXIV, P8038, Sigma) in PBS, 3×5 min washes in ice-cold TBS and were then placed with coverplates in sequenza racks (Thermo Shandon, Pittsburgh, USA)]. Following a 5 min TBS wash, nonspecific binding of antiserum was blocked by incubation in 50% v/v swine serum in TBS for 10 min. Slides were incubated for 15–18 h at 4°C with the antibody against cMb diluted 1:500 in 20% swine serum in TBS. The slides were washed for 5 min in TBS and then incubated for 30 min at room temperature with swine anti-rabbit IgG (Dakocytomation, Glostrup, Denmark) diluted 1:100 in 20% v/v swine serum in TBS. A further 5 min TBS wash was followed by 30 min incubation at room temperature with rabbit PAP (Dakocytomation) diluted 1:100 in 20% v/v swine serum in TBS. After additional washing with TBS outside the sequenza racks, slides were incubated with stirring for 10 min with 0.5 mg ml^{-1} 3,3′ diaminobenzidine tetrahydrochloride (DAB,

Fluka, Buchs, Switzerland) and 0.01% v/v H_2O_2 (perhydrol 30%, Fisher Scientific) in 0.1 mol l^{-1} imidazole buffer, pH 7.1. Sections were counterstained for 30 s in Papanicolaou's haematoxylin (1:20 v/v in distilled water; Merck Eurolab GmbH, Darmstadt, Germany), followed by rinsing in running tap water for 5 min, dehydrated in ascending alcohols, cleared in xylene and mounted with DPX mountant (VWR International, Poole, UK) and cover slipped. Consecutive sections to those used for protein expression analysis were stained using normal rabbit serum at the same dilution to act as a negative control. In addition, other sections were incubated with the diluted antibody against cMb that had been preincubated with excess g42pMb peptide (10 μg ml^{-1}) for 1 h at 37°C, to test for residual protein binding activity.

Preparation of full-length cMb1 construct for recombinant expression in a zebrafish cell line

The full-length open reading frame for liver *cMb1* was PCR-amplified using the N-terminal (*Eco*RI) primer 5′gagaattcatggccgatcacgaactggttctgaaatgc3′ and C-terminal (*Bam*HI) primer 5′acggatccttaaccggcgaatccgatctccttgtagta3′ that included restriction sites at the 5′ end to allow subsequent directional cloning. 1 μl of an archived bacterial clone of *cMb1* (Williams et al., 2008) served as a template in a 50 μl PCR reaction using 0.5 μl Platinum Taq (5 units μl^{-1}; Invitrogen, Carlsbad, USA) and the supplied 10× buffer, together with the primers (0.5 μmol l^{-1} each), dNTPs (0.2 mmol l^{-1}) and $MgCl_2$ (1.5 mmol l^{-1}). Thermocycling was performed as follows: 1 cycle at 95°C for 2 min followed by 27 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 1 min 30 s and finally one extended polymerisation step at 72°C for 4 min. The resulting single PCR product was purified (Nucleospin, Machery Nagel, Germany), double-digested with *Eco*RI and *Bam*HI (New England Biolabs, Hitchin, UK) and subcloned into the vector pIRES2-EGFP (Clontech, Mountain View, CA, USA). We then used this vector to stably transfect the zebrafish cell line PAC-2, a fibroblast-like line derived from 24-h-old, trypsin-dissociated embryos. Transfection was performed using electroporation, and then stable clones were selected for neomycin resistance (Vallone et al., 2004).

Preparation of riboprobes for carp myoglobin transcripts

PCR primers capable of distinguishing between the two *cMb* isoforms were used to amplify short (<200 bp) PCR products for the production of riboprobes for RNA-ISH. Isoform-specific PCR primers for *cMb1* and *cMb2* were designed to two regions that varied between the cMb isoforms. Forward primers were designed to the DNA sequences encoding the N-terminal peptides MADHELV (*cMb1*; atggccgatcacgaactggtt) and MADYERF (*cMb2*; atggctgattacgagcggttt). Reverse primers were designed to the antisense DNA encoding the peptide sequences between positions 53 and 61, NAAVKAHG (cMb1; gccgtgggccttcaccgctgcgtt) and DTLVASHG (cMb2; accgtgggacgccaccaacgtgtc). These PCR products were ligated into pCRII cloning vector (Invitrogen), according to the manufacturer's instructions. Ligations were transformed into TOPO-OneShot chemically competent cells (Invitrogen). The resulting bacterial clones served as templates for checking the orientation of the gene in the cloning vector to determine which vector primer (M13F or M13R) was required to produce sense and antisense strands. By using single gene-specific primers in combination with a single vector primer, it was possible to determine the orientation of the cDNA insert by PCR. The identity of selected clones was confirmed by sequencing (Lark Technologies, Takeley, Essex, UK). PCR products from these clones were gel extracted (Nucleospin, Machery Nagel, Germany) to provide templates (1 μg) for dioxygenin riboprobe production (Roche, following the manufacturer's instructions). Before use in the ISH, a simple dot blot was performed to titre the riboprobes using an anti-dioxygenin antibody and alkaline phosphatase detection (see below).

RNA-ISH for *cMb1* and *cMb2*

RNA-ISH was performed on tissue sections from carp and zebrafish, as described previously (Kipar et al., 2005). Sections were xylene treated to remove paraffin and digested in proteinase K (1 μg ml^{-1}) at 37°C for 15 min. This was followed by post-fixation, acetylation and pre-hybridisation incubations. Hybridisation was undertaken at 37°C for 18 h with the *cMb1* (carp and zebrafish tissue sections) and *cMb2* (carp tissue sections only) riboprobes at a concentration of 1 μl/500 μl hybridisation mix (Zurbriggen et al., 1993). After hybridisation, slides were washed and stained with anti-DIG-AK-AP antibody (1:200, v/v) (Boehringer, Berkshire, UK) and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich, Poole, UK) [25 μl and 20 μl per 10 ml, with levamisole (Sigma-Aldrich) at 5 mg/10 ml]. Sections were counterstained for 10 s in Papanicolaou's haematoxylin (1:20 v/v in distilled water), followed by rinsing in tap water for 5 min. Slides were mounted with glycergel (Dakocytomation) and sealed with a coverslip. For each tissue, subsequent sections were incubated with the forward and reverse riboprobe, respectively. The reverse probe served as the negative control.

Statistical analysis

All data are presented as means ± s.d. for the stated number of independent observations. Statistical significance was determined using Student's *t*-tests.

RESULTS

Western analysis of myoglobin amounts

We generated an anti-peptide antibody directed against the most C-terminal peptide of cMb2. This antibody was tested in two ways. First, we demonstrated that prior adsorption of the g42pMb peptide to the antibody ablated any binding on the immunoblot against carp liver proteins (data not shown), demonstrating that there was no other detectable protein-specific binding activity in the antibody. Second, we tested antibody performance against supernatants from PAC-2 zebrafish cell lines expressing recombinant carp cMb1; g42pMb antibody (g42pMbAb) detected a single, distinct band by western analysis in induced cells but no binding in uninduced or control untransformed cells (data not shown). The estimated molecular mass of this band matched that expected from the translated gene sequence and corresponded with the single band for carp liver, heart and gill at 16 kDa (Fig. 1A). For brain, we identified a band of slightly greater molecular mass, and in some blots this was accompanied by a less dense band with a size that matched the 16 kDa bands for the other tissues. We conclude that the antibody detected both isoforms.

We first compared the Mb amounts in different tissues using slot blot analysis with supernatant preparations for each tissue from five replicate carp. Each band was quantified densitometrically, and the results for each tissue were calculated relative to the reference heart preparation (Fig. 1B). Heart supernatants were diluted by a factor of 100 to provide comparable band densities for all tissues. By serial dilution of a standard heart preparation, we demonstrated that the densities shown in Fig. 1B lay within the linear range of the detection curve (data not shown). Taking the heart over-dilution into account,

the amount of Mb in liver was 0.39±0.10% of that in heart tissue (100±10%). The corresponding value for gill was 0.74±0.08% and for brain was 0.60±0.12%.

Second, we determined the effects of chronic hypoxia on Mb amounts relative to the same tissues from normoxic control specimens (Fig. 1C). Hypoxia was associated with a 1.94±0.30-fold increase in brain Mb, a 4.06±1.21-fold increase in gill Mb and a 2.80±0.25-fold increase in liver Mb. Values for brain, liver and gill were significant (two-tailed Student's *t*-test; $P<0.05$). Hypoxia caused a nonsignificant reduction of 0.73±0.37 fold in heart Mb expression in part because the very low amounts of protein required could not be corrected for loading.

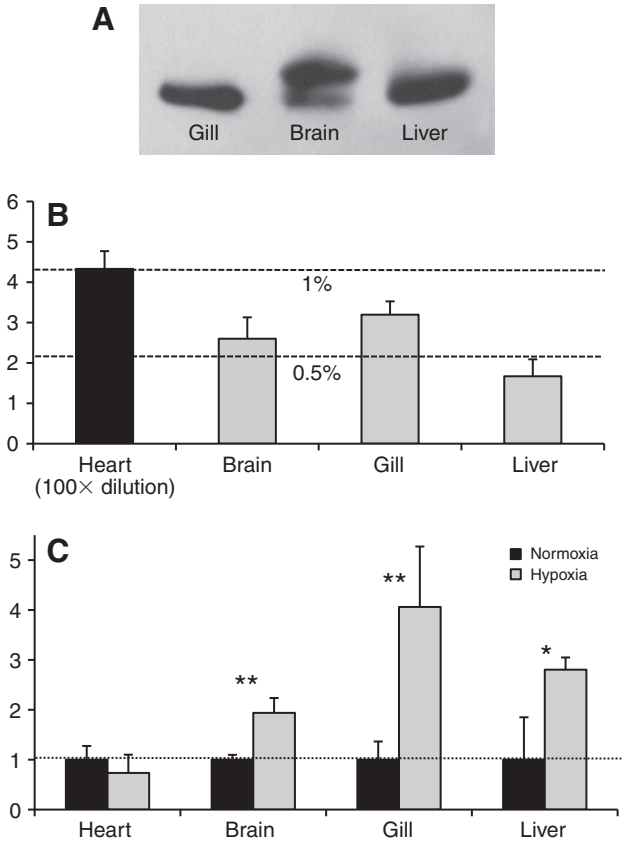

Fig. 1. (A) Representative western analysis of tissue Mb proteins. Gill and liver display an identical molecular mass, which, based on the sequenced cDNA (Fraser et al., 2006), has a predicted molecular mass of 15.744 kDa. Brain displays a larger protein, consistent with a predicted molecular mass of 16.174 kDa, together with a band at lower molecular mass that migrates alongside the single band (cMb1) observed in other tissues. The higher abundance of cMb2 compared with cMb1 protein in brain correlates closely to estimated transcript levels that have been reported previously (Fraser et al., 2006). Note that the presence of the less-abundant cMb1 isoform in brain is only visible in brain samples abundant in myoglobin. Protein loading was not equivalent between tissues. (B) Quantitative comparison of Mb protein expression in tissues of the common carp determined using immuno-detection on a dot-blot. The vertical axis represents arbitrary densitometric values. The heart supernatant was diluted 100× in order to provide for band densities that were similar to the undiluted supernatants of the other tissues. The horizontal dashed lines thus represent 1 and 0.5% of the mean heart Mb levels (N=4, where each sample was taken from a different specimen). (C) The effects of chronic (5 day) hypoxia treatment upon the tissue Mb levels relative to those in normoxia controls. Samples were subjected to western analysis, and each immunoblot contained normoxia- and hypoxia-treated specimens for one tissue, together with a 100× diluted heart extract as a positive control. Bands were analysed densitometrically and values were expressed relative to the mean for the normoxia control for each tissue (dashed line, N=5, except liver where N=4; *$P<0.01$ and **$P<0.001$).

Localisation of Mb expression within tissues

We employed immunohistology (IH), using the g42pMbAb, and RNA-ISH, using a riboprobe that detected both *cMb* genes (*cMb1/2*) and a second that was specific to *cMb2*, to identify Mb-expressing cells in formalin-fixed, paraffin-embedded tissue sections from carp and zebrafish. Again, we confirmed the *in situ* specificity of the antibody against cMb by pre-adsorption of the g42pMb peptide to the antibody, which ablated IH staining. Also, negative-control sections incubated with normal rabbit serum instead of the antibody against Mb failed to show any reaction (data not shown). For RNA-ISH, incubation of consecutive sections with the corresponding reverse probes served as negative controls, which exhibited no signals (data not shown).

Fig. 2 shows the expression of cMb protein and *cMb* mRNA in heart and skeletal muscle from carp. Cardiac myocytes (Fig. 2A) generally showed strong diffuse Mb expression, whereas *cMb1/2* mRNA signals were mainly seen focally within myofibres (Fig. 2B). Capillary vascular endothelial cells were also seen to express cMb protein (Fig. 2A). In the epaxial skeletal muscle, myofibres exhibited variable levels of cMb protein expression, represented by weak-to-intense diffuse positive staining (Fig. 2C). In some fields, numerous negative myofibres were seen. *cMb1/2* mRNA expression was again patchy within the myofibres, with signals mainly restricted to the cytoplasm close to myoseptae (Fig. 2D). In both tissues, the *cMb2* riboprobe did not yield any signals in the RNA-ISH (data not shown).

In carp liver, cMb protein expression was most intense in endothelial cells lining the sinusoids but was also seen in endothelial cells of some larger vessels (Fig. 3A). Hepatocytes generally exhibited faint-to-weak cytoplasmic and occasional nuclear protein expression. In hypoxic carp, however, multifocal areas of hepatocytes with strong cMb staining were observed (Fig. 3A, inset). Expression of *cMb1/2* mRNA was also variable in hepatocytes. It appeared absent in many animals, whereas, in others, strong hepatocyte signals were observed (Fig. 3B). Occasional endothelial cells exhibited signals (Fig. 3B, inset). In carp, the pancreas (Fig. 3A,B) is dispersed throughout the liver (Kong et al., 2002; Nakamura and Yokote, 1971; Youson et al., 2006). It exhibited a generally weak cMb protein and moderate *cMb1/2* mRNA expression within epithelial cells (Fig. 3). The *cMb2* probe yielded a signal of moderate intensity in some pancreatic epithelial cells (data not shown).

In the carp brain, the staining of endothelial cells, mainly in capillaries was again a consistent feature (Fig. 4). However, neurones were also identified as a source of cMb (Fig. 4). cMb protein expression was observed within the cytoplasm and the cell processes of neurons, including large motor neurones (Fig. 4A,C). The distribution and number of positive neurones varied between animals. The most-consistent expression was seen in the cerebellum, where cell processes in the molecular layer and a variable number of Purkinje cells were generally positive (Fig. 4A). The cortex exhibited occasional weakly positive neurones, and large motor neurones in the brain stem showed a variable expression (Fig. 4C). In the optic tectum, however, neurones were generally negative. The *cMb1/2* mRNA expression pattern was generally similar to the cMb protein expression (Fig. 4B,D), although with differing numbers of cells. Again, the distribution and number of positive cells varied between animals. In the cerebellum, Purkinje cells were occasionally positive and some neurones in the molecular layer often appeared

positive as well, whereas neurones in the granular layer were strongly positive in some animals (Fig. 4B) and weakly positive in others. The cortex also exhibited neurones positive for *cMb1/2* mRNA. Cells in the periventricular layer of the optic tectum were often positive, and positive neurones were also seen in the thalamus and the brain stem (Fig. 4D). Cells showing a signal for *cMb2* mRNA (representing the brain-specific Mb isoform) were generally sparse. Some neurones in the cortex (Fig. 4E) and some neurones in the

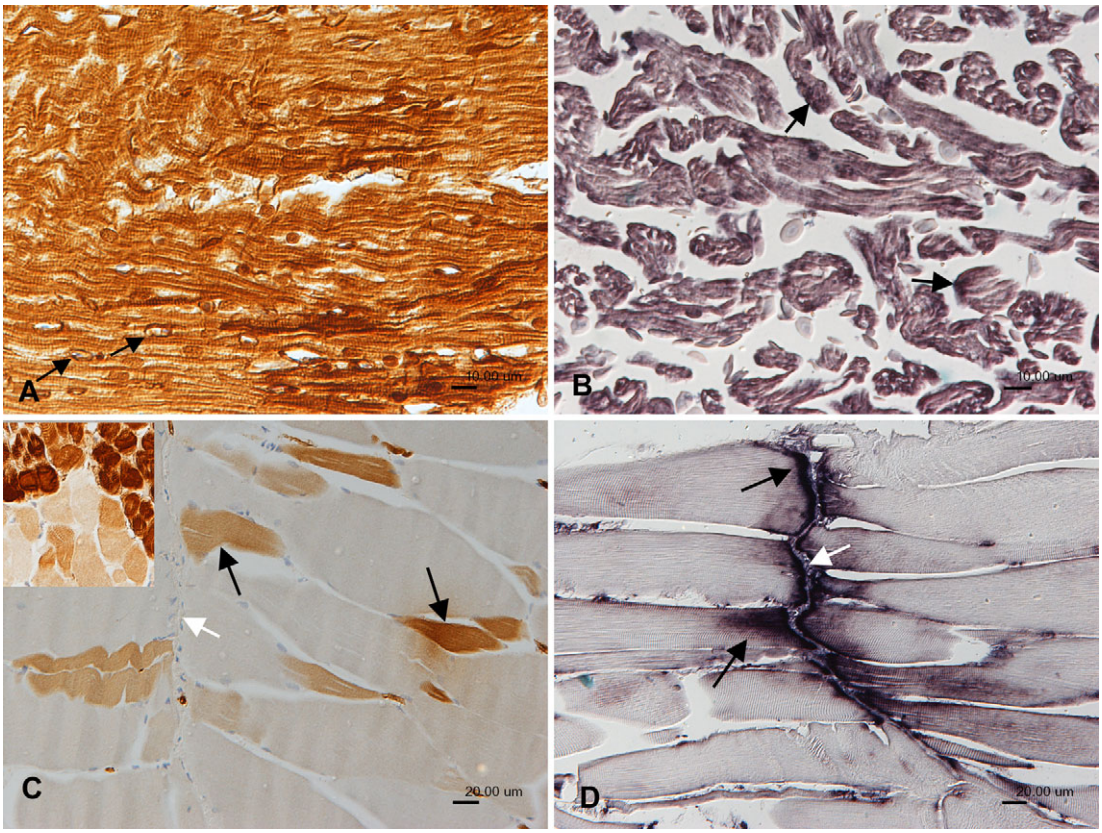

Fig. 2. Carp myoglobin protein and mRNA expression in heart (A,B) and epaxial muscle (C,D). (A) All cardiac myocytes exhibit strong, diffuse cMb protein expression. In addition, endothelial cells in small capillaries stain positive for cMb (arrows). (B) *cMb* mRNA expression is seen in all myocytes, but often as a focal cytoplasmic signal (arrows). (C) In the epaxial skeletal muscle, a small proportion of myofibres exhibit diffuse cytoplasmic protein expression (black arrows). White arrow: myoseptum. Inset: a cross-section of muscle shows the high variation in staining intensity between myofibres. (D) *cMb* mRNA expression is seen as a focal cytoplasmic signal, mainly at the periphery of the fibre (black arrows), close to the myoseptum (white arrow). In A and C, rabbit anti-cMb peptide was used; in B and D, RNA-ISH with the *cMb1/2* riboprobe was used. Scale bars indicate 10 μm for A and B, and 20 μm for C and D.

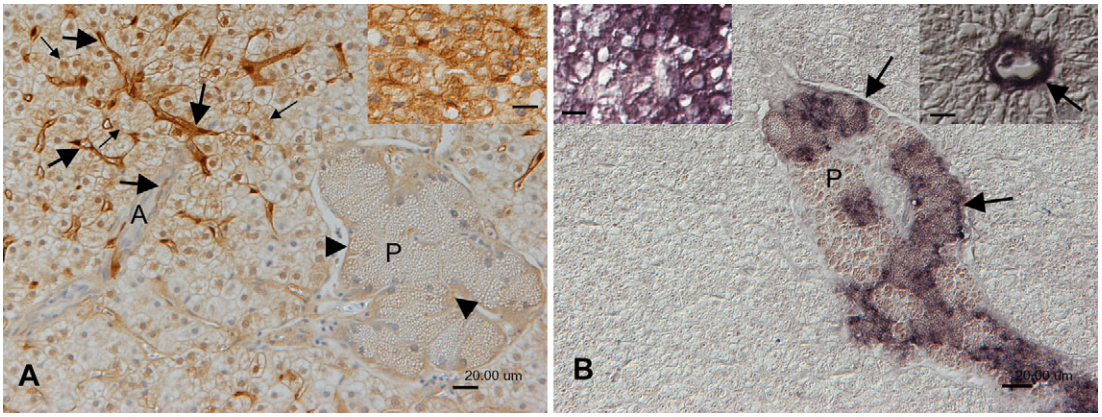

Fig. 3. Myoglobin protein and mRNA expression in liver and pancreas of carp. (A) cMb protein expression is most intense in endothelial cells lining the sinusoids (large arrows) but is also seen in endothelial cells of some larger arteries (large arrow labelled A). Hepatocytes generally exhibit faint-to-weak cytoplasmic and occasional nuclear staining (small arrows). In particular, in hypoxic carp, multifocal areas of hepatocytes that exhibit strong cMb staining are apparent (inset). The exocrine pancreatic epithelial cells (P) generally show faint cytoplasmic cMb expression (arrowheads). (B) *cMb* mRNA expression is variable in hepatocytes, represented by an apparent lack of signal up to a strong cytoplasmic signal (left inset). Occasional endothelial cells, in particular of central veins, exhibit a signal (right inset, arrow). The exocrine pancreatic epithelial cells (P) generally show moderate cytoplasmic mRNA expression (arrows). In A, rabbit anti-cMb peptide was used; in B, RNA-ISH with the *cMb1/2* riboprobe was used. Scale bars indicate 20 μm.

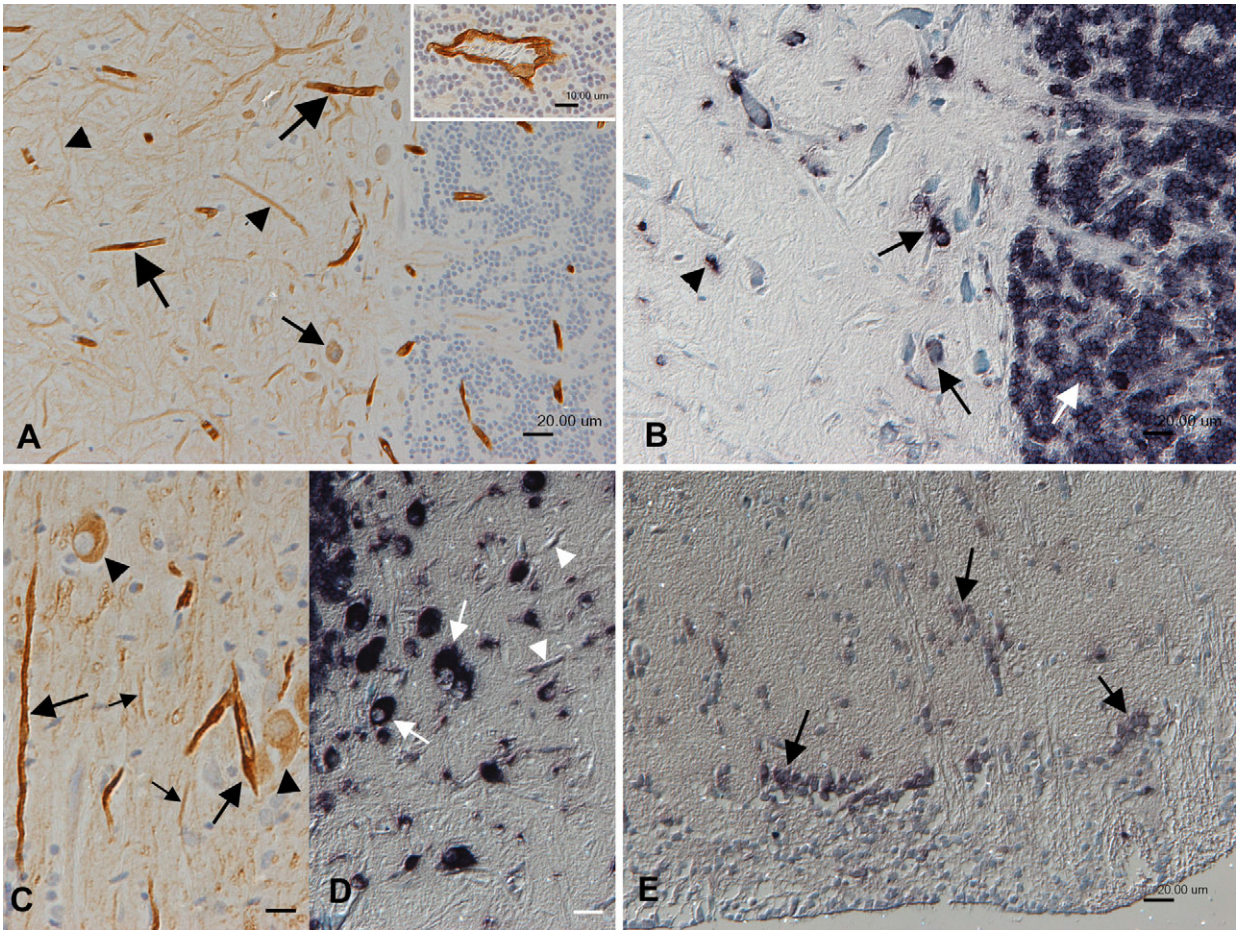

Fig. 4. Myoglobin protein (A,C) and mRNA (B,D: *cMb1/2*, E: *cMb2*) expression in the carp brain. (A,B) Cerebellum. (A) cMb protein expression is most intense in endothelial cells of capillaries (large arrows) and small blood vessels (inset). Weak-to-moderate cytoplasmic staining is seen in some Purkinje cells (small arrow) and in neuronal cell processes (arrowheads). Erythrocytes are negative for cMb (inset). (B) *cMb1/2* mRNA expression is seen in occasional neurons in the molecular layer (arrowhead), in some Purkinje cells (arrows) and in all cells in the granular layer (white arrow). (C,D) Brain stem. (C) cMb protein expression is seen in vascular endothelial cells (large arrow), in the cytoplasm of large motor neurones (arrowheads) and in cell processes (small arrows). (D) Large motor neurones (arrows) and vascular endothelial cells (arrowheads) are also positive for *cMb1/2* mRNA. (E) Cortex. *cMb2* mRNA expression is very limited and restricted to small aggregates of neurons (arrows). In A and C, rabbit anti-cMb peptide was used; in B and D, RNA-ISH with the *cMb1/2* riboprobe was used; in E, RNA-ISH with the *cMb2* riboprobe was used. Scale bars indicate 20 μm for A, B and E; those in the inset to A, and in C and D are 10 μm.

periventricular layer of the optic tectum exhibited positive signals. There was no evidence of glial cell Mb expression. Furthermore, erythrocytes were negative for both cMb protein and *cMb1/2* and *cMb2* RNA (Fig. 4A, inset).

In the eye, vascular endothelial cells were shown to express cMb protein and the *cMb1/2* RNA (data not shown). In the retina, a moderate number of cells in the outer granular layer and rods and cones exhibited weak-to-moderate cMb protein expression, but were generally negative for *cMb1/2* RNA. The *cMb2* riboprobe did not yield any signal.

In the gills (Fig. 5), we observed strong cMb protein and *cMb1/2* RNA expression within pillar cells, where cMb protein was located both in the main part of the cells and in the flanges that extend around the capillary spaces (Fig. 5A). cMb was also evident in epithelial cells covering the lamellae. While cMb protein was expressed less intensely in cells in areas between secondary lamellae, *cMb1/2* mRNA signals in these cells were generally strong (Fig. 5B). This is the site of chloride cell accumulation, although we were unable to discriminate these from other epithelial cells. Chondrocytes of the branchial arches appeared negative or only weakly positive for cMb protein but showed strong *cMb1/2* mRNA signals (Fig. 5A,B). The *cMb2* probe did not yield any signals in the RNA-ISH (data not shown).

In the head kidney, variably intense cMb protein expression was evident in nephron tubules, ranging from a weak-moderate to a strong, diffuse cytoplasmic reaction in individual to all epithelial cells in tubular cross-sections (Fig. 6A,B). There was no evidence of segment-specific (e.g. proximal or distal tubules) Mb protein expression or expression specific to certain nephrons, in which the whole tubule expressed Mb. RNA-ISH for *cMb1/2* mRNA showed a similar staining pattern (Fig. 6C,D). Glomerula were generally negative for both Mb protein and *cMb1/2* mRNA (Fig. 6A,B). The associated haematopoietic tissue exhibited variable numbers of cMb protein and *cMb1/2* mRNA-positive cells (Fig. 6B,D), and cMb protein expression by capillary endothelial cells was observed as well (Fig. 6B). RNA-ISH with the *cMb2* probe yielded a signal in occasional tubular epithelial cells (data not shown).

Cross-sections through the small intestine (Fig. 7) revealed cMb protein expression within capillary endothelial cells in all layers of the intestinal wall, including the rugae, submucosa and muscle layers

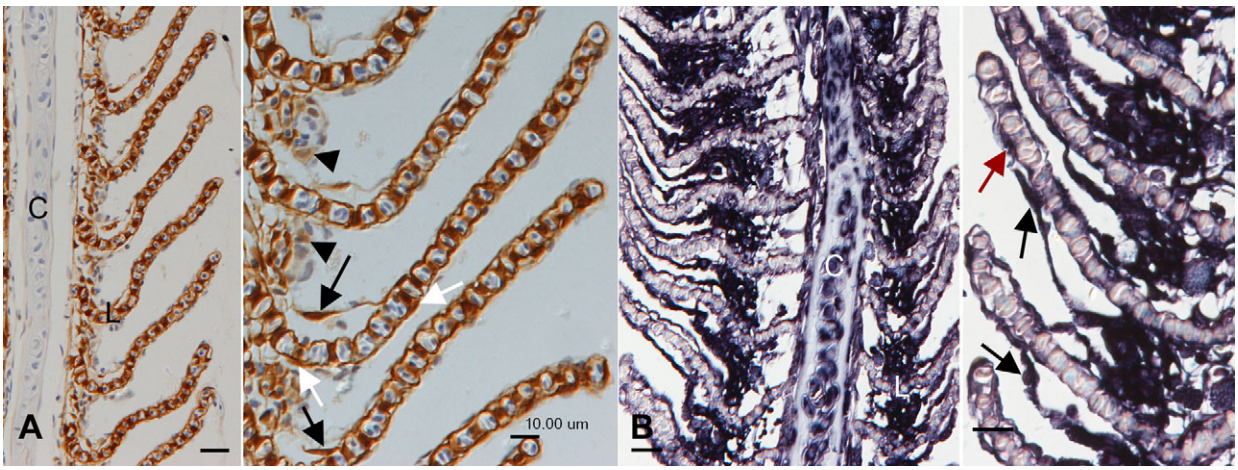

Fig. 5. Myoglobin protein (A) and mRNA (B) expression in the carp gill. (A) On the left, an overview shows cMb protein expression in the lamellae (L), whereas the cartilage (C) is negative (scale=20 μm). On the right, a magnified view of the secondary lamellae highlights intense diffuse cytoplasmic cMb protein expression by pillar cells (white arrows) and lamellar epithelial cells (black arrows), whereas staining of chloride cells is generally less intense (arrowheads; scale=10 μm). (B) On the left, an overview shows *cMb mRNA* expression in the lamellae (L) and the chondrocytes in the cartilaginous (C) skeleton (scale=20 μm). On the right, a closer view of the secondary lamellae highlights intense diffuse cytoplasmic *cMb* mRNA expression by lamellar epithelial cells (black arrows) and chloride cells (arrowheads), whereas the signals in pillar cells are predominantly in the flanges (red arrow; scale=10 μm). In A rabbit anti-cMb peptide was used; in B, RNA-ISH with the *cMb1/2* riboprobe was used.

(Fig. 7A,B). In addition, smooth muscle cells exhibited faint staining (Fig. 7B). RNA-ISH confirmed *cMb1/2* mRNA expression by capillary endothelial cells but also identified strong signals in neurones of both the submucosal and intermyenteric plexi (Fig. 7C,D) and in intestinal epithelial cells (Fig. 7C). The *cMb2* probe did not yield any signals in the RNA-ISH (data not shown).

Fig. 8 shows the expression of Mb protein and mRNA in corresponding tissues from zebrafish. As with carp, cardiac myocytes (Fig. 8A) generally showed strong, diffuse protein expression, and, in the epaxial skeletal muscle, myofibres exhibited the same variable Mb protein expression, represented by weak-to-intense diffuse cytoplasmic staining (Fig. 8B). mRNA expression was present and similar in its distribution to that found in carp (data not shown). Capillary vascular endothelial cells generally showed strong cMb expression both in muscle and liver (Fig. 8B,C). In the liver, hepatocytes were mainly negative for both cMb protein (Fig. 8C) and mRNA (data not shown). In gills, both pillar cells and lamellar epithelial cells were found to contain cMb protein and mRNA, again in a pattern similar to that of the carp (Fig. 8D). In the brain, both cMb protein and mRNA expression were observed in neurones and capillary endothelial cells (Fig. 8E,F), as seen in carp brain. In the intestine, Mb protein expression was also detected in vascular endothelial cells and, weakly, in smooth muscle cells, and, like in carp, *cMb* RNA was present in neurones in mural plexi and in some epithelial cells (data not shown).

DISCUSSION

Quantification of protein amounts in tissues

We have developed a homologous and very effective anti-peptide cMb antibody. The binding of this to cMb protein was demonstrated by the detection of the recombinant Mb1 in an induced zebrafish PAC-20 cell line but not in uninduced cells, with a product that conforms to the predicted molecular mass. Western analysis of tissue protein yielded just one band in all tissues and two bands in brain. All of these bands conformed to the predicted molecular masses for Mb1 and Mb2, respectively, which is consistent with the antibody specifically detecting only the Mb proteins.

Using this antibody preparation, we have quantified the relative amounts of Mb protein expression across tissues of the carp. We found considerable variation between tissues. Thus, gill and brain possessed 1.9- and 1.5-fold, respectively, more Mb protein than liver, but all non-muscle tissues, including gill, liver and kidney, expressed very small amounts of Mb (~0.5%), relative to that of the heart. All these data were normalised to soluble protein in post-mitochondrial supernatants. The antibody detected both isoforms in brain, and we were unable to quantify their separate contributions, but the western analysis indicates that the amount of cMb2 was several fold greater than that for cMb1.

Our immunohistological analysis of cellular Mb location indicates that the low levels of tissue expression relate to the restriction of Mb to a similarly small proportion of cells within these tissues. This and the resulting trace levels of tissue/organ expression are perhaps the principal reasons why non-muscle Mb lay undiscovered for so long, particularly as the Mb concentration would be too low to affect tissue colouration, as in cardiac and skeletal muscle. Another reason is the widespread but evidently mistaken belief that this well-known protein has such a clearly defined oxygen-binding function and restricted tissue distribution as to remove the need to question its role. The highly cell-specific pattern of expression means that, although gross tissue/organ concentrations might be low, the cellular concentration of Mb in expressing cells might not be different from those of the heart, where 100% of the cardiac myocytes contain Mb at concentrations of 100–400 $\mu mol\,l^{-1}$. This conclusion greatly affects the discussion of potential functions of Mb in non-muscle tissues, given the potential for both oxygen buffering and enzymatic roles.

Cellular localisation of Mb in non-muscle tissues

Using immunohistology, we report the localisation of cMb in a surprising diversity of cell types within different non-muscle tissues, results that were both confirmed and extended by RNA-ISH. Cells expressing cMb include neurones in brain and intestinal plexi, hepatocytes, the respiratory epithelium and pillar cells of the gill secondary lamella, and in the nephron, where some but not all tubular

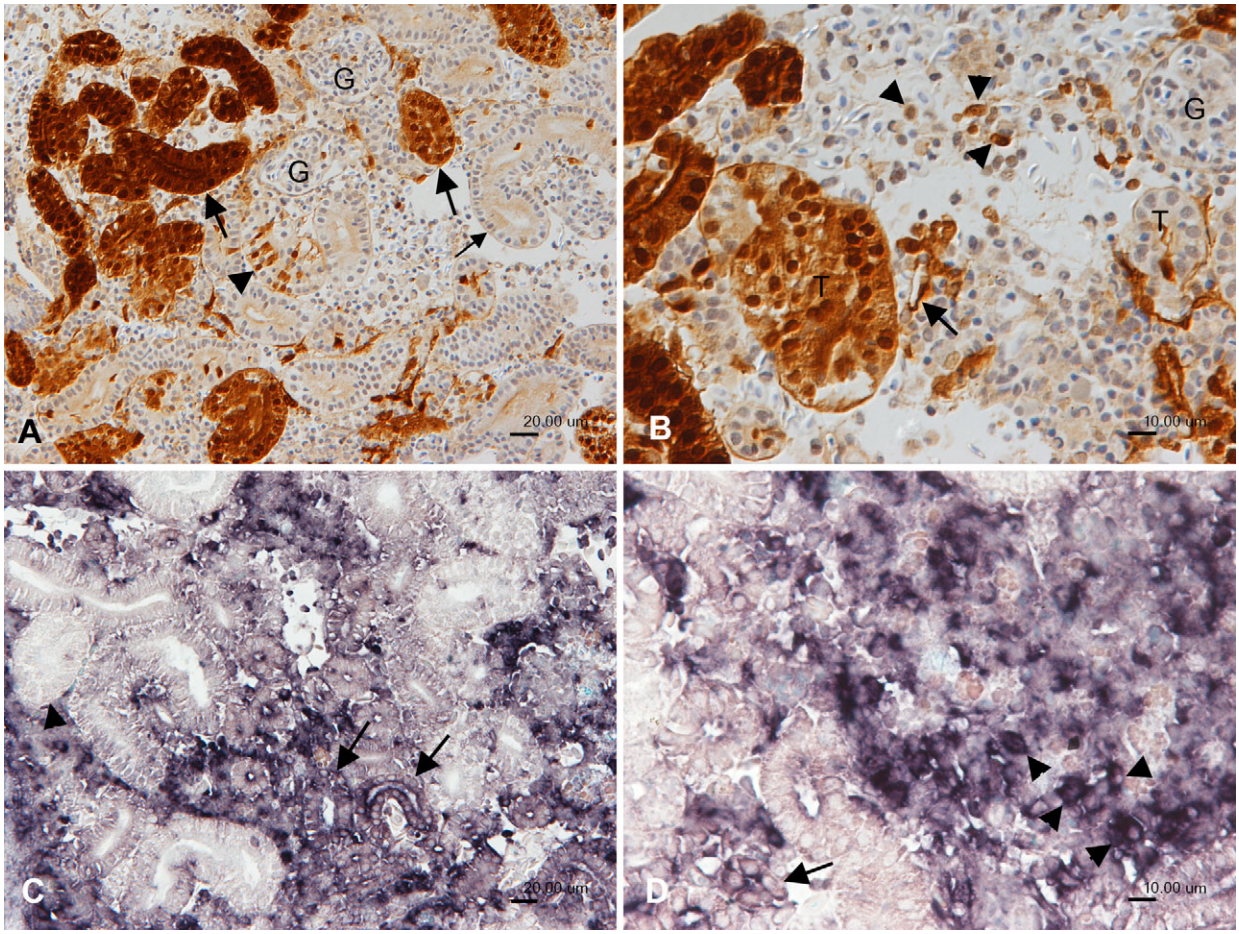

Fig. 6. Myoglobin protein (A,B) and mRNA (C,D) expression in the carp kidney. (A,B) Mb protein expression is seen in tubular epithelial cells and was represented by a diffuse cytoplasmic and often nuclear staining of individual (arrowheads) or all epithelial cells (arrow) in a tubular cross-section. Glomerula (G) were negative for Mb. (B) An area of haematopoietic tissue is included where several haematopoietic cells expressed Mb protein (arrowheads). Capillary endothelial cells expressing Mb protein were also observed (arrow). T, tubule; G, glomerulum. (C,D) RNA-ISH showed a pattern of *cMb* mRNA expression similar to the pattern of protein expression. In addition to tubular epithelial cells (arrows), haematopoietic cells (arrowheads) exhibited a diffuse cytoplasmic signal. In A and B, rabbit anti-Mb peptide was used; in C and D, RNA-ISH with the *cMb1/2* riboprobe was used. For A and C scale bars indicate 20 μm and for B and D they indicate 10 μm.

epithelial cells exhibited intense cMb protein expression. Finally, we have produced evidence of *cMb1/2* transcript expression in the mucosal epithelium of the intestine, although we were not able to link this to protein expression.

Certainly the most consistent and surprising observation among all tissues examined was the expression of cMb in endothelial cells lining the capillary bed and small blood vessels. These observations were supported by both immunohistology and RNA-ISH for *cMb1/2*, and essentially identical results were observed in the zebrafish. This points to a broad role for cMb1 in microvasculature function in capillaries with continuous (such as in muscle and brain), fenestrated (intestinal mucosa) as well as discontinuous (liver) endothelial linings (Pavelka and Roth, 2005). Brain expresses two *Mb* genes, but, because the *cMb2* riboprobe failed to bind to capillaries, we conclude that only *cMb1* was expressed by endothelial cells.

Does Mb function in microvascular regulation?

Regarding a microvascular function in fish, it is perhaps significant that the pillar cells of the gill were strongly Mb positive. These cells are modified endothelial cells that bridge the two sheets of epithelial cells that form the sides of the plate-like secondary lamella (Wilson and Laurent, 2002). They line and thereby define the spaces through which blood flows beneath the effective respiratory surface of the gill (Evans et al., 2006; Wilson and Laurent, 2002). Compared with pavement cells, pillar cells are rich in mitochondria and thus would be expected to be metabolically active. They also possess microfilaments, which are thought to have contractile properties by which they regulate blood flow (Bettex-Galland and Hughes, 1973), and there is evidence of paracrine control *via* adjacent neuro-epithelial cells (Jonz and Nurse, 2003). In these various respects, the pillar cells are similar to the mammalian pericytes described by Attwell and colleagues (Peppiatt et al., 2006).

Given that Mb now has an established role in nitric oxide metabolism (Brunori, 2001) and that NO is involved in vascular control (Lowenstein et al., 1994; Moncada et al., 1991), the presence of Mb in endothelial cells suggests a function in the regulation of blood flow. NO produced by the vascular endothelium is well known to diffuse to the underlying smooth muscle of pre-capillary arterioles, where it causes vasodilation. However, recent work in the mammalian brain indicates that the regulation of blood flow might also be effected locally at the level of the capillary, through the action of contractile pericytes that are positioned at regular intervals along the capillary (Peppiatt et al., 2006). These cells are under autonomic control (glutamate), which, by stimulating NO

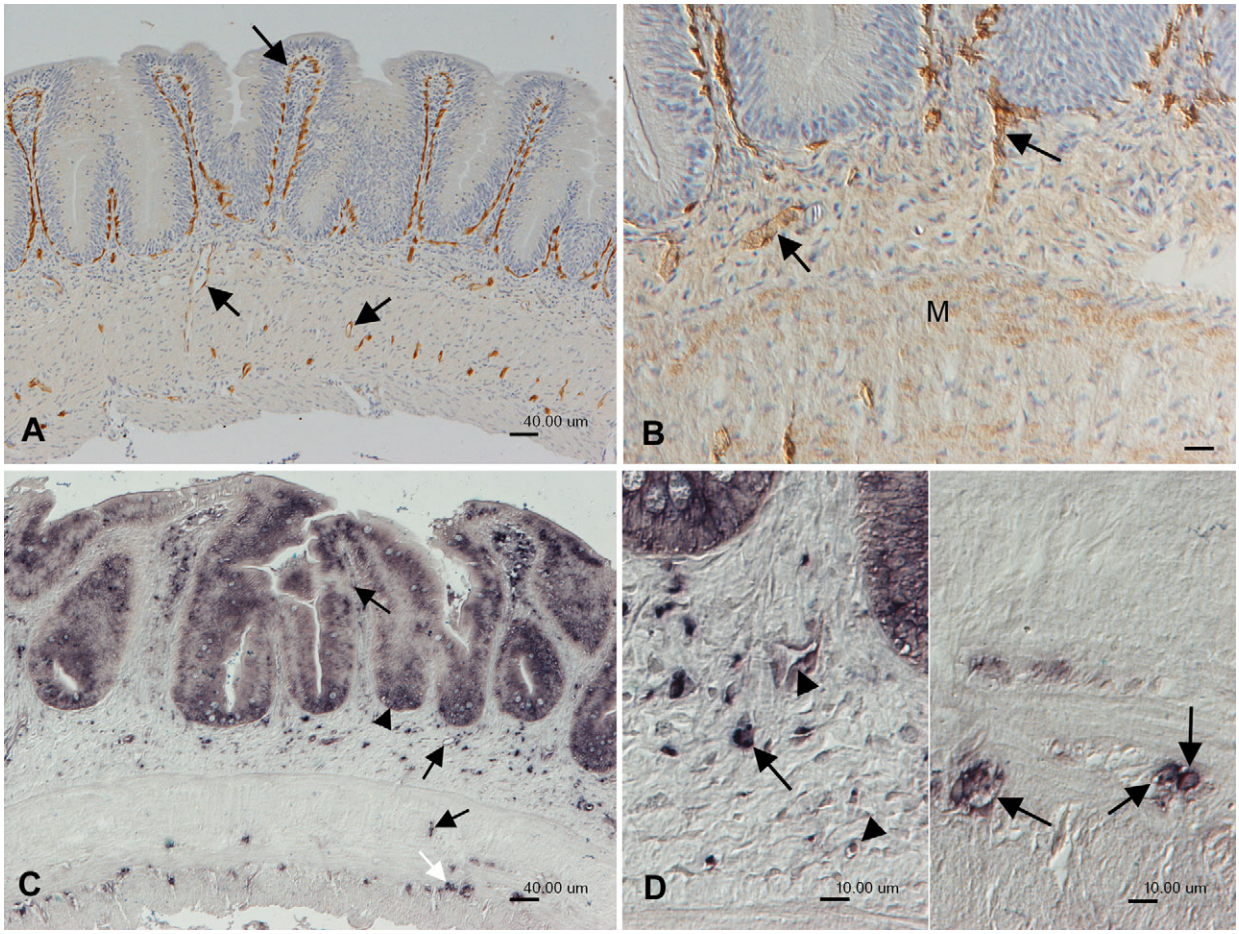

Fig. 7. Myoglobin protein (A,B) and mRNA (C,D) expression in the carp intestine. (A) Intense cMb protein expression is seen in endothelial cells of capillaries in all layers of the intestinal wall (rugae and smooth muscle layers, arrows). (B) Higher magnification of submucosa and muscle layers (M) confirms endothelial cMb protein expression (arrows) and weak staining for cMb in smooth muscle fibres. (C) Endothelial cells of capillaries in all layers of the intestinal wall exhibited *cMb* mRNA (black arrows). In addition, neurones in the myenteric plexus (white arrows) and intestinal epithelial cells (arrowhead) stain positive. (D) Higher magnifications of the submucosa and smooth muscle layers confirmed *cMb mRNA* expression by neurones in submucosal (arrow on left picture) and intermyenteric (arrows on right picture) plexi and capillary endothelial cells (arrowheads on left picture). In A and B, rabbit anti-cMb peptide was used; in C and D, RNA-ISH with the *cMb1/2* riboprobe was used. Scale bars indicate 40 μm for A, 20 μm for B, 40 μm for C and 10 μm for D.

production, might cause pericyte relaxation and capillary dilation (Peppiatt et al., 2006). Indeed, capillaries possess a more intense noradrenergic innervation compared with that of arterioles (Cohen et al., 1997), and, in pulmonary capillaries of mammals, the application of circumferential stretch induces the production of nitric oxide by the endothelial nitric oxide synthase (Kuebler et al., 2003).

Fish capillaries also appear to possess local control systems, and these might well involve a signalling mechanism based on the production and breakdown of NO (Fritsche et al., 2000b; Söderström et al., 1995). The endothelial Mb thus might act as a nitrite reductase, with nitrite acting as an endocrine NO reservoir. A conversion of nitrite to NO does occur in zebrafish (Jensen, 2007), and NO generated by endothelial cells affects vascular function in zebrafish larvae (Fritsche et al., 2000a). Finally, fish experience environmental fluctuations in nitrite levels that might have physiological effects (Jensen, 2007; Jensen, 2003).

Addressing questions regarding the microvascular functions of *Mb* will require the production of a *Mb*-knockout fish for comparison with a wild-type fish. Currently, this is not feasible for carp, but it is increasingly possible for the zebrafish. We demonstrate here that zebrafish Mb displays the same tissue distribution and microvascular location as in carp, and so examination of vascular function in zebrafish lacking *Mb* presents a viable direction for future work.

Brain Mb and functions of cMb2

RNA-ISH of the brain using the *cMb2* riboprobe suggests that cMb2 is expressed only in neurones, whereas the *cMb1/2* riboprobe labels both neurones and capillary endothelial cells. We therefore conclude that the endothelial cells are exclusively populated with cMb1 protein. Overall, the distribution of neurones expressing *cMb2* is very restricted (some neurones in the cortex and in the periventricular layer of the optic tectum), whereas *cMb1/2* and cMb protein expression was more widespread (cerebellum, cortex, brain stem, optic tectum). Interestingly, the number of positive neurones varied greatly between individual fish. Although we previously found using microarray assessment that *cMb2* transcription was not upregulated by chronic hypoxia (Fraser et al., 2006), Roesner and colleagues (Roesner et al., 2006) using RT-PCR found a two-fold increase in goldfish Mb2. We now show a similar upregulation at the level of protein in carp, this being a more definitive indication of a functional tissue response to hypoxia. Owing to their closely matched molecular masses, we have not been able to quantify separately responses of

the cMb1 and cMb2 isoforms at the protein level, but, in those gels where separation of bands was observed, the relative amount of cMb2 was substantially higher than cMb1. Again, the most direct means of addressing the function of *Mb2* is *via* comparison of a *Mb2* mutational knockout with the wild-type control. However, generation of knockout lines is not currently a feasible proposition with either of the two species currently known to possess two *Mb* isoforms, namely carp and goldfish. As a result, progress in defining a function for Mb2 is likely to be less rapid than for Mb1.

cMb2 has an additional, externally positioned cysteine group compared with cMb1 (Fraser et al., 2006). This might endow NO binding and buffering functions, as proposed for cysteine 13 in

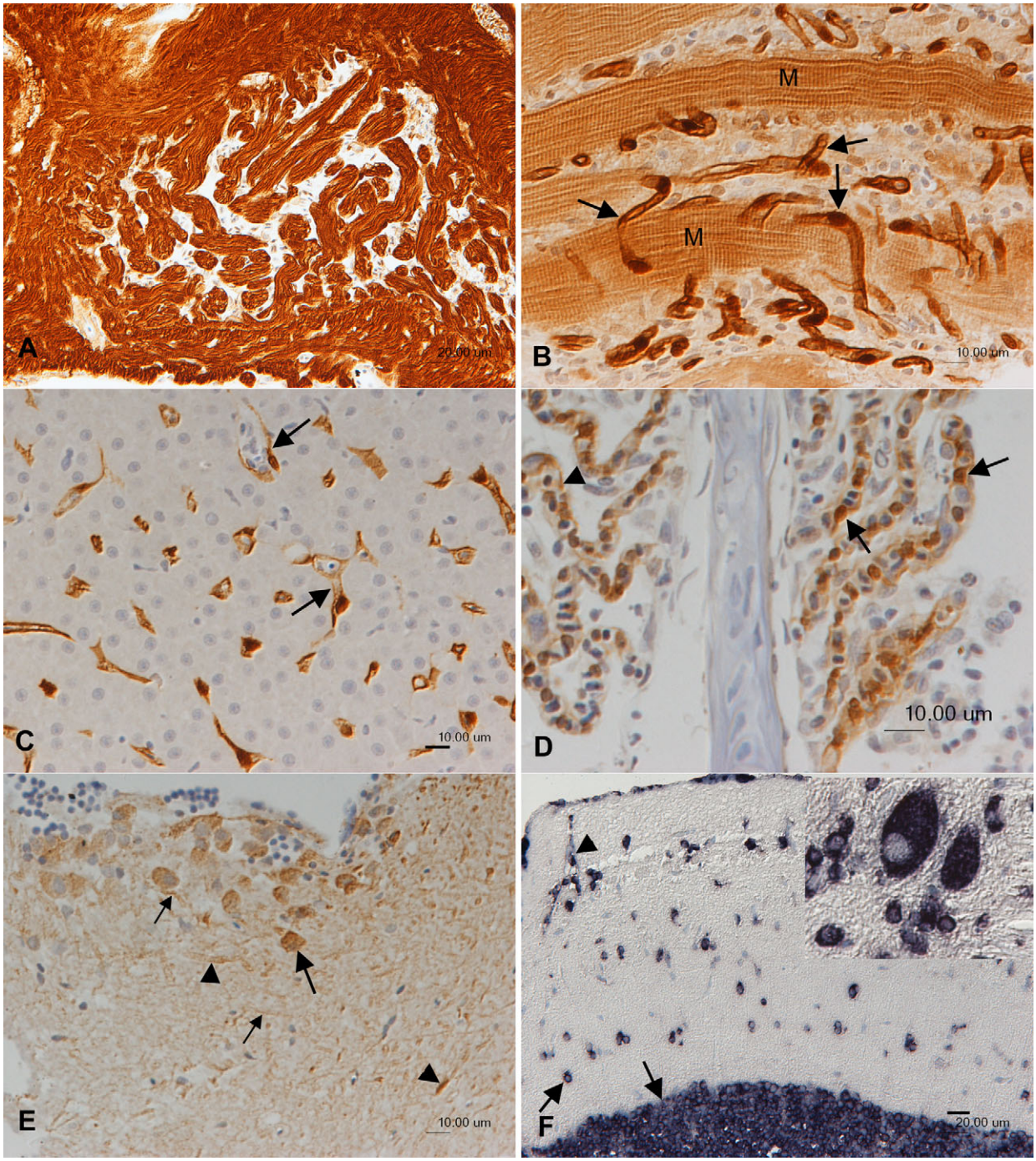

Fig. 8. Myoglobin protein and mRNA expression in various tissues of zebrafish. (A) All cardiac myocytes exhibit strong Mb protein expression. (B) In the epaxial skeletal muscle, myofibres (M) exhibit diffuse cytoplasmic Mb protein expression. Capillary endothelial cells stain strongly positive (arrows). (C) In the liver, strong cMb protein expression is seen in endothelial cells lining the sinusoids (arrows). Hepatocytes stain negative. (D) The gills show cMb protein expression by pillar cells (arrow) and lamellar epithelial cells (arrowhead). (E) Brain cortex. cMb protein is seen in neuronal cell bodies (large arrow) and cell processes (small arrows) as well as in capillary endothelial cells (arrowheads). (F) RNA-ISH identifies *cMb* mRNA expression in several types of neurones, such as Purkinje cells in the granular layer in the cerebellum (arrows), large motor neurones in the hypothalamus (inset) and in capillary endothelial cells (arrowhead). In A–E, the PAP method and rabbit anti-cMb peptide were used; in F, RNA-ISH with the *cMb1/2* riboprobe was used. Scale bars indicate 20 μm for A and F, 10 μm for B–E.

human Mb (Rayner et al., 2005), to constitute an additional brain-specific store for vasoactive NO or other small ligands, analogous to the mechanism proposed for cysteine 93 in the β-subunit of human haemoglobin (Hare and Stamler, 2005; Luchsinger et al., 2003). This latter cysteine is highly conserved in the haemoglobins of homeotherms (mammals and birds), yet we find it absent from most fish haemoglobins, which might make the presence of the extra cysteine in cMb2 especially significant. If this structural feature of cMb2 is acting as a small ligand buffer or reservoir then this function should be sensitive to ablation of this second cysteine site. Testing the *in vivo* function of these positional substitutions in Mb function will require production of fish strains in which the endogenous gene has been substituted by an engineered gene coding for a modified protein. Alternatively some metabolic functions of substitutions can be addressed by *Mb* expression in cell cultures.

So far as we know, carp and goldfish are the only vertebrates known to possess more than one Mb isoform. The brain-specific isoform is likely to arise from a genome duplication event in the cyprinid lineage within the past 12–15 million years, after the divergence with the zebrafish lineage (David et al., 2003). We show here that the single zebrafish gene appears to provide the same expression profile in the brain as the two isoforms in the carp, which suggests that the novel carp isoform takes over at least part of the neuronal expression pattern already evident in the common ancestor of the carp and zebrafish. At this stage, we have no evidence that the *cMb2* offers an expression pattern that is different from that in zebrafish.

Effects on cellular respiration

In the mammalian heart, NO inhibits cellular respiration and limits the generation of reactive oxygen species (ROS) after ischaemia-reperfusion (IR) injury, thereby protecting myocytes from oxidative injury. Again, nitrite has been invoked as the bio-available source of this NO, and nitrite treatment reduces IR injury in the isolated rat heart. There is now good evidence that this NO can be generated by Mb. Thus, ectopic expression of the gene encoding Mb in rat liver offers protection against IR injury (Nitta et al., 2003). Second, the heart of the $Mb^{-/-}$ knockout mouse is more sensitive to infusion of ROS than that of wild-type controls and also releases significantly more ROS during an IR protocol (Flogel et al., 2004). Third, the knockout has also demonstrated that Mb is responsible for nitrite-dependent NO production and that nitrite reduces myocardial infarction (Hendgen-Cotta et al., 2008).

These key observations were interpreted entirely within the context of the conventional, muscle-only location of Mb, but it could apply just as well to non-muscle tissues. So these vasodilative and antioxidant effects of NO might be important in brain neurones and the nephron tubule, both of which possess an intense metabolism that might be damaged by the ROS formed following recovery from environmental hypoxia or ischaemia. Indeed, the well-known environmental hypoxia-tolerance of both carp and zebrafish might well be linked to the tissue pattern of Mb expression, and Burmester and colleagues have shown increased Mb protein expression in hypoxically exposed goldfish (Roesner et al., 2008). This is also consistent with Mb expression being highly restricted to situations demanding fine vascular control (capillaries) or in cells subjected to IR injury, as in the metabolically active brain and kidney tubule.

This body-wide distribution and functions of Mb has to be interpreted within the context of the distribution within carp of the other globin genes (Burmester et al., 2002b). Neuroglobin (*Nrgb* transcript, Nrgb protein) is expressed predominantly in nervous tissue in human, mouse and zebrafish (Burmester et al., 2002b; Burmester et al., 2000) but is not regarded as being hypoxia inducible. Fuchs and colleagues (Fuchs et al., 2004) found that gill, brain and eye in zebrafish were Nrgb-positive using immunoblotting but that muscle was negative. They also found that *Nrgb* transcripts were distributed widely in brain neurones, but also in the olfactory system, the inner segments of retinal rod cells and the mitochondria-rich chloride cells of the gills.

By contrast, cytoglobin (*Cygb* transcript, Cygb protein) is expressed in a wide variety of human tissues (Trent and Hargrove, 2002), including liver, lung, adipose tissue, kidney, thyroid and thymus, pancreas and various brain regions. It has been particularly located in connective tissue fibroblasts and related cell types (Schmidt et al., 2004), where it has been linked with the production of extracellular matrix proteins by fibroblasts, hepatic stellate cells, osteoblasts and chondroblasts (Hankeln et al., 2004). Although cMb was found in some neuronal cells, its wide distribution in liver, pancreas and intestine, and appearance in capillaries in these tissues, makes it distinct from both *Nrgb* and *Cygb*. Moreover, of these globins, only Mb is found in cardiac and skeletal myocytes. Nevertheless, the function of Nrgb protein has been discussed in terms of NO metabolism, cytoprotection from ischaemia, *et cetera* (Brunori, 2006), and distinguishing the new wider cell-specific distribution of Mb from that of Nrgb is clearly a prerequisite for separating and understanding their respective functions, at least in the brain.

Conclusions

We account for the low level of expression of Mb in non-muscle tissues and reveal a surprisingly diverse range of non-muscle cellular sites for myoglobin expression. This, together with new data on the role of myoglobin in handling nitric oxide and reactive oxygen species, calls for a substantial re-evaluation of the physiological role of this protein in the carp and zebrafish, and it is not unlikely that this applies across the vertebrates, including mammals. In fish, the presence of Mb in pillar cells of gills and in capillary endothelial cells of all other tissues examined points to a role in the regulation of capillary function and thus in the supply of oxygen. The location of myoglobin in central and peripheral neurones and in epithelial cells of the kidney tubules is puzzling but might relate to the differing metabolic activity levels of each region. This is perhaps consistent with a role in cytoprotection from the damaging effects of ROS. Both roles are consistent with the upregulation of Mb protein expression in all tissues except the heart following chronic hypoxia. The unique brain-specific Mb isoform has been localised exclusively within neuronal cells, and a priority is to relate this distribution to that of other globin proteins, including cytoglobin and neuroglobin. Finally, the distribution of this expression property across the vertebrates and, particularly, how expression relates to life style and environment (e.g. breath-hold diving, high altitude and intense exercise) remain to be addressed.

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