

Isolation of a novel aquaglyceroporin from a marine teleost (*Sparus auratus*): function and tissue distribution

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Summary

The aquaporins (formerly called the major intrinsic protein family) are transmembrane channel proteins. The family includes the CHIP group, which are functionally characterised as water channels and the GLP group, which are specialised for glycerol transport. The present study reports the identification and characterisation of a novel GLP family member in a teleost fish, the sea bream *Sparus auratus*. A sea bream aquaporin (sbAQP) cDNA of 1047 bp and encoding a protein of 298 amino acids was isolated from a kidney cDNA library. Functional characterization of the sbAQP using a *Xenopus* oocyte assay revealed that the isolated cDNA stimulated osmotic water permeability in a mercury-sensitive manner and also stimulated urea and glycerol uptake. Northern blotting demonstrated that sbAQP was expressed at high levels in the posterior region of the gut, where two

transcripts were identified (1.6 kb and 2 kb), and in kidney, where a single transcript was present (2 kb). *In situ* hybridisation studies with a sbAQP riboprobe revealed its presence in the lamina propria and smooth muscle layer of the posterior region of the gut and in epithelial cells of some kidney tubules. sbAQP was also present in putative chloride cells of the gill. Phylogenetic analysis of sbAQP, including putative GLP genes from *Fugu rubripes*, revealed that it did not group with any of the previously isolated vertebrate GLPs and instead formed a separate group, suggesting that it may be a novel GLP member.

Key words: aquaporin, GLP, *in situ* localisation, multiple transcripts, *Fugu rubripes*, gastrointestinal tract, kidney, teleost fish, sea bream, *Sparus auratus*.

Introduction

The aquaporin family [formerly called the major intrinsic protein (MIP) family] consists of transmembrane channel proteins (AQP), which function as channels for non-ionic compounds (Heymann and Engel, 1999). In vertebrates two principal groups have been identified, the CHIP (CHannel forming Integral Protein) and GLP (GLYcerol intrinsic Protein) proteins (Froger et al., 1998; Heymann and Engel, 1999; Park and Saier, 1996; Reizer et al., 1993) and phylogenetic studies suggest that the groups arose from two divergent bacterial paralogues. Members of the CHIP cluster function exclusively as water channels (aquaporins), while members of the GLP group have a much broader function and, although considered to be primarily glycerol transport proteins (aquaglyceroporins; Park and Saier, 1996), they are also permeable to water and other small solutes (for reviews, see Ishibashi et al., 1998a,b; Verkman et al., 1996; Wintour, 1997).

Several CHIP homologues have been identified in mammals: AQP0, AQP1 and AQP2 (Lanahan et al., 1992; Preston and Agre, 1991; Yang et al., 1999), AQP4, AQP5 and

AQP6 (Krane et al., 1999; Raina et al., 1995; Sobue et al., 1999; Turtzo et al., 1997; Yasui et al., 1999) and AQP8 (Ishibashi et al., 1997a; Koyama et al., 1998). Most of them have been shown to stimulate osmotic water permeability of plasma membrane when expressed in *Xenopus* oocytes. Four aquaporin homologues belonging to the GLP group have been identified in mammals: AQP3 (Echevarria et al., 1994; Ishibashi et al., 1994; Ma et al., 1994), AQP7 (Ishibashi et al., 1997b), AQP9 (Kuriyama et al., 1997; Tsukaguchi et al., 1998) and AQP10 (Hatakeyama et al., 2001; Ishibashi et al., 2002). Members of the GLP group have a widespread distribution in tissues associated with water transport. For example, AQP3 is abundantly expressed in the principal cells of the mammalian kidney (Ecelbarger et al., 1995; Echevarria et al., 1994), in the large airways, urinary bladder, conjunctiva, epidermis (King et al., 1997; Nielsen et al., 1997) and gastrointestinal tract (Koyama et al., 1999; Ramirez-Lorca et al., 1999). AQP7 is abundantly expressed in the rat seminiferous tubules and plasma membrane of late spermatids and is also present in rat

heart, skeletal muscle and kidney (Ishibashi et al., 1997a). AQP9 is expressed at high levels in liver and testis and at lower levels in brain (Tsukaguchi et al., 1998) and AQP10 is expressed in the small intestine (Ishibashi et al., 2002).

Relatively few reports about AQP proteins in non-mammalian vertebrates exist. In amphibians AQP1–AQP4 (Abrami et al., 1994; Ma et al., 1996; Schreiber et al., 2000) have been identified. In fish, a CHIP protein (AQP0) has been identified in *Fundulus heteroclitus* (Virkki et al., 2001) and recently an AQP3 homologue was reported in eel *Anguilla anguilla* (Cutler and Cramb, 2002). Fish are a useful group in which to study aquaporin evolution as they inhabit diverse aquatic environments and face a constant osmotic and ionic challenge, which varies according to the waters they inhabit (freshwater <0.1 mOsmol kg^{-1} ; seawater approximately 1000 mOsmol kg^{-1}). The importance of water and ion transport by renal and extrarenal epithelial tissues for maintenance of osmotic homeostasis in fish is well documented (Karnaky, 1998). However, the importance of the aquaporins in water movements and associated osmotic homeostasis in fish has received surprisingly little attention. The present paper reports the cloning and functional characterisation of an aquaglyceroporin cDNA from a marine teleost, the gilthead sea bream (*Sparus auratus* L.). In addition, northern blotting and *in situ* hybridisation were used to characterise its tissue and cellular distribution.

Materials and methods

Animals and tissues

Adult gilthead sea bream *Sparus auratus* L. (approximately 350 g) were maintained in through-flow seawater tanks at $17\pm 2^\circ\text{C}$ under natural photoperiod for winter in the Algarve, Portugal. To obtain tissues for mRNA extraction fish were killed by stunning and decapitation, and liver, kidney, gastrointestinal tract (GIT), skin, gills and skeletal muscle were frozen in liquid nitrogen or fixed overnight at 4°C in 4% paraformaldehyde (PFA). Frozen material was stored at -80°C until utilised for RNA extraction and PFA-fixed material was washed with PTw (phosphate-buffered saline+0.1% Tween-20) and immediately processed for preparation of sections for *in situ* hybridisation.

Construction and screening of sea bream kidney cDNA library

Total RNA was extracted from the collected tissue using 'TRI reagent' (Sigma-Aldrich, St Louis, MO, USA). The poly(A)⁺ RNA fraction was obtained from total RNA by chromatography on columns of oligo-dT cellulose (Amersham Biosciences, Lisbon, Portugal).

A cDNA library was constructed from 5 μg of gilthead sea bream kidney poly(A)⁺ RNA using the UNI-ZAP XR cDNA cloning kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The double-stranded cDNA was ligated into the corresponding vector and packaged into Gigapack Gold III packaging extracts (Stratagene). The sea

bream kidney library was screened with a 700 bp [α - ^{32}P]dCTP-labelled sea bream AQP cDNA probe (Rediprime, random labelling kit, Amersham Biosciences) generated by RT-PCR. Filters were hybridised overnight at 65°C in Church–Gilbert buffer (1 mmol l^{-1} EDTA, 0.25 mol l^{-1} NaH_2PO_4 , 7% SDS) and washed 2×10 min at 65°C in $0.1\times$ SSC/0.1% SDS (1/200 dilution of a solution of 3 mol l^{-1} NaCl, 0.3 mol l^{-1} sodium citrate, pH 7.0 – $20\times$ SSC). One positive plaque was isolated out of 400 000 recombinants, automatically excised into pBluescript (Stratagene) and sequenced to give threefold coverage. The sequence has been deposited in GeneBank (accession number AY363261).

Osmotic water permeability, glycerol permeability and urea uptake assays in Xenopus oocytes

Capped RNA (cRNA) transcripts corresponding to *S. auratus* AQP were synthesized *in vitro* with T3 RNA polymerase (Promega, Madison, WI, USA) from *Xho*I-digested pBluescript vector containing the sbAQP cDNA. The isolation and microinjection of oocytes have been described previously (Deen et al., 1994). In brief, pieces of the ovary from *Xenopus laevis* were treated with collagenase A (2 mg ml^{-1} ; Roche, Holland) in modified Barth's culture medium (MBS), 0.33 mmol l^{-1} $\text{Ca}(\text{NO}_3)_2$, 0.4 mmol l^{-1} CaCl_2 , 88 mmol l^{-1} NaCl, 1 mmol l^{-1} KCl, 2.4 mmol l^{-1} NaHCO_3 , 10 mmol l^{-1} Hepes, pH 7.5, 0.82 mmol l^{-1} MgSO_4 , for 2 h at room temperature. After repeated washing with fresh MBS, ovarian follicles at stages V–VI were selected and equilibrated in MBS at 18°C for 24 h. The resulting intact follicles and partially denuded oocytes were then injected with either 50 nl of distilled water (negative control), 50 nl of distilled water containing 10 ng cRNA of sbAQP, or with 1 ng cRNA of human AQP1 as a positive control (data not shown; Denker et al., 1988), and incubated in MBS at 18°C for another 24 h. Subsequently oocytes were completely defolliculated with watchmaker forceps, equilibrated again for 24 h at 18°C , and then used in water and solute transport assays.

The osmotic water permeability (P_f) was measured from the time course of osmotic oocyte swelling in a standard assay. Oocytes were transferred from 200 mOsm MBS to 20 mOsm MBS medium at room temperature, and the swelling of the oocytes was followed by video microscopy using serial images at 2 s intervals during the first 20 s period. The P_f values were calculated taking into account the time-course changes in relative oocyte volume $[d(V/V_o)/dt]$, the oocyte surface area (S), and the molar volume of water ($V_w=18$ cm^3 ml^{-1}), using the formula $V_o[d(V/V_o)/dt]/[S\times V_w(\text{Osm}_{\text{in}}-\text{Osm}_{\text{out}})]$. To determine glycerol permeability, the oocytes were transferred to an isotonic solution containing 160 mmol l^{-1} glycerol and complemented with 40 mOsm MBS to adjust the solution to 200 mOsm. The apparent glycerol permeability coefficient (P'_{gly}) was calculated from oocyte swelling using the equation $[d(V/V_o)dt]/(S/V_o)$ (Verkman and Ives, 1986). A slight shrinkage of control eggs was observed as a consequence of a slight deviation from isotonicity of the incubation medium.

To examine the effect of mercury on the P_f , the oocytes were

incubated in MBS containing 1 mmol l⁻¹ HgCl₂ for 15 min before the swelling assay, which was also performed in the presence of HgCl₂. To determine if the mercurial effect was reversible, the same oocytes were rinsed 3 times in MBS, incubated with 5 mmol l⁻¹ mercaptoethanol for 15 min, and subjected to the swelling assays 2 h later.

Urea transport activity mediated by sbAQP was measured by uptake of [¹⁴C]urea (Amersham Biosciences). Injected oocytes were transferred to 1 mmol l⁻¹ urea containing 2 µCi ml⁻¹ (74 kBq ml⁻¹) in MBS for 20 min, rapidly rinsed 3 times with ice-cold MBS, and lysed in 10% SDS at room temperature followed by liquid scintillation counting.

Northern blot analysis

Between 2 and 5 µg of mRNA from sea bream liver, kidney, gastro-intestinal tract (duodenum, midgut, hind-gut and rectum), skin, gill and skeletal muscle were fractionated on a 5.5% formaldehyde/1.5% agarose gel in MOPS, transferred to a nylon filter (Hybond-N, Amersham) with 10× SSC and cross-linked at 80°C for 2 h. Prior to hybridisation, the filter was washed at 60°C for 20 min in 1× SSC, 0.1% SDS and pre-hybridized in Church–Gilbert buffer for 2 h at 58°C. Hybridisation was allowed to proceed overnight at 58°C in fresh pre-hybridization solution containing the full-length sea bream AQP as the probe, isolated from the kidney library labelled with [α-³²P]dCTP (Rediprime, random labelling kit, Amersham Biosciences). Stringency washes were carried out for 2× 15 min at 60°C in 1× SSC, 0.1% SDS and then at 65°C for 10 min in 0.1× SSC, 0.1% SDS. The blot was exposed to a Phosphoimager (Biorad, Hercules, CA, USA) for 7 days. In order to evaluate the relative amounts of mRNA loaded onto the filter for each tissue, the blot was also hybridized with a sea bream β-actin probe (Santos et al., 1997) following the same protocol as before but reducing exposure time to 2 h. Exposure data was analyzed using the Multi-Analyst/PC software package (Biorad, Lisbon, Portugal).

In situ hybridisation

The distribution of sbAQP mRNA along the gastrointestinal tract, in the gill and kidneys was further investigated by *in situ* hybridisation. The full-length sbAQP cDNA cloned in pBluescript was digested with *Bam*HI (Promega, Madison, WI, USA) at 37°C for 1.5 h in order to linearise the DNA. The linearised vector was purified and *in vitro* transcription was carried out using 20 units of T7 RNA polymerase in transcription buffer (Amersham Biosciences) with 2 µl of digoxigenin-RNA labeling mix (Roche, Lisbon, Portugal), for 2 h at 37°C. The riboprobe synthesis was stopped with 2 µl of 0.2 mol l⁻¹ EDTA, precipitated with sterile sodium acetate (0.1 volume, 3 mol l⁻¹; pH 5.2) and 75% ethanol (2.5 volumes) and resuspended in 50 µl of water. Riboprobe purity and concentration were determined by electrophoresis in a 1% agarose gel containing ethidium bromide.

Tissue sections were dewaxed, rehydrated and then pre-hybridised at 55°C for 4 h in hybridisation solution (50%

formamide, 4× SSC, 1 mg ml⁻¹ torula RNA, 0.1 mg ml⁻¹ heparin, 1× Denhart's, 0.1% Tween 20, 0.04% CHAPS). Tissues were then hybridised overnight in a humidified box at 55°C in 40 µl per section of hybridisation solution containing 15 µl ml⁻¹ of riboprobe. Controls were pretreated with RNase prior to hybridization with riboprobe, or the riboprobe was

Table 1. List of CHIP and GLP proteins used for the phylogenetic analysis

Species	Protein	Tree code	Accession number	
Mammals				
<i>Homo sapiens</i>	AQP1	HsAQP1	P29972	
	AQP2	HsAQP2	P41181	
	AQP3	HsAQP3	BC013566	
	AQP4	HsAQP4	P55087	
	AQP5	HsAQP5	P55064	
	AQP8	HsAQP8	BAA34223	
	AQP9	HsAQP9	O43315	
	AQP10	HsAQP10	AB066105	
	<i>Mus musculus</i>	AQP1	MmAQP1	Q02013
		AQP2	MmAQP2	AAD21017
AQP3		MmAQP3	ADD20305	
AQP4		MmAQP4	P55088	
AQP5		MmAQP5	ADD32491	
AQP7		MmAQP7	BAA24537	
AQP8		MmAQP8	P56404	
AQP9		MmAQP9	AB037180	
<i>Ratus norvegicus</i>		AQP2	RnAQP2	P34080
	AQP3	RnAQP3	P47862	
	AQP4	RnAQP4	P47863	
	AQP5	RnAQP5	P47864	
	AQP6	RnAQP6	AAD29856	
	AQP7	RnAQP7	P56403	
	AQP8	RnAQP8	P56405	
	AQP9	RnAQP9	AAC36020	
	<i>Bos taurus</i>	AQP4	BtAQP4	BAA36505
<i>Ovis aries</i>	AQP1	OaAQP1	AF009037	
<i>Cannis familiaris</i>	AQP1	CcAQP1	AB011373	
Amphibians				
<i>Xenopus laevis</i>	AQP3	XlAQP3	CAA10517	
<i>Bufo marinus</i>	AQP1	BmAQP1	AF020620	
	AQP2	BmAQP2	AF020621	
	AQP4	BmAQP4	AF020623	
<i>Rana esculenta</i>	AQPA	ReAQP	L24754	
Fish				
<i>Fundulus heteroclitus</i>	AQP0	FhAQP	AF191906	
<i>Anguilla anguilla</i>	AQP3	AaAQP3	AJ319533	
<i>Sparus auratus</i>	AQP	SbAQP	AY363261	
<i>Danio rerio</i>	AQP3	DrAQP3	BC044188	
Bacteria	GDFP	EcGLPf	ECU13915	
Plants	AQP1	AcAQP1	AF255795	

The acronyms used in the phylogenetic tree and public database sequence accession numbers are also indicated.

excluded from the hybridization. High stringency washes were carried out for 3×5 min at 55°C with 2×SSC. Tissue sections were then washed 2×5 min with 2×SSC:0.12% CHAPS at 22°C, followed by a wash for 5 min in 2×SSC:PTw (1:1, v/v) and finally 5 min in PTw. Detection of hybridised probe was carried out using anti-digoxigenin-alkaline phosphatase (AP) Fab fragments (1/100) (Roche). The chromagens for colour detection were NBT (4-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro 3-indolylphosphate) and colour development was carried out over 2–17 h at 30°C. Stained sections were rinsed in PBS, fixed for 15 min in 4% paraformaldehyde at room temperature, rinsed in PBS and mounted in glycerol gelatine. Sections were analysed using a microscope (Olympus BH2) coupled to a video attachment (Sony 3CCD DXF-930P) linked to a computer for digital image analysis.

Phylogenetic and protein structure analysis

A frequent problem in phylogenetic analysis is the limited representation of fish sequences. For a more robust phylogenetic analysis, the genome of the model organism *Fugu rubripes* (<http://fugu.hgmp.mrc.ac.uk>) was searched for GLP members using the sbAQP. *Fugu* scaffolds containing GLP protein amino acid signatures were then analysed using NIX, a Web tool to view the results of a suite of DNA analysis programs (<http://menu.hgmp.mrc.ac.uk>). The predicted coding regions were extracted and further confirmed using BLAST (Altschul et al., 1990) against SWISSPROT and non-redundant TREMBL. The human genome was also analysed for the presence of GLP homologues. The deduced sbAQP amino acid sequence was aligned with other available aquaporin amino acid sequences in public databases (see Table 1) using CLUSTAL X Version 1.64b (Thompson et al., 1997) and the percent identity between different AQP forms was determined (Table 2). Phylogenetic trees were generated in PAUP* Version 4.0b (Swofford, 1998) to identify maximum parsimony trees for different hypothesis of relationships using *Allium cepa* AQP1 (AF255795) as an outgroup in this analysis. The sbAQP deduced protein structure was analysed using HMMTOP V.2.0 software (Tusnady and Simon, 2001) and a hydropathy profile obtained using the Kite and Doolittle method (Kyte and Doolittle, 1982).

Results

Cloning of cDNA and amino acid sequence analysis

The clone isolated from the newly prepared kidney cDNA library (Fig. 1) spans 1047 bp and encodes a protein of 298 amino acids (aa). The 5′ untranslated region (UTR)

-84	ggc acg agc tgc gac cca cct gat gac ggc gtc ccg cct ctg aaa	-40
-39	gct gac cag tgg ccg gac ggt gca gag gaa acc tcg ggg	ATG GAG
		M E
7	AGG CTG CTG AGG AAG TGT CAG ATC AGA AAC CAG CTG GTC AGG GAG	51
3	R L L R K C Q I R N Q L V R E	17
52	TGT TTG GCT GAG TGC CTC GGA GTC TAT GTC CTG ATT CTG TTC GGA	96
18	C L A E C L G V Y V L I L F G	32
97	TGT GGC TCT GCC GCC CAG GTG ACC ACG AGC CAA GAC AAG AAC GGG	141
33	C G S A A Q V T T S Q D K N G	47
142	CAG TAC CTG TCG ATC AAT CTG GGC TTT GCT CTG GGA GTG ACA TTC	186
48	Q Y L S I N L G F A L G V T F	62
187	GGG GTG TTT GTG TCC CGC GGA GTC TCA GGG GCC CAT CTG AAC CCG	231
63	G V F V S R G V S G A H L N P	77
232	GCT VTG TCT CTG AGC TTG TGC GCT TTG GGC CGA CAT CCC TGG ATG	276
78	A V S L S L C A L G R H P W M	92
277	AAG CTG CCA TTC TAC GTC TTC TTC CAG GTG ATG GGA GCC TTT CTG	321
93	K L P F Y V F F Q V M G A F L	107
322	GCA GCA GCC ACC GTT GGT CTG CAG TAC GAT GCC ATC CAG GCG	366
108	A A A T V G L Q Y Y D A I Q A	122
367	TAC AGC GGA GGT GAG CTG ACG GTG ACA GGT CCC ACC GCC ACC GCC	411
123	Y S G G E L T V T G P T A T A	137
412	GGC ATA TTC TCC ACC TAC CCA GCT GCC TAC CTC AGT TTG TGG GGA	456
138	G I F S T Y P A A Y L S L W G	152
457	GGT GTC GTG GAC GTG ATA GGC ACA GGC GCC CTG CTG ATG TGT	501
153	G V V D Q V I G T A A L L M C	167
502	GTC CTG GCT CTG GGG GAC CAG AGG AAC AGT CCC CTC CCT GAT GGT	546
168	V L A L G D Q R N S P L P D G	182
547	CTG CAG CCG GTC CTG GTG GGA GCA GCC GTG CTG GTA ATC GGC GTC	591
183	L Q P V L V G A A V L V I G V	197
592	TCG ATG GGC TCC AAC AGC GGC TAC GCC CTC AAC CCA GCC AGG GAT	636
198	S M G S N S G Y A L N P A R D	212
637	TTG GGA CCT CGA CTG TTC ACG TAC ATC GCC GGC TGG GGA GTG GAT	681
213	L G P R L F T Y I A G W G V D	227
682	GTT TTC AAG GCC GGA GGT GGC TGG TGG GTG CCC ATA GTG gCC	726
228	V F K A G G G W W W V P I V A	242
727	CCC TGT GTC GGG GCG CTG GGA ACA CTG ATC TAC ATG CTG ATG	771
243	P C V G A L L G T L I Y M L M	257
772	ATT GAA GTG CAC CAC CCC AGT CTG TCT GAG CTC CAC ACG TCA TGT	816
256	I E V H H P S L S E L H T S C	272
817	CAA GAG GCT ACT GAG GAG AAG GCG GGG GTG GAG CTG GAG GGG GTG	861
273	Q E A T E E K A G V E L E G V	287
862	AAG CCA CAC TGT GTA AAA CCC AGC ACT AAA ATG TAG tga gaa ggt	906
288	K P H C V K P S T K M *	298
907	taa aga agc tgt gat tat aca gta aag gtt att gat ggt aaa aaa	951
952	aaa aaa aaa aaa	963

Fig. 1. Sea bream AQP cDNA and deduced amino acid sequence. Two MIP family signatures (NPA) were identified and are indicated in bold at positions 76 and 208. The six transmembrane domains predicted using the Kite and Doolittle method are underlined in italics.

comprises 84 bp and the 3′UTR comprises 69 bp ending in poly(A)⁺. However, as no polyadenylation signal could be identified this suggests that the 3′UTR of the clone was truncated. Sequence comparison revealed that the encoded protein was most similar to members of the GLP group and shared 48% identity with the recently identified human AQP10, 47% identity with human AQP3, 43% identity with mouse AQP7 and 42% identity with mouse AQP9 (Table 2). The sequence identity between the cloned sbAQP and eel AQP3 (46%) was lower than that with mammalian and amphibian AQP3 (50%) and this may be partly explained by the phylogenetic distance between these fish. The sbAQP also exhibited low sequence conservation with CHIPs (<20%, Table 2). None of the previously isolated AQP showed a higher sequence similarity with the sbAQP (Table 2). This is in contrast to eel AQP, which shared 67% identity with human AQP3, and makes it difficult to unambiguously identify sbAQP on the basis of sequence similarity, suggesting that it may be a novel form of AQP.

In common with other aquaporins, six transmembrane

domains were predicted in sbAQP from the hydropathy profile and are underlined in the deduced aa sequence (Fig. 1). The MIP family signature, Asn-Pro-Ala (NPA), was present at aa 76–78 and at aa 208–210 of sbAQP, which are located, respectively, in the second (B) and fifth (E) connecting loop. Both the N terminus and C terminus were predicted to be in the cytosol, as for other AQPs. Amino acid residues 8, 18, 33, 84, 167, 244, 272 and 291 were cysteine. No potential N-linked glycosylation site [NX(S/T)] was found in sbAQP. The deduced molecular mass of the protein is 31.6 kDa and the theoretical pI 6.5.

Functional characterisation of sbAQP

Xenopus laevis oocytes injected with cRNA encoding sbAQP had a sevenfold larger P_f than that of oocytes injected with water alone (control, Fig. 2A). Moreover, oocytes expressing sbAQP showed increased P_{gly} and P_{urea} (measured, respectively, using volumetric and radiotracer methods) relative to the values found with water-injected oocytes (Fig. 2B,C). Comparison of the relative permeability of sbAQP to water, glycerol and urea revealed that its functional characteristics are similar to mammalian AQP3 and AQP10: it is most permeable to water and its permeability to glycerol and urea is several-fold lower (Echevarria et al., 1996). The water channel function of sbAQP was sensitive to mercuric chloride (HgCl₂). In the presence of 1 mmol l⁻¹ HgCl₂ there was a ~70% decrease in osmotic water permeability (P_f). However, it was not possible to reverse this effect by mercaptoethanol treatment. This is in contrast to the observations of human AQP3 and AQP10, where at 1 mmol l⁻¹ HgCl₂ there is significant inhibition of water transport, which is partially reversed in the presence of mercaptoethanol (Ishibashi et al., 2002; Kuwahara et al., 1997b).

SbAQP expression and tissue distribution

Two transcripts of 2 kb and 1.6 kb were identified by northern blotting in the gastrointestinal tract (mid-gut, hind-gut and rectum) and in all these tissues the relative abundance of both transcripts was similar (Fig. 3A). In kidney only the large transcript of 2 kb and in gill only the 1.6 kb transcript were detected. No signal was detected in duodenum or in any of the other tissue analysed. Comparison of the expression of sbAQP with that of β -actin (Fig. 3B) indicated that hind-gut and kidney are the tissues with the highest expression of sbAQP followed by the rectum, mid-gut and gill.

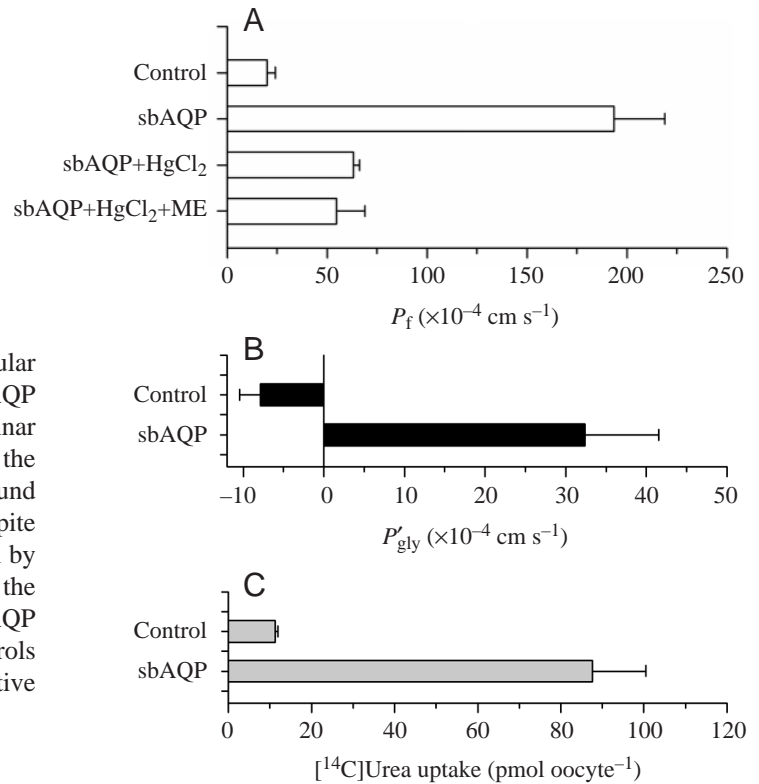
In situ hybridisation was carried out to characterise the cellular expression of sbAQP in the gastrointestinal tract, gills and kidney (Fig. 4). An intense sbAQP signal was found in the epithelial cell lining some of the kidney tubules (Fig. 4A,C). In gill sbAQP expression was most intense in the primary filaments, where the chloride cells are normally found, and expression of sbAQP was also observed at the proximal ends of some of the secondary lamellae (Fig. 4D,F). The expression of sbAQP in the hind-gut was principally in cells scattered in the lamina propria (Fig. 4G), although the identity of these cells remains to be established. Further expression of sbAQP

Table 2. Percentage identity between GLP proteins showing that SbAQP has greatest similarity to AQP3 and AQP10

	% Identity																			
	SbAQP	FhAQP	Mm	Mm	Mm	Mm	Mm	Mm	Mm	Mm	Hs									
			AQP1	AQP2	AQP3	AQP3	AQP3	AQP4	AQP5	Rn	AQP6	Mm	AQP7	Mm	AQP8	Mm	AQP9	Mm	AQP10	Hs
SbAQP	298	18	20	22	47	46	16	19	19	43	17	42	48	42	17	42	48	48	48	48
FhAQP	263	263	44	52	20	20	34	43	49	20	25	20	19	20	25	20	20	20	19	19
Mm	269	269	269	39	18	17	34	38	41	18	20	17	17	17	20	17	17	17	17	17
Mm	271	271	271	271	20	19	35	49	61	23	24	21	17	21	24	21	21	21	17	17
Hs	292	292	292	292	292	67	20	17	20	41	19	44	44	41	19	44	44	44	44	44
Aa	296	296	296	296	296	296	18	16	18	38	16	39	43	38	16	39	39	39	43	43
Mm	323	323	323	323	323	323	323	34	37	17	21	19	15	17	21	19	19	15	15	15
Mm	265	265	265	265	265	265	265	49	20	20	23	21	16	20	23	21	21	16	16	16
Rn	276	276	276	276	276	276	276	276	276	276	276	276	276	276	276	276	276	276	276	276
Mm	303	303	303	303	303	303	303	18	18	18	23	21	15	18	23	21	21	15	15	15
Mm	261	261	261	261	261	261	261	15	15	15	15	15	15	15	15	15	15	15	15	15
Mm	295	295	295	295	295	295	295	18	18	18	18	18	18	18	18	18	18	18	18	18
Mm	301	301	301	301	301	301	301	18	18	18	18	18	18	18	18	18	18	18	18	18
Hs	301	301	301	301	301	301	301	18	18	18	18	18	18	18	18	18	18	18	18	18

Lengths of proteins in amino acid residues are shown in bold type. Code names are given in Table 1.

Fig. 2. (A) Osmotic water permeability (P_f), (B) apparent glycerol permeability (P'_{gly}) and (C) urea uptake of *Xenopus* oocytes expressing sbAQP. Oocytes were injected with 50 nl of water containing 10 ng cRNA of sbAQP or 50 nl of distilled water only (control oocytes) 48 h prior to the experiments. P_f and P'_{gly} were calculated from the time course of osmotic swelling of the oocyte in a hyposmotic- or glycerol-containing medium and urea transport activity was measured by [14 C]urea uptake. Values are means \pm S.E.M. of 10 oocytes from different batches. ME, mercaptoethanol.



was also found in cells localised at the interface of the circular and longitudinal muscle layer in the hind-gut (Fig. 4I). sbAQP was not found to be expressed in either the columnar enterocytes of the villi or in the numerous goblet cells of the hind-gut. A similar pattern of sbAQP distribution was found in the mid-gut, although the signal was far weaker. Despite being unable to detect sbAQP transcripts in the duodenum by northern blot a very weak signal was found in cells of the mucosa by *in situ* hybridisation (data not shown). No sbAQP signal was detected in the liver (Fig. 4J) and the controls utilised to verify probe specificity were also negative (Fig. 4B,E,H).

Phylogenetic analysis

Analysis of the *Fugu* genome led to the identification *in silico* of six scaffolds containing putative GLP genes. The extracted *Fugu* genes were introduced in a multisequence alignment (Fig. 5) used in the phylogenetic analysis. Two *Fugu* genes (from scaffolds M000233 and M004004) were

excluded because they were extremely truncated. The first phylogenetic analysis done showed two principal clades that corresponded, respectively, to GLP and CHIP proteins. To better exemplify the relationship between the GLP family

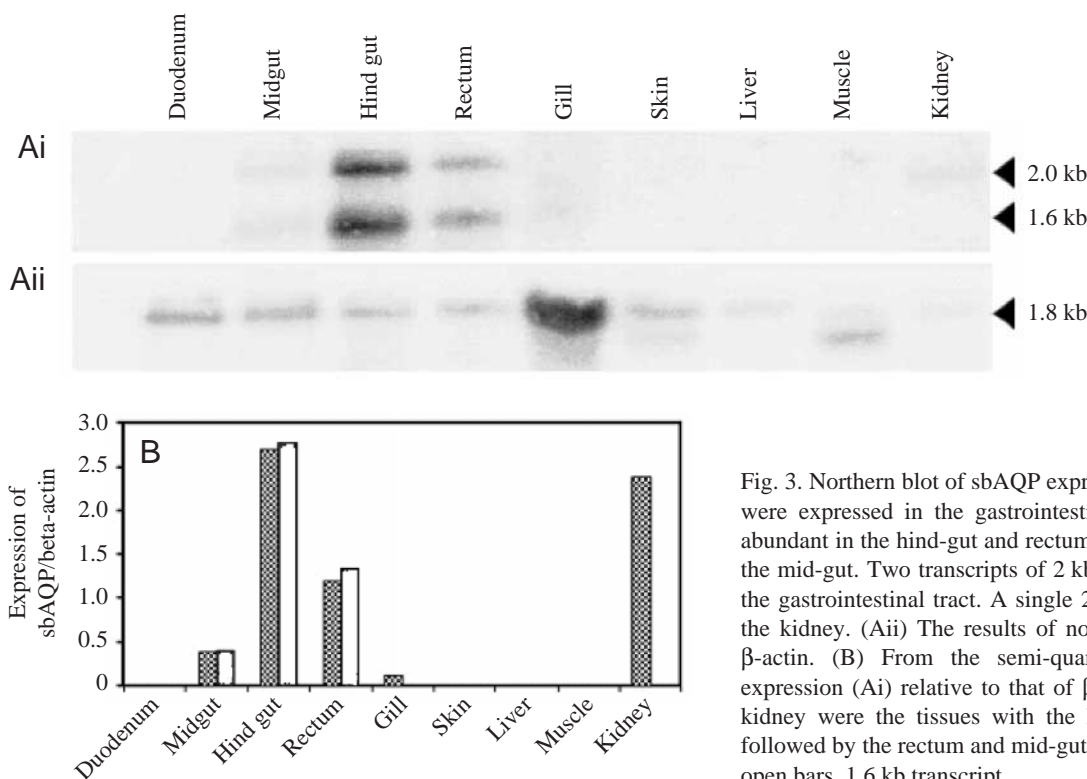


Fig. 3. Northern blot of sbAQP expression. (Ai) sbAQP transcripts were expressed in the gastrointestinal tract, where it was most abundant in the hind-gut and rectum, with lower levels detected in the mid-gut. Two transcripts of 2 kb and 1.6 kb were identified in the gastrointestinal tract. A single 2 kb transcript was detected in the kidney. (Aii) The results of northern blot hybridisation with β -actin. (B) From the semi-quantitative analysis of sbAQP expression (Ai) relative to that of β -actin (Aii), the hind-gut and kidney were the tissues with the highest expression of sbAQP followed by the rectum and mid-gut. Hatched bars, 2 kb transcript; open bars, 1.6 kb transcript.

members, phylogenetic analysis was performed with only members of this group and *Allium cepa* AQP1 was the outgroup (Fig. 6). The GLP clade was composed of separate groups that corresponded to AQP3, AQP7, AQP9 and AQP10. The *Fugu* gene in scaffold M001883 grouped with AQP3 and was most similar to the available fish sequences. The genes in scaffolds M000386 and M001042 grouped, respectively, with mammalian AQP7 and AQP10. AQP9 was the only GLP in which there was no match with a predicted *Fugu* GLP gene. The sbAQP formed an independent group clustering with the *Fugu* gene from scaffold M000648. Extensive searches of the human genome failed to identify a sbAQP paralogue.

Discussion

We report the cloning of an aquaglyceroporin from a sea bream kidney cDNA library. Its amino acid composition and certain structural features, namely, the presence of two MIP family signatures (NPA) and longer amino acid sequences in the second and third extra-cellular loops (Ishibashi et al., 1998a), support its classification as a GLP protein.

Sequence similarity and phylogenetic analysis incorporating extracted *Fugu* genes, and the identification of a *Fugu* gene that clustered in an independent group with sbAQP, strongly suggest that sbAQP is a novel GLP member. Extensive searches of the human genome failed to identify a paralogue, suggesting that the gene found in sea bream and *Fugu* may have evolved only in the fishes after divergence from the tetrapod lineage.

Characterisation of sbAQP function using the *Xenopus* oocyte water and solute transport assay indicates that it is a functional fish aquaglyceroporin and transports water, glycerol and urea. Moreover, the osmotic water permeability is mercury (HgCl_2) sensitive. However, treatment with mercaptoethanol fails to reverse the latter effect and it is unclear if this is an artefact caused by non-specific effects, such as the toxicity of the high-dose of HgCl_2 (1 mmol l^{-1}), on *Xenopus* oocyte viability or reflects a characteristic of the sbAQP protein (Kuwahara et al., 1997b). Mercurial reagents are thought to block the aqueous pore of AQPs by binding specifically to cysteine residues, and Cys-11 in mammalian AQP3 and Cys-212 in mammalian AQP2 have been shown to confer this characteristic (Kuwahara et al., 1997a). The absence of Cys-11 but presence of Cys-8 in sbAQP may indicate that the latter is responsible for the observed mercury sensitivity, although candidate cysteine residues are present nearby at positions 18, 22 and 33.

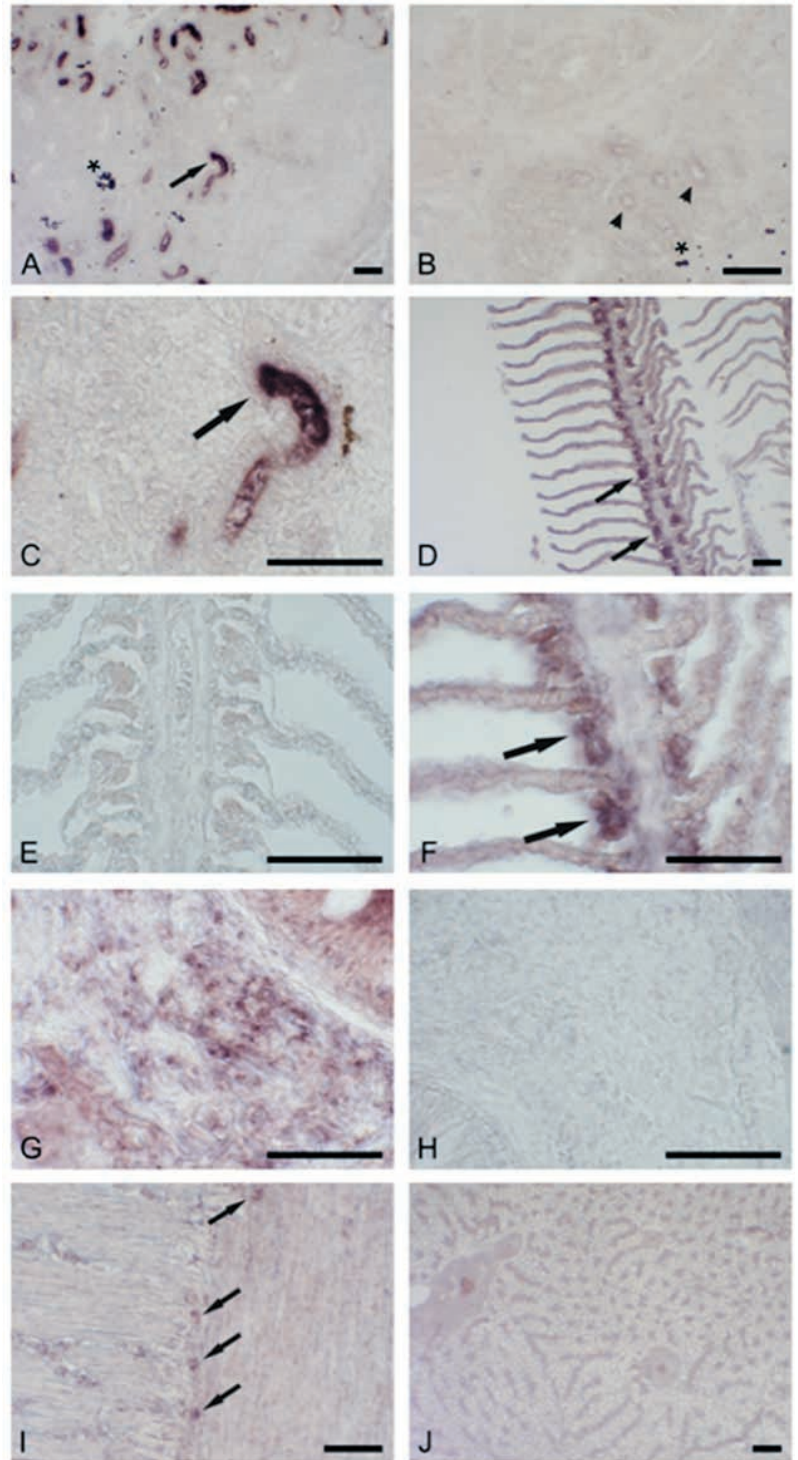


Fig. 4. *In situ* hybridisation localises sbAQP expression in the kidney, gills and gastrointestinal tract. sbAQP was highly expressed in some of the kidney tubules (A) and (C). In the gill, expression was detected along the length of the lamellae with the most intense signal present in the apical cellular region of lamellae (D,F) where the chloride cells (arrows) were localised (F). An intense expression was found in the hind-gut in cells localised in the *lamina propria* (G) and in cells (arrows) at the interface between the two muscle layers of the gut (I). No sbAQP expression was observed in the liver (J) or in tubules (arrowheads) in control sections of the kidney (B), gills (E) or gut (H). Scale bars, $1 \mu\text{m}$. Asterisks indicate haem deposits in the kidney.

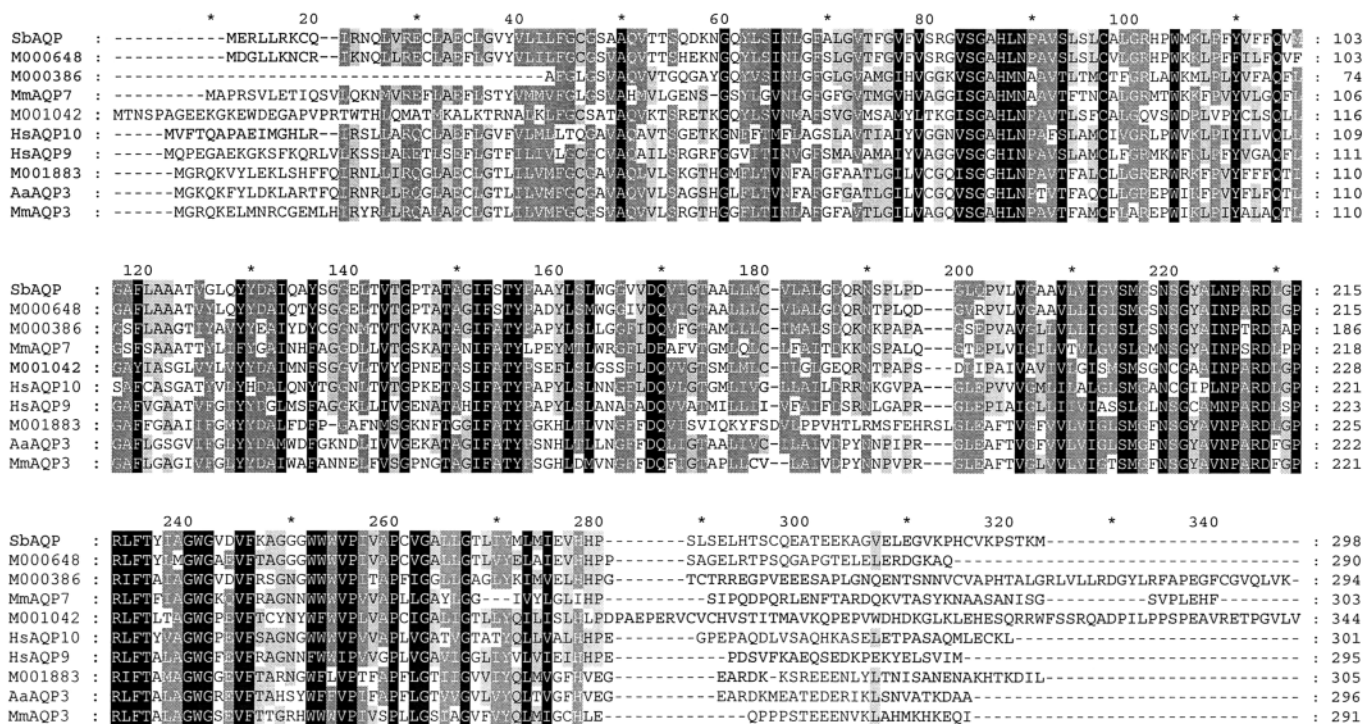
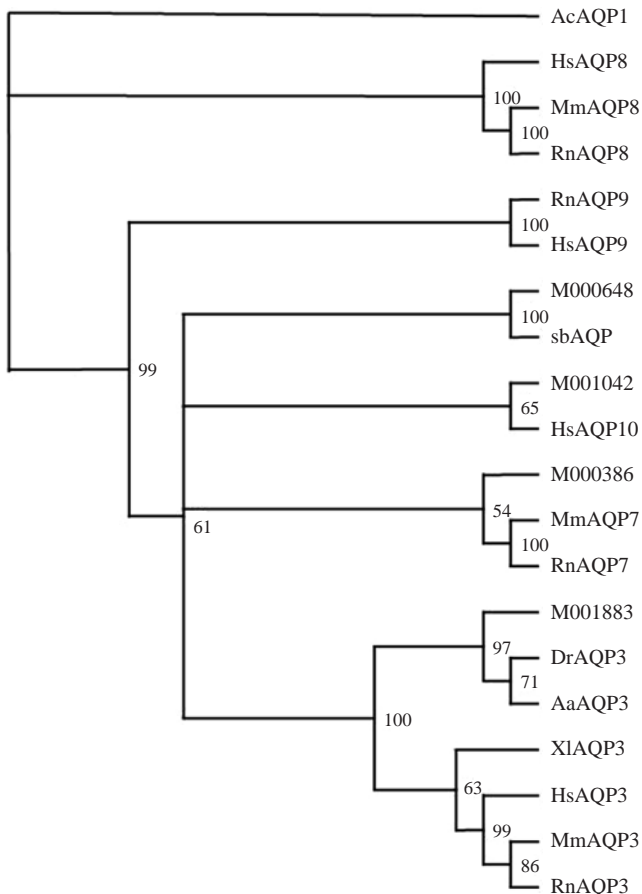


Fig. 5. Multiple sequence alignment of sbAQP, putative *Fugu* GLPs extracted *in silico* from the genome and GLP proteins selected to cover the principal groups. Species identification is indicated in Table 1. *Fugu* sequences (M00...) are identified by the scaffolds from which they were obtained. Identical sequences are highlighted in black. Dark and light tints indicate partial identity.



Analysis of sbAQP tissue distribution in adult sea bream, demonstrated that it is widely distributed in water-transporting epithelia. The gastrointestinal tract, but more specifically the hind-gut and the kidney, express the highest levels of sbAQP mRNA. The origin of the two transcripts identified in the sea bream gastrointestinal tract has not been determined, although the relatively limited conservation of sbAQP with other members of the GLP group isolated so far in vertebrates (Table 2), and the high stringency conditions utilised for the northern blot, appear to rule out probe cross-hybridisation with these forms. However, identification *in silico* in the *Fugu* genome of at least nine AQP raises the possibility that the probe used in the present study could have cross-hybridised with an as-yet-unknown isoform and further work will be required to resolve this question. In human, two forms of AQP10 have been identified, a ~2 kb transcript and a splice variant of 2.3 kb that lacks the sixth transmembrane domain (Hatakeyama et al., 2001; Ishibashi et al., 2002). Multiple transcripts of another GLP family member, AQP7, have also

Fig. 6. Consensus tree with corresponding bootstrap values (from sampling 1000 trees) obtained by Parsimony analysis of vertebrate GLP proteins using the sequence of *Allium cepa* (Ac) AQP1 as outgroup. sbAQP fails to cluster with any of the previously isolated GLPs and instead cluster with a gene predicted from the *Fugu* genome. Species identification is indicated in Table 1. *Fugu* sequences (M00...) are identified by the scaffolds from which they were obtained.

been reported in rat with two transcripts found in kidney, heart and skeletal muscle (Ishibashi et al., 1997a). It will be of interest to determine the nature of the two transcripts of AQP identified in sea bream and to characterise their function *in vivo*. For example, in man the 2.3 kb splice variant of AQP10 lacking the sixth transmembrane domain is permeable to water but not to glycerol or urea, unlike the full-length 2 kb transcript.

In man the duodenum and jejunum are the major sites of expression of AQP10, and these are also the sites for entrance and secretion of large volumes of water (Hatakeyama et al., 2001). A similar observation is made with AQP3, which is expressed in the basolateral membrane in the collecting duct of kidney (Ecelbarger et al., 1995; Echevarria et al., 1994; Ma et al., 2000) and in the rectum and hind-gut (Ramirez-Lorca et al., 1999). The general presence of AQPs in membranes important for water movement has led to the suggestion that transcellular as well as paracellular pathways of water movement exist in this tissue in man. The behaviour of aquaporins reported in mammals and their response to movements of water driven by osmotic gradients, fits in well with experimental observations of water movement in the fish intestine, where it has been linked to ion movements similar to those reported in ouabain-sensitive water absorption in Atlantic salmon (Usher et al., 1991) and *Fundulus heteroclitus* (Marshall et al., 2002a,b). In the latter situation it has been demonstrated that the intestine not only absorbs water but also secretes fluid by mechanisms linked, at least in part, to cystic fibrosis transmembrane conductance regulator-like (CFTR-like) ion channel. More studies will be required to determine if sbAQP is directly involved in water absorption in the intestine in order to establish whether it provides a mechanism by which drinking and water absorption can be independently regulated in the fish intestine (Fuentes and Eddy, 1997).

In teleost fish the only members of the MIP family identified are an AQP0 homologue in the killifish *Fundulus heteroclitus* (Accession number, AF191906), AQP3 homologues in the European eel *Anguilla anguilla* (Accession number, AJ319533) and zebra fish *Danio rerio* (Accession number, BC044188), and an AQP in *Tribolodon hakonenis* (Accession number, AB055465) (Cutler and Cramb, 2002; Virkki et al., 2001). In killifish AQP0 is restricted to the lens and is present as two transcripts of 2.8 and 1.8 kb, which are suggested to correspond to alternative splice variants. AQP3 in the European eel has a wider tissue distribution, being present in low abundance in the eye, oesophagus and intestine as a single transcript of 2.4 kb and in high abundance in the gills, where an additional transcript of 7 kb is also identified. Moreover, in the eel the level of expression of AQP3 appears to be regulated by the composition of the bathing medium, so that the expression of AQP3 in the gills of freshwater-adapted eel is several-fold higher than in those adapted to saltwater. Comparison of the eel AQP3 and sbAQP tissue distribution reveals an overlap, although the relative abundance of transcripts seems to differ. The gill in freshwater fish plays an important role in total body water influx and is suggested to

account for up to 90% of water uptake (Haywood et al., 1977). However, this is not true in marine fish where the presence of leaky epithelia ensures high water loss through the gills, in particular the chloride cell complex. The distribution of sbAQP mRNA in the gills of the sea bream coincided with that described by immunohistochemistry for AQP3 in seawater-adapted European eel (Lignot et al., 2002). The mRNA of AQP in seabream and protein of AQP3 in European eel is principally localised in the chloride cells within the primary filaments of the gills and at the proximal ends of the secondary lamellae. The physiological role of sbAQP in the gill remains to be established, whether it is directly involved in water movement and/or also associated with elimination of nitrogenous waste products (Isaia, 1984).

The tissue distribution of sbAQP coincides with that of the mammalian GLP proteins, AQP3, AQP7 and AQP10. Surprisingly the expression of eel AQP3 in the kidney is very low and does not coincide with the abundant expression in the basolateral membrane of the collecting duct (Ecelbarger et al., 1995; Echevarria et al., 1994; Ma et al., 2000) in mammals. In seawater fish such as the sea bream, the kidney is unable to produce highly concentrated urine and instead produces low quantities of urine containing abundant quantities of divalent ions such as Mg^{2+} , Ca^{2+} or even SO_4^{2-} . The relatively high expression of sbAQP in the kidney may be related to functional adaptations in the kidney of seawater fish, where water movements are thought to be linked to monovalent ions such as Na^+ , Cl^- resorption and secretion in the tubules (Beyenbach and Liu, 1996).

In conclusion, a sbAQP cDNA was isolated from a kidney cDNA library and its primary structure was found to be homologous to other GLP proteins, with the same functional characteristics. Phylogenetic analysis of sbAQP, including putative GLP genes from *Fugu*, demonstrated that it did not group with any of the previously isolated GLP and instead formed a separate group, suggesting that it may be a novel GLP member.

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