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Expression of two isoforms of the vacuolar-type ATPase subunit B in the zebrafish *Danio rerio*

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Summary

In the present study we tested the hypothesis that two isoforms of the regulatory subunit B of vacuolar-type ATPase (V-ATPase) are expressed in the zebrafish *Danio rerio*. The complete coding sequences for both isoforms, vatB1 and vatB2, were cloned and sequenced. BLASTX analysis revealed the greatest similarity to amino acid sequences of B subunits from the European eel *Anguilla anguilla* and rainbow trout *Oncorhynchus mykiss*. The isoforms were expressed in a bacterial system and the recombinant proteins verified using isoform-specific antibodies directed against vatB isoforms of the eel. The distribution of both isoforms in zebrafish tissues was investigated using reverse transcriptase-polymerase chain reaction and western blot analysis. The results revealed that at the RNA level both isoforms were expressed in all tested organs, i.e. the gills, swimbladder, heart, kidney, liver, spleen, intestine and skeletal muscle. At the protein level, however, there were tissue-specific variations in the levels of the two vatB isoforms expressed. The highest amounts of V-ATPase were detected in total protein preparations from gill, heart and liver tissue. In liver tissue, however, the western blot analysis indicated that vatB1 was not as prominent as vatB2, and immunohistochemistry revealed that antibodies directed against vatB1 yielded a very weak staining in a number of cells, while an antibody directed against vatB1 and vatB2 yielded a strong staining in virtually every cell. Similarly, neurosecretory cells of the small intestine were stained with an antibody directed against vatB1 and vatB2, but not with an antibody specific for vatB1. Therefore we conclude that the differential expression of two isoforms of the V-ATPase subunits, which may serve different functions as in several mammalian species, may also be a common phenomenon in teleost fish.

Key words: V-ATPase, subunit B, isoform, hepatocyte, expression, zebrafish, *Danio rerio*.

Introduction

In eukaryotic organisms vacuolar-type ATPase (V-ATPase) is a proton pump located in cytoplasmatic membranes and in membranes of various organelles such as lysosomes, vacuoles or microvesicles (Futai et al., 2000; Moriyama et al., 2000). The transporter is oriented such that protons are pumped out of the cytoplasm either into the organelle or out into the extracellular space. It thus not only regulates acidity of organelles, but also contributes to pH homeostasis of the cytoplasm. In epithelial cells V-ATPase may also contribute to ionic control and osmoregulation (Harvey et al., 1998).

V-ATPases are multi-subunit enzyme complexes that translocate protons across membranes using the free energy of ATP hydrolysis. They consist of two main parts, the so-called head region or the peripheral sector (V_1) for ATP hydrolysis, and the V_o domain, which includes the part of the protein necessary for proton translocation (Wieczorek et al., 1999). For some of the subunits, e.g. a, E and G, tissue- or cell-specific isoforms have been described, and the different isoforms appear to be involved in different cellular processes (Murata et al., 2002; Sun-Wada et al., 2002; Oka et al., 2001; Mattsson et al., 2000; Toyomura et al., 2000).

The head region includes three A and three B subunits, and two isoforms of the B subunit, which is part of the regulatory/catalytic domain of the enzyme, have been described in *Homo sapiens*, *Bos taurus* and *Rattus rattus* (Breton et al., 2000; Van Hille et al., 1994; Puopolo et al., 1992). According to tissue-specific expression patterns the two isoforms are the so-called ‘kidney isoform’ (vatB1) and the ‘brain isoform’ (vatB2), and the kinetic properties of the mature enzymes appear to be different (Gluck, 1992; Wang and Gluck, 1990). The expression of different subunits with different properties may of course be related to different physiological functions, and one possibility, for example, could be that one isoform is related to proton transfer through epithelial membranes, while the second isoform is involved in the acidification of intracellular vesicles.

In a previous study we cloned and sequenced two isoforms of the B-subunit of V-ATPase in swimbladder tissue of the European eel *Anguilla anguilla* (Niederstätter and Pelster,

2000), but in trout gill cells only one isoform of the B subunit has been detected (Perry et al., 2000). Comparison of the conserved amino acid residues of the two isoforms of eel with the mammalian proteins led to the conclusion that the two eel isoforms may both be related to the mammalian 'brain' V-ATPase B subunit (Cutler and Cramb, 2001). On the other hand, comparison of the more variable amino acid residues of the isoforms clearly revealed that eel vatB1 has a higher homology to the 'kidney' isoform of mammals. If both eel subunits were homologous to the 'brain' subunit of mammals, this would have some interesting implications. Given the fact that fish are older in evolutionary terms than mammals, the implication is that the kidney isoform evolved after separation of the fishes, either as a new isoform, or by modification of one of the two brain isoforms. However, homology of the single isoform sequenced from trout with the kidney isoform of mammals has also been shown (Perry et al., 2000). Thus two isoforms may also exist in other fish species, despite the fact that only one isoform has been detected in trout. Furthermore, immunohistochemical observation of gas gland cells from the eel swimbladder revelaed that the subunit vatB1 is mainly located in the apical region, while the vatB2 is also found in basolateral membranes (Boesch et al., 2003), which confirms the idea that these two isoforms may indeed serve partially different functions. The expression of both isoforms in eel was not limited to the swimbladder and was detectable by reverse transcriptase polymerase chain reaction (RT-PCR) in other organs such as gills and kidney (H. Niederstätter and B. Pelster, unpublished results).

The present study was therefore designed to test the hypothesis that two isoforms of the B-subunit exist in the zebrafish *Danio rerio*, a member of the large family of cyprinid fish. First, molecular evidence for the expression of two isoforms of the V-ATPase subunit B in the zebrafish was found by cloning and sequencing RNA fragments of both isoforms using degenerate primers by RT-PCR. After determination of the complete coding sequences, zebrafish subunit vatB1 and vatB2 were expressed in a bacterial system. We then investigated the tissue distribution of these two isoforms using RT-PCR, western-blot analysis and immunohistochemistry. The data provide evidence that expression of both isoforms of the vacuolar-type ATPase subunit B is not only restricted to fish species that experience severe changes in water osmolarity, such as the catadromous eel, but may also be a common phenomenon in freshwater fish like the zebrafish.

Materials and methods

Adult wild-type zebrafish *Danio rerio* Hamilton 1822 were obtained from our fish breeding facilities. Animals were kept in small aquaria at 28°C and fed daily. Prior to tissue dissection adult zebrafish were anesthetized with buffered tricaine (MS222, final concentration 170 mg l^{-1}) and killed by cerebral translocation.

RNA isolation

Total RNA was extracted from 50–100 mg tissue samples according to the procedure described by Chomczynski and Sacchi (1987) using Trizol Reagent (Life Technologies, Gibco, BRL, Karlsruhe, Germany). After DNAse treatment the concentration of total RNA was determined using RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR, USA).

RT-PCR amplification of V-ATPase subunits B1 and B2 fragments

In the first step, degenerate oligonucleotide primers (HATP-1 forward and reverse) for RT–PCR were used. Approximately 1 μg of total RNA extracted from zebrafish gill tissue was used in reverse transcription to generate the first strand cDNA. Then a 1 μl portion of the cDNA was used in a standard PCR reaction containing 20 nmol l^{-1} of each primer, 10 μmol l^{-1} dNTPs and 1× Advantage-2 Polymerase (Clontech, Palo Alto, CA, USA) at an annealing temperature of 50°C over 30 cycles. The resulting PCR fragment was purified using a GeneClean kit according to the manufacturer's instructions (BIO101, Carlsbad, CA, USA) and ligated into a pCR®4-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA). The plasmid was transfected and subsequently amplified in *E. coli* strain TOPO10 (Invitrogen) according to standard protocols. DNA sequencing was carried out as described below using M13 forward and reverse primers (Invitrogen).

RLM-RACE of V-ATPase subunits B1 and B2

Following cloning and DNA sequencing of the first RT–PCR products, gene specific primers (GSP) for use in 5′-RACE and 3′-RACE (specific for each isoform) were designed. An RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) Kit (GeneRacer™, Invitrogen, Carlsbad, CA, USA) was used to transcribe RNA with AMV reverse transcriptase and GeneRacer™Oligo dT primers to full-length 5′ and 3′-end cDNA. RACE-PCR was performed in a volume of 25 μl consisting of 1× PCR buffer (Clontech), 0.2 μmol l^{-1} of each primer, dNTPs and 0.5 μl Advantage 2 Polymerase (Clontech) using a touch-down protocol in a Gene Amp PCR System 9700 (PE Applied Biosystems, Norwalk, CT, USA). After gel purification with GeneClean (BIO-101) PCR fragments were routinely cloned into pCR®4-TOPO vector and sequenced using M13 forward and reverse primers and sequence-specific primers where necessary.

DNA sequencing and analysis of the cloned PCR products

For sequencing we used the dye terminator system (PE Applied Biosystems, Foster City, CA, USA) and an automated sequencer (373A DNA Stretch Sequencer; PE Applied Biosystems). The templates for sequencing were generated by purification of plasmid DNA using the alkaline lysis method (QuiaPrep, Quiagen, Hilden, Germany). DNA sequences were analyzed using ABI Prism Sequencing Analysis Software (Version 3.0, PE Applied Biosystems) and aligned with the Sequence Navigator AC software package (Version 1.01, PE Applied Biosystems) in combination with the GenBank

Table 1. *Primer sequences*

Primer	Sequence
Degenerate primers	
HATP-for-1 Clayborne	5′GRT GNG TDA TRT CRT CRT NNG G-3
HATP-rev-1 Clayborne	5′GGN GTN AAY ATG GAR CAN GC-3′
RACE PCR primers	
GeneRacer™ 5′Primer	5′-CGA CTGG AGC ACG AGG ACA CTG A-3′
GeneRacer™ 3′Primer	5′-GCT GTC AAC GAT ACG CTA CGT AAC G-3′
Zf vatB1-5′Race	5′-ACG GGC AGC AGA CAC CTC TCT C-3′
Zf vatB1-3′Race	5′-CTG AAC CTG GCC AAC GAT CCT ACC-3′
Zf vatB2-5′Race	5′GTA AGG CCT CGG CAT AAG AGC TCA T-3′
Zf vatB2-3′Race	5′GGC CAA TGA TCC CAC GAT TGA A-3′
Sequencing primers	
M 13 forward	5′CTGGCCGTCGTTTTAC-3′
M 13 reverse	5′CAGGAAACAGCTATGA-3′
T7 forward	5′-TAATACGACTCACTATAGGG-3′
V5C-term reverse	5′-ACCGAGGAGAGGGTTAGGGAT-3′
ZF 3′vatB1 vorne	5′GAT GAC TTG CTG TAC CTG-3′
Zf 3′vatB1 hinten	5′TAT TTC CCT TTG AGA TGT-3′
Zf 3′Race vatB2 vorne	5′AAA GAT GTC CAG GCA ATG-3′
Zf 3′Race vatB2 hinten	5′TGT AAA CTC TGT CTG GCC-3′
Amplification of ORFs	
Zf vatB1 99/22	5′-ATG TCG ACG TTA GTA GCG AAC C-3′
Zf vatB1 1595/22 rc	5′-AGA CTC TCT CGG GTA GAA TTC G-3′
Zf vatB2 149/23	5′-ATG AAG GCT CTT AGG GGA ATG GT-3′
Zf vatB2 1648/22 rc	5′-AGC CAG AGT GCT CTG AGG AAT C-3′
Reverse transcriptase PCR	
Zf vatB1 123/18	5′-CCC GTG GAT CTA AAT GGC-3′
Zf vatB1 576/23 rc	5′-TCT CCT CTG GGT AGA TAC GAC AC-3′
Zf vatB2 172/24	5′-GAG CGG AGC CGT GAG CGA AAT ATC-3′
Zf vatB2 580/24 rc	5′-CAC CGC AGG TCC TCG ATC AAT TGG-3′
Zf β2-microglobulin 963/23*	5′-ACC AAA CAC CCT GAT CTG CTA TG-3′
Zf β2-microglobulin 1669/21 rc*	5′-CCC CTG CAC ACC AAG AAC AAG-3′

RACE, rapid amplification of cDNA ends; ORF, open reading frame; PCR, polymerase chain reaction; Zf, zebrafish.
*Primers were designed and named on the basis of a sequence of β2-microglobulin (GenBank accession number L05384; Ono et al., 1993).

and Swiss-Prot databases for comparison with other known sequences.

Determination of tissue-distribution by RT-PCR

Total RNA from several tissues (muscle, heart, intestine, liver, spleen, gills, swimbladder) of the zebrafish was isolated as described above. First-strand cDNA synthesis was performed with 500 ng total RNA of each sample, random hexamer primers and MLLV Reverse Transcriptase (Power Script™, Clontech). Quality of cDNA was controlled by PCR for the house-keeping gene β2-microglobulin (for primers, see Table 1). Using two specific primer pairs for the two V-ATPase subunit B isoforms, the presence of V-ATPase and the tissue-specific distribution of these subunits were analyzed. Sequencing of the resulting PCR products was performed in order to verify the specificity of the primers. The PCR products obtained were visualized by agarose gel electrophoresis and ethidium bromide staining.

Bacterial expression of recombinant zebrafish vatB1 and vatB2

The open-reading frames (ORFs) including the native start codons, but not the stop codons, were amplified using specific primer pairs for each isoform of the B subunit of V-ATPase (see Table 1). PCR was performed at a volume of 25 µl consisting of 1× PCR buffer (Clontech); 25 pmol of the corresponding primer, 25 µmol of each dNTP and 0.5 µl Advantage HF-2 mix (Clontech) in a Gene Amp PCR system 9700 thermocycler (Applied BioSystems). cDNA made from zebrafish gill tissue served as template. The purified PCR-products were ligated into a pCR® T7/CT TOPO® vector using T/A cloning strategy (Invitrogen) and cloned into TOPO 10F′ cells (Invitrogen). The plasmids were analyzed in both directions by sequencing using sequencing primers T7-forward and V5C-term reverse (Invitrogen). Expression and detection of both isoforms in BL21(DE3)pLysS (Invitrogen) cells followed the procedures described by Boesch et al. (2003). The presence of the induced

protein was verified by western-blot using specific antibodies that were made against peptides of the two V-ATPase B subunit isoforms of the European eel. One antibody was directed against an amino acid sequence close to the 5′ end specific for vatB1 (#1035), and a second antibody (#1034) was specific for a conserved amino acid sequence that is identical for both isoforms (vatB1 and vatB2). Whereas peptide sequences of zebrafish and eel were identical for the binding site of antibody #1034, in the binding site of antibody #1035 at position 3 asparagine was replaced by proline and at position 5 glutamic acid was replaced by aspartic acid (see Fig. 1).

Determination of tissue specific distribution by western-blot analysis

Tissue samples from zebrafish were excised and immediately soaked in Trizol (Life Technologies) containing 0.16% of β-mercaptoethanol (Sigma-Aldrich, Vienna, Austria) following the instructions of the manufacturer. The resulting protein pellets were solved in 1× SDS sample buffer containing 1% proteinase inhibitor cocktail (Sigma-Aldrich), 0.05% PMSF (Sigma-Aldrich) and 1% β-mercaptoethanol. Proteins were separated under reducing conditions (125 μmol l^{-1} dithiothreitol) using a 10% Bis-Tris gel and Mops buffer (Novex, San Diego, USA), and blotted onto PVDF membranes (BioRad, Hercules, CA, USA) using a constant voltage of 25 V for 1 h. Membranes were blocked for 1 h with 0.2% I-block (Tropix, USA) and 0.1% Tween 20 (Sigma-Aldrich) in 0.1 mol l^{-1} PBS at room temperature. Primary antibody incubation was performed overnight at 4°C in blocking buffer. Use of various antibody concentrations for the western blot revealed that the optimal signal was obtained at a dilution of 1:250 for antibody #1034, and of 1:50 for antibody #1035. After additional washing steps the membranes were probed for 1 h with a horse radish peroxidase (HRP)-conjugated second antibody (HRP-conjugated anti-rabbit IgG; Sigma-Aldrich). Finally, proteins were visualized using the enhanced chemiluminescence ECL detection reagents (Amersham, Buckinghamshire, UK).

Preparation of histological specimens and immunohistochemistry

Tissue preparation and immunohistochemical localization of V-ATPase subunits B basically followed the procedure described by Boesch et al. (2003). Briefly, dissected tissue samples were fixed in 4% buffered paraformaldehyde at 4°C overnight. The samples were dehydrated in a series of ethanol baths and finally embedded in paraffin by sequential incubations in methyl benzoate (1× overnight, 3× 3–12 h), benzene (2× 30 min), benzene/paraffin (1× 2 h at 60°C) and paraffin (three changes within 12–16 h).

For the immunocytochemical localization paraffin sections (4–5 μm thickness) from various tissues were cut using an Autocut 2040 (Reichert, Vienna, Austria) and mounted on coated glass-slides (Dimethylsilane, Sigma-Aldrich-Chemie, Vienna, Austria). The sections were dewaxed by series of xylene and ethanol. After antigen-retrieval by proteinase-K digestion and acetylation with 10 min incubation in 0.5% anhydrous acetic acid in 0.1 mol l^{-1} Tris-HCl, pH 8.0, non-specific bindings were blocked with 10% foetal calf serum in Tris-buffered saline (TBS). Incubation with the appropriate (optimal) dilution of the primary antibodies in blocking buffer was undertaken at 4°C overnight. After five washes with TBS slides were incubated with a polyclonal biotinylated anti-rabbit/mouse IgG (Duett-ABC Kit Solution C; Dako, Glostrup, Denmark) for 20 min. Then additional washes were performed and the sections were probed with an anti-biotin alkaline-phosphatase antibody (dilution 1:100; Dako) for 1 h. Finally a purple color reaction was developed at 4°C in a solution of 4-nitro blue tetrasodium chloride (Roche Molecular Biochemicals, Mannheim, Germany) and 5-bromo-4-chloro-3-indolylphosphate-4-toluisin salt (Roche). The sections were washed three times with TBS, mounted in Gel Mount (Lipshaw Immunon, Pittsburgh, PA, USA) and coverslips placed on top. Sections were observed and photographed using bright-field light microscopy (Polyvar, Reichert, Vienna, Austria; Zeiss, Jena, Germany).

Results

Determination of sequences of V-ATPase subunits B1 and B2 in zebrafish

Using degenerate primers a 388 bp product was amplified from cDNA of zebrafish gill tissue and cloned. Sequencing of about 15 clones of this product revealed the presence of two different amplicons (vacuolar-type ATPase subunit B1 and B2) with a sequence identity of 82%. BLASTN analysis showed that both products had a high degree of identity with sequences of the subunit B isoforms of the European eel and of rainbow trout. Based on the resulting sequence information, specific primers were designed for 5′ and 3′-RACE-PCR. Using the RLM-RACE technique, cloning and sequencing, we determined the whole sequence of both products including leader sequences and the polyadenylation signal. BLASTN revealed 88.7% similarity for the total nucleotide sequences. Translation of vatB1 sequence starting at position 99 and ending at position 1619 (UAA) resulted in a predicted amino acid sequence of 506 residues with a calculated molecular mass of 55.9 kDa and an isoelectric point of 4.32. The open reading frame of vatB2 is located between positions 149 and 1678, resulting in a predicted amino acid sequence of 509 residues with a calculated molecular mass of 56.5 kDa and an isoelectric point of 5.53. Using BLASTX we found that the deduced amino acid sequence of zebrafish vatB1 (GenBank accession number AF 472614) showed 95% identity to eel vatB1 and 94% identity to trout vatB1. Zebrafish vatB2 (GenBank accession number AF 472615) showed the highest degree of identity (95%) to eel vatB2 (see Fig. 1 for alignment of deduced amino acid sequences).

Expression of recombinant zebrafish V-ATPase subunits B1 and B2 in E. coli

The open reading frames of zebrafish vatB1 and vatB2 including native start codons were cloned and expressed in *E.*

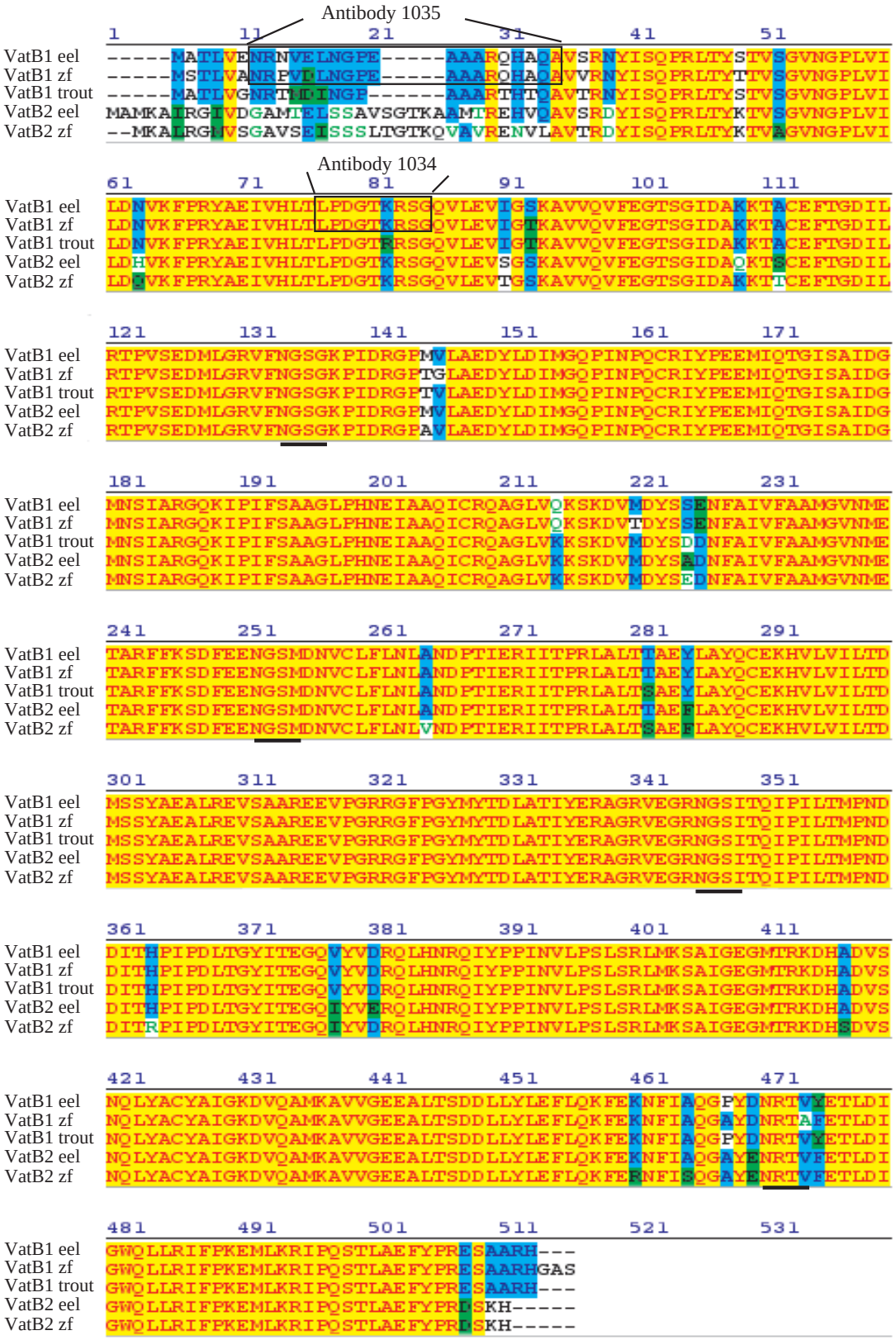

Fig. 1. Amino acid alignment (single letter code) of *D. rerio* (zf) V-ATPase subunits vatB1 and vatB2 (GenBank accession numbers AF472614 and AF472615), *A. anguilla* (eel) V-ATPase subunits vatB1 and vatB2 (Niederstätter and Pelster, 2000) and *O. mykiss* (trout) V-ATPase subunit isoform vatB1 (Perry et al., 2000). Yellow background color indicates identical residues, blue background color indicates most frequent residues. The origin of the sequence is indicated on the left. Possible N-glycosylation sites within the conserved region are underlined. The binding sites of antibodies 1035 and 1034 are indicated.

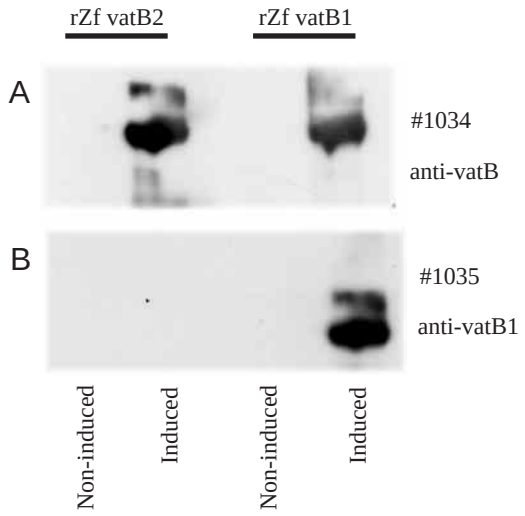

Fig. 2. Determination of recombinant zebrafish (rZf) vatB1 and vatB2 by western blot analysis. Non-purified bacterially expressed zebrafish vacuolar ATPase subunit B isoforms vatB1 and vatB2 were probed with (A) antibody #1034, which recognizes both isoforms of the B subunit, and (B) antibody #1035, which is specific for vatB1.

coli BL21(DE3)pLysS cells. The bacteria were harvested by centrifugation and directly transferred into sample buffer prior to SDS-gel electrophoresis. Expression was carried out at 25°C, and addition of 0.5 mmol l^{-1} isopropylthiogalactoside (IPTG) clearly induced the expression of a 60 kDa protein after 2 h, corresponding to the molecular mass of both recombinant isoforms, which was increased using 6-Histag by approximately 5 kDa. Western blot analysis of the recombinant zebrafish vatB1 and vatB2 proteins with antibodies directed against vatB (#1034) and vatB1 (#1035) showed that the expressed proteins indeed were vatB1 and vatB2. Recombinant zebrafish vatB1 was recognized by both antibodies, whereas vatB2 was recognized only by antibody 1034 (see Fig. 2).

Tissue distribution of V-ATPase subunits B1 and B2 in zebrafish

The tissue distribution of V-ATPase subunits B1 and B2 was assessed at the RNA and the protein levels. Samples of different tissues from several animals were dissected and either RNA or the protein isolated. Each experiment was replicated with at least four different animals. RT-PCR for vatB1 and vatB2 revealed that both isoforms are expressed in all tested tissues (Fig. 3). The sequence of the amplified PCR product was verified by sequence analysis.

The distribution of vatB1 and vatB2 at the protein level was assessed by western blot analysis. Total protein was isolated, and 30 μg of each protein sample used for each blot. V-ATPase was expressed in all tissues tested, but there were differences in the V-ATPase content of the different tissues. The highest level of expressed protein was detected in gill, liver and heart tissues. Whereas in most tissues the band produced by the two antibodies was similar in size, in the liver the band obtained with antibody #1035 (vatB1) was not as prominent as that obtained with antibody #1034 (vatB1 and vatB2). A high content of both B-subunit isoforms was also found in the intestine, the spleen and the kidney. Very little, however, was detected in swimbladder, although western blot analysis with an increased amount of protein (60 μg) revealed presence of the B-subunit of V-ATPase in this tissue as well. To avoid false positive results due to the expression of V-ATPase in blood cells we also tested samples of zebrafish blood. These samples showed almost no reactivity when probed with the antibodies. We therefore concluded that the discovered B-subunits of V-ATPase indeed did represent protein from the tested tissues (Fig. 4).

Immunohistochemical localization of the two B subunits in the gills, heart, and kidney cells gave similar results for both antibodies. In the liver, however, the two antibodies yielded very different results. While antibody #1035 gave a very weak signal, antibody #1034 gave a strong signal in virtually all cells (Fig. 5). Controls without primary antibody showed no staining. In the small intestine, localization of the B subunit yielded weak staining in a number of cells, but the antibody #1034 generated a strong signal in large cells located near the lamina propria, which according to their location and size are thought to be neurosecretory cells (enterochomaffin cells, APUD cells; Fig. 6). These cells could not be identified with the antibody #1035, which only binds to isoform vatB1.

Discussion

The results of the present study demonstrate the expression of two isoforms of the B-subunit of V-ATPase in the zebrafish, a member of the large family of cyprinids, and this is also true for the goldfish, another cyprinid fish (S. T. Boesch and B.

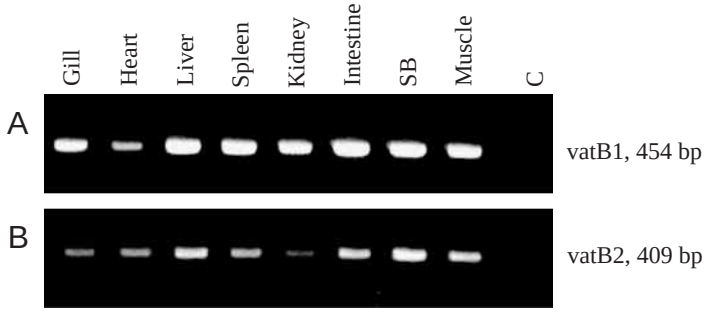

Fig. 3. Tissue distribution of vatB1 and vatB2. RT-PCR analysis of V-ATPase subunit B isoforms 1 and 2 in different tissues of *D. rerio*. 500 ng of total RNA were transcribed to cDNA using MLVV-reverse transcriptase and Random N6 Primers PCR was performed with isoform-specific primers. PCR-products were run on a 1% agarose gel and stained with ethidium bromide. Both isoforms are expressed in all tested tissues (A, vatB2 PCR product of 454 bp; B, vatB2 PCR products of 409 bp). SB, swim bladder; C, control (no template).

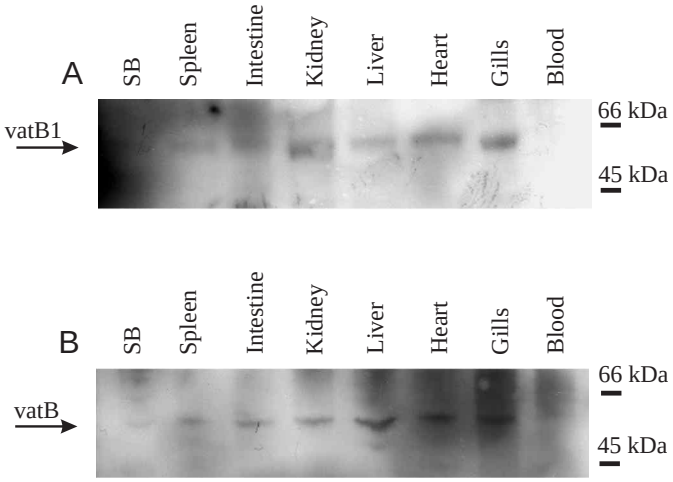

Fig. 4. Western blot analysis of zebrafish tissues with antibodies specific for vacuolar ATPase subunit B isoforms. 30 μg of total protein were applied to each lane. Antibody #1034 recognizes both isoforms (B) whereas antibody #1035 (A) is specific for subunit isoform vatB1. Both antibodies revealed bands of approximately 55 kDa. SB, swimbladder tissue.

Pelster, unpublished observations). BLASTX analysis revealed that the deduced amino acid sequence of one of these isoforms shows highest sequence identity with the isoform vatB1 (possibly the so-called kidney isoform), which has already been found in gas gland cells of the European eel, while the second isoform shows the highest sequence identity with vatB2 (possibly the brain isoform), also present in the eel (Niederstätter and Pelster, 2000). This suggests that the expression of two isoforms of the B-subunit of V-ATPase is widespread among fishes, although in the salmonid *Oncorhynchus mykiss* only the isoform vatB1 has been identified so far (Perry et al., 2000).

The B-subunit of the V-ATPase is part of the head region of the protein, and it is not a transmembrane protein. VatB1, mainly expressed in specialized proton-translocating intercalated cells (B-cells) in the rat kidney, carries a C-terminal binding motif (DTAL motif) that enables interaction with the PDZ domain of the Na^+/H^+-exchanger regulatory factor (NHE-RF) (Breton et al., 2000). The vatB2 isoform, also expressed in various cell types of the kidney, lacks this binding domain and therefore cannot interact with members of the PDZ protein family. Thus, interaction between vatB1 and NHE-RF or other PDZ proteins may contribute to a differential localization of V-ATPases containing either the B1 or the B2 subunit. NHE-RF has also been shown to interact with actin filaments of the cytoskeleton (Murthy et al., 1998). Holliday et al. (2000) demonstrated that both isoforms of the B subunit of mammalian osteoclasts, vatB1 and vatB2, include actin binding sites within the first 106 or 112 amino acid residues of the amino-terminal region. Thus, the B subunit appears to be involved in an interaction of V-ATPase with the cytoskeleton. Given the high homology between the eel isoforms and the two

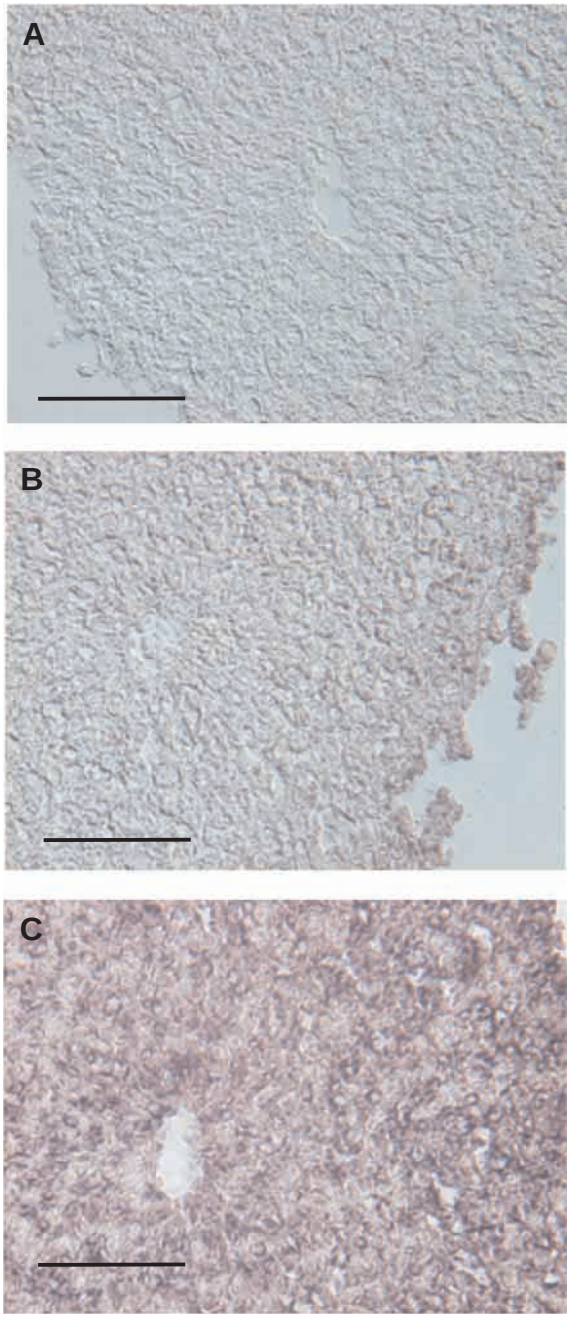

Fig. 5. Immunocytochemical staining of vacuolar ATPase B subunit isoforms vatB1 and vatB2 in zebrafish liver. (A) No background staining was observed in the negative control without primary antibody. (B) Antibody #1035, which is specific for the vatB1 isoform, resulted in a weak staining of hepatocytes. (C) Antibody #1034, which binds to vatB1 and vatB2, resulted in a very strong signal in most hepatocytes. Scale bars, 50 μm.

mammalian isoforms (see Niederstätter and Pelster, 2000) it is quite likely that these binding sites have been conserved.

The two isoforms apparently differ not only in their kinetic characteristics, but also in the way they interact with other proteins or subunits, which suggests that the mammalian isoforms of the B-subunit included in the V-ATPase may

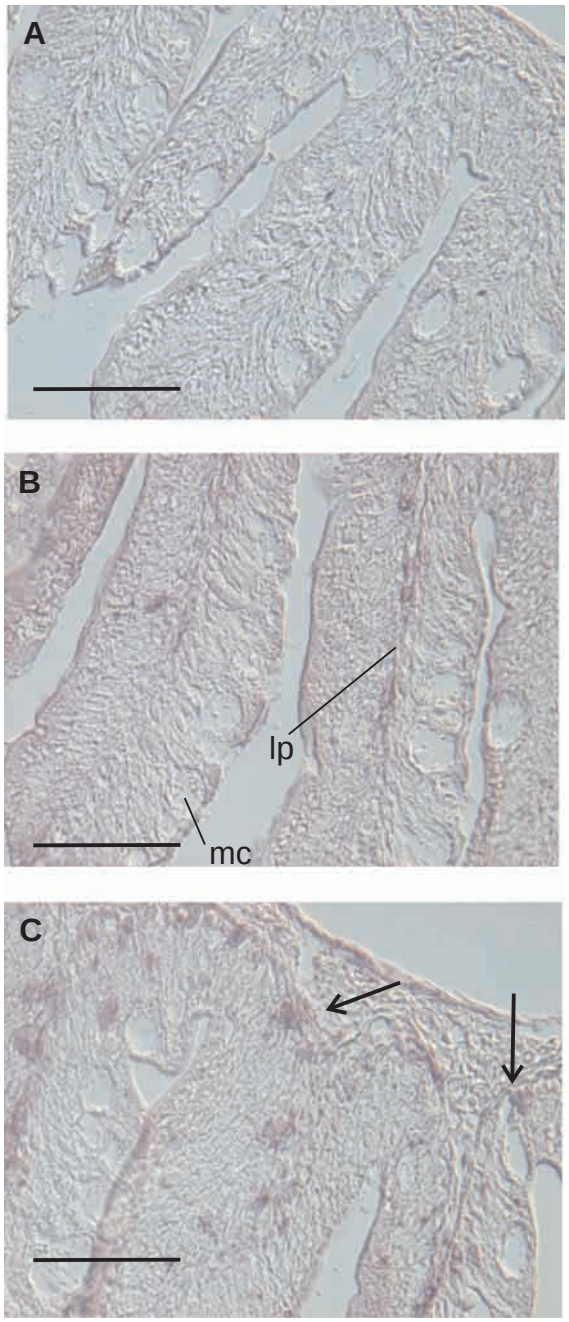

Fig. 6. Immunocytochemical staining of vacuolar ATPase B subunit isoforms vatB1 and vatB2 in the small intestine of the zebrafish. (A) No background staining was observed in the negative control without primary antibody, except in the region of the microvilli, which contains levamisol-resistant alkaline phosphatase. (B) Antibody #1035, which is specific for the vatB1 isoform, resulted in a weak staining of cells. The microvilli showed unspecific staining, and some cells in the central region of the villi near the blood vessels of the lamina propria showed a positive reaction. (C) Antibody #1034, which binds to vatB1 and vatB2, resulted in a very strong signal in large cells at the base of the villi, which according to their position and shape most likely represent neurosecretory cells. mc, mucus cells; lp, lamina propria. Arrows identify large positive cells. Scale bars, 50 μm.

modify the physiological properties of the ATPase. None of the vatB1 isoforms of fish sequenced so far includes the DTAL motif, which is present in the mammalian kidney isoform (see Fig. 1), a possible indication that the physiological function of fish vatB1 might differ from the function of the mammalian kidney isoform.

The expression of enzymes with different properties implies that they may serve different functions, and this in turn would result in a distinct localization of the isoforms. In the swimbladder of the European eel we indeed observed that the vatB1 was located mainly in the apical membrane and in lamellar bodies, while vatB2 appeared to be mainly located in basolateral membranes (Boesch et al., 2003). In the zebrafish we found that mRNA of both isoforms is present in all tested tissues. RT-PCR, however, is a very sensitive tool and it cannot be excluded that in some tissues only a few copies of the mRNA were present, so that the protein in fact is barely expressed.

On the protein level we indeed observed that the expression of the B-subunit is variable between the different tissues analyzed. The gills, for example, which play an important role in osmoregulation, had a relative high expression of V-ATPase.

We also found expression of the B subunit in the liver and the spleen, both organs with a high metabolic activity. In the liver, however, western blot analysis indicated that the vatB1 subunit was not as abundant as vatB2. This was confirmed by immunohistochemistry. Localization of the subunits within the tissue clearly revealed that vatB2 was present in all hepatocytes, while antibodies directed specifically against vatB1 produced only a very weak signal, indicating that this subunit was barely expressed. In the liver, V-ATPase is mainly involved in the acidification of intracellular organelles; there is no epithelial proton transport as, for example, observed in fish gill cells or in tubular kidney cells. The predominance of subunit vatB2 in this tissue therefore suggests that vatB2 might mainly be involved in intracellular proton transport and contribute to an acidification of lysosomes and other cell vesicles.

The small intestine provided a second example of differential localization of the isoforms, since neurosecretory (enterochomaffin cells, APUD cells) could be stained with antibody #1034, but not with the vatB1-specific antibody antibody #1035. Neurosecretory cells are secretory cells, and from our results vatB2 is the predominant isoform in these cells.

As V-ATPases are ubiquitous enzymes it is not surprising that they are also present in skeletal muscle tissue and the heart. Here the V-ATPase may be located in the membrane of synaptic vesicles, where it pumps protons from the cytoplasm into the synaptic vesicle (Nelson and Harvey, 1999; Kanner and Schuldiner, 1987). Hong (2002) showed in a pharmacological study that the release of neurotransmitters

from synaptic vesicles at the nerve-muscle junction is inhibited by bafilomycin A, a selective inhibitor of the V-ATPase.

In summary, the results of our study demonstrate that the existence of two isoforms of subunit B of V-ATPase is widespread among fishes, despite the fact that in rainbow trout only one isoform has been detected. The differential localization of the two isoforms observed in the swimbladder of the European eel (Boesch et al., 2003), in the liver and in small intestine neuroscretory cells of zebrafish suggest that the two isoforms may serve partly different functions.

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