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Expression of carbonic anhydrase, cystic fibrosis transmembrane regulator (CFTR) and V-H$^+$-ATPase in the lancelet *Branchiostoma lanceolatum* (Pallas, 1774)

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ABSTRACT

Sequencing of the amphioxus genome revealed that it contains a basic set of chordate genes involved in development and cell signaling. Despite the availability of genomic data, up till now no studies have been addressed on the comprehension of the amphioxus osmoregulation. Using primers designed on *Branchiostoma floridae* carbonic anhydrase (CA) II, cystic fibrosis transmembrane regulator (CFTR) and V-H$^+$-ATPase, a 100 bp long region, containing the protein region recognized by the respective antibodies, has been amplified and sequenced in *B. lanceolatum* indicating the presence of hortologous V-ATPase, CFTR and carbonic anhydrase II genes in *Branchiostoma lanceolatum*. Immunohistochemical results showed that all three transporting proteins are expressed in almost 90% of epithelial cells of the skin in *B. lanceolatum* adults with a different degree of positivity in different regions of body wall and with a different localization in the cells. The comparison of results between young and adult lancelets showed that the distribution of these transporters is quite different. Indeed, in the young specimens the expression pattern of all tested molecules appears concentrated at the gut level, whereas in adult the gut loses its key role that is mostly supported by skin.

Introduction

Teleosts and Chondrichthyes regulate body fluid pH and ionic composition mainly through the integument, gills, kidney and gut (Morris, 1960; Claiborne et al., 2002; Evans et al., 2005; Gilmour and Perry, 2009; Wood et al., 2010). In the early ontogenetic stages, before the development of gills and kidney, the skin is believed to be the major structure responsible for pH and osmotic regulation (Kaneko et al., 2002; Varsamos et al., 2002, 2005; Falk-Petersen, 2005; Lin et al., 2006; Bodinier et al., 2009). In larval and adult epithelia of the regulatory organs, principal cells and specific cells, such as mitochondria-rich cells (MRC) or ionocytes or chloride cells, express many ion transport-related transmembrane proteins and enzymes, including H$^+$V-ATPase and carbonic anhydrase, that are involved in acid–base and ionic regulation (Piermarini and Evans, 2001; Claiborne et al., 2002; Marshall and Singer, 2002; Piermarini et al., 2002; Hirose et al., 2003; Lin et al., 2006; Grosell et al., 2009; Gilmour and Perry, 2009).

Hagfish are considered the most ancient of craniates and, among marine fish, they are a unique living *taxon* that is exclusively marine and osmoconformer (Morris, 1960). They do not need osmotic regulation though they can compensate for volume loading in slightly hypo-osmotic environments or after hypo-osmotic fluid loading during feeding (Cobb et al., 2004). Nevertheless, their gill epithelium is rich in MRC cells, probably involved in pH regulation (Elger and Hentschel, 1983; Mallat et al., 1987; Choe et al., 2002; Tresguerres et al., 2006a, 2007a; Parks et al., 2007).

Cephalochordata, commonly known as amphioxus or lancelet, represent a small *taxon* of Chordata consisting of about 30 marine species. It was traditionally considered the sister *taxon* of craniates (Holland et al., 1996), but more recently molecular phylogenetic analyses have indicated that tunicates, as the sister *taxon* of the vertebrates and cephalochordates as the most basal chordate group (Holland et al., 2008; Putnam et al., 2008).

Up till now, the mechanisms of acid–base or ionic regulations have not been studied in amphioxus and specialized cells in transporting epithelia have never been described. It has been suggested that *Branchiostoma nigeriense* is isosmotic with the environment or osmoregulates since it can survive in salinities from 13 to 58 parts per thousand (Webb and Hill, 1958). Moreover, the morphology of the *Branchiostoma floridae* kidney does not change if lancelets are bred in artificial sea water at different salt

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concentrations suggesting that amphioxus is either isosmotic or is osmoregulating by means of other organs (Moller and Ellis, 1974).

The publication of the wholly sequenced amphioxus genome suggested that it contains a basic set of chordate genes involved in development and cell signaling, including duplication genes proposed to function in innate immunity and endocrine systems (Holland et al., 2008). Despite the availability of genomic data, up till now no studies have been made to understand osmoregulation in the amphioxus.

The aim of this research was to investigate the presence and localization of some signal proteins involved in osmoregulation and pH regulation processes, such as carbonic anhydrase (CA) II, cystic fibrosis transmembrane regulator (CFTR) and V-H^+-ATPase, by molecular and immunohistochemical techniques, taking young and adult *Branchiostoma lanceolatum* lancelets as an experimental model and comparing the obtained results with data reported in the literature on these proteins in marine fish.

Materials and methods

Animals

Twenty young (metamorphosed sexually immature) specimens (10–12 mm in length) of *B. lanceolatum* (Pallas, 1774) were obtained from sediments of Elba Island (Italy, Mediterranean Sea). An additional ten adult lancelets (5–6 cm in length) were collected from the neighboring Ligurian Sea.

Molecular methods

DNA extraction was performed from a 1 cm long portion of three *B. lanceolatum* specimens using the Wizard® SV Genomic DNA Purification System (Promega, Madison, WI, USA). DNA samples have been quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc, Wyman St. Waltham, MA, United States).

In order to favor the choice of useful antibodies, an internal 100 bp long portion of genes coding for V-ATPase, CFTR and carbonic anhydrase II genes has been amplified by PCR and sequenced. In particular, the amplified regions corresponded to protein portions recognized by the antibodies. The primer couples were F_{CFTR} (5′-GCGGTCGAGTCAGTGTTGT)/R_{CFTR} (5′-TCCTCCAAGCCTTCTTGTTG), F_{CAII} (5′-ATCGCAGTTGAGGTGCATTT)/R_{CAII} (5′-GAAAAGGTAGGACTGTCGCTTG) and F_{VATP} (5′-TCCCTAGACATTGGCTGGTC)/R_{VATP} (5′-GGCTGTCCCTGGGGTAGTAT) respectively for the CFTR, carbonic anhydrase II and V-H^+-ATPase peptides. The amplification mix contained 100 ng genomic DNA, 1 μM of each primer, 200 μM dNTPs and 2 U of DyNAZyme II polymerase (Finnzymes Oy, Vantaa, Finland). Amplifications were performed using a Hybaid thermalcycler. The reaction conditions were the same for the three primer couples: 94 °C for 90 s, and a total of 25 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. Amplified bands have been analyzed by 1.5% agarose gel electrophoresis, according to standard procedures. DNA sequencing was performed at BMR Genomics (Padua, Italy) using a Sanger sequencing technique.

Immunohistochemical methods

Samples were fixed in 4% paraformaldehyde, embedded in paraffin wax and cut in 7 μm transverse sections. For the immunohistochemical tests the following polyclonal antibodies were used: anti-V-H^+-ATPase (B1/2 (L-20) sc-31464 Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:50 and 1:100), anti-CFTR (C-19 036 sc-8910 Santa Cruz, 1:100) and anti-carbonic anhydrase II (039100-401-136 Rockland, Gilbertsville, PA, USA, 1:50 and 1:100). For the biotin–avidin immunohistochemistry technique (BAS) slides were rinsed three times in 0.01 M PBS, pH 7.4 and incubated for 30 min in 0.3% H_2O_2 to block endogenous peroxidase activity, then placed in PBS containing 0.3% Triton X-100 and blocked with 5% normal serum (Rockland) for 30 min. The slides were incubated with the primary antibody in a humid chamber at 4 °C overnight. Subsequently, they were rinsed with PBS and incubated for 30 min at room temperature with the secondary biotinylated antibody (titer 1:300) (Rockland). After rinsing in PBS and 0.1 M Tris pH 7.6, the preparations were incubated with avidin–biotin complex (ABC, Vector, Vectastain, Burlingame, CA, USA) in Tris for 45 min at room temperature. The reaction was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB: 10 mg/15 ml Tris) (Sigma–Aldrich, St. Louis, MO, USA). Reactions were allowed to develop for 4–10 min with 10–12 μl 30% H_2O_2. All sections were rinsed in Tris and *aqua fontis*, dehydrated and mounted in Eukitt (O. Kindler GmbH & Co., Freiburg, Germany), and observed with Zeiss Axioscop microscope equipped with a Canon camera Power Shot A640.

For the immunofluorescence slides were rinsed three times in 0.01 M PBS, pH 7.4 and incubated with 10% normal serum for 20 min. The slides were incubated with the primary antibody in a humid chamber at 4 °C overnight. Subsequently, they were rinsed with PBS and incubated with secondary fluoresceinated antibody (FITC, Rockland) diluted 1:20 for 1 h at room temperature. After rinsing in PBS the slides were mounted with DABCO anti-fading solution and observed by laser confocal microscope Leica TCS SP5 at the "Centro Interdipartimentale Grandi Strumenti" (CIGS) of Modena and Reggio Emilia University.

The specificity of the reactions was checked by negative controls incubating the sections with (1) PBS instead of the primary antibody, (2) normal serum instead of the primary antibody, (3) PBS instead of the secondary antibody, and (4) primary antibody preadsorbed in liquid phase with the homologous antigen (50 μg/ml diluted antiserum).

Results

Molecular results

Bioinformatic analyses, performed in the *Metazome* (http://www.metazome.net/) and *OrthoDB* (http://cegg.unige.ch/orthodb5) databases showed the presence of genes coding for V-ATPase, CFTR and carbonic anhydrase II in the *B. floridae* genome. These three genes have been annotated, but not studied so that a BLAST analysis in GenBank has been made in order to confirm their proper identification.

The predicted *B. floridae* aminoacidic sequence fgenesh2_pg.scaffold_205000018 (protein ID XP_002609084) consisted of 508 residues, contained the pfam01992 domain previously identified in several ATP synthase (C/AC39) subunit and showed a high similarity with the *Apis mellifera* (XP_624112), *Bos taurus* (NP_001001146) and *Mus musculus* (AAC52411) V-ATPase peptides. The V-ATPase coding gene sequence (Gene ID 7219325) is reported to be expressed in the embryonic, larval and adult stages of lancelets.

The annotated amino acidic sequence estExt_fgenesh2_pg.C_2610037 (protein ID XP_002601262) consisted of 322 residues, contained the pfam00194 domain identified in the eukaryotic-type carbonic anhydrase and showed a strong similarity with the *Gallus gallus* (XP_415218.3), *Xenopus tropicalis* (XP_002931952) and *Bos taurus* (NP_001179451) carbonic anhydrase II peptides. The respective gene sequence (Gene ID 7248027) is reported to be expressed in the neurula and larval stages of lancelets, whereas its expression in adults has been not evaluated.