Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na\(^+\) uptake in zebrafish H\(^+\)-ATPase-rich cells

Tzung-Yi Lin, Bo-Kai Liao, Jiun-Lin Horng, Jia-Jian Yan, Chung-Der Hsiao, and Pung-Pung Hwang

1Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan; 2Institute of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan; 3Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan, Republic of China

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Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na\(^+\) uptake in zebrafish H\(^+\)-ATPase-rich cells. Am J Physiol Cell Physiol 294: C1250–C1260, 2008. First published March 5, 2008; doi:10.1152/ajpcell.00021.2008.—H\(^+\)-ATPase-rich (HR) cells in zebrafish gills/skin were found to carry out Na\(^+\) uptake and acid-base regulation through a mechanism similar to that which occurs in mammalian proximal tubular cells. However, the roles of carbonic anhydrases (CAs) in this mechanism in zebrafish HR cells are still unclear. The present study used a functional genomic approach to identify 20 CA isoforms in zebrafish. By screening with whole mount in situ hybridization, only zca2-like a and zca15a were found to be expressed in specific groups of cells in zebrafish gills/skin, and further analyses by triple in situ hybridization and immunocytochemistry demonstrated specific colocalizations of the two zca isoforms in HR cells. Knockdown of zca2-like a caused no change in and knockdown of zca15a caused an increase in H\(^+\) activity at the apical surface of HR cells at 24 h postfertilization (hpf). Later, at 96 hpf, both the zca2-like a and zca15a morphants showed decreased H\(^+\) activity and increased Na\(^+\) uptake, with concomitant upregulation of znhe3b and downregulation of zap6v1a (H\(^+\)-ATPase A-subunit) expressions. Acclimation to both acidic and low-Na\(^+\) fresh water caused upregulation of zca15a expression but did not change the zca2-like a mRNA level in zebrafish gills. These results provide molecular physiological evidence to support the roles of these two zca isoforms in Na\(^+\) uptake and acid-base regulation mechanisms in zebrafish HR cells.

ionocytes; Na\(^+\)/H\(^+\) exchanger; skin; gill; embryo

Carbonic anhydrase (CA, EC 4.2.1.1) is a zinc metalloenzyme that catalyzes the reversible reaction: CO\(_2\) + H\(_2\)O ⇌ H\(_2\) + CO\(_2\) ⇌ HCO\(_3\)\(^-\) + H\(^+\). This reaction forms the basis for the regulation of the acid-base balance in organisms. The 15 CA isoforms identified in mammals so far have been found to differ in their kinetic properties, susceptibility to inhibitors, and specific tissue distributions (29). CA2 is the only soluble form of carbonic anhydrase in renal epithelial cells. On the other hand, CA4 is expressed on either the apical brush-border membrane and/or the basolateral membrane of proximal tubule cells (29). The kidney reabsorb all the bicarbonate that is filtered by the glomeruli. Most, 70%−85%, of the bicarbonate is reabsorbed in proximal tubules and only 10%−20% in the thick ascending limb of Henle. In those segments, intracellular CA2 and apical CA4 are both responsible for net transepithelial bicarbonate transport. In proximal tubules, an apical Na\(^+\)/H\(^+\) exchanger, NHE3, and the vacuolar H\(^+\)-ATPase secrete protons into the lumen. Through the function of CA4, these secreted H\(^+\) ions combine with luminal-filtered HCO\(_3\)\(^-\) to form CO\(_2\) and H\(_2\)O. Then the CO\(_2\) thus formed diffuses into the cell through the apical cell membrane to form H\(^+\) and HCO\(_3\)\(^-\) through cytosolic CA2 enzymatic activity. The protons are recycled back into the lumen by NHE3 and the vacuolar H\(^+\)-ATPase, while HCO\(_3\)\(^-\) diffuses passively across the basolateral membrane via a Na\(^+\)-HCO\(_3\) cotransporter (29).

Fish gills are well documented as being a principal organ for ion uptake and acid-base regulation mechanisms, and mitochondrion-rich cells, the major ionocytes, and/or pavement cells have been proposed as achieving these functions via the transport of H\(^+\) and/or HCO\(_3\)\(^-\) by exchange with Na\(^+\) and/or Cl\(^-\) (9, 17, 20, 28). CA has been demonstrated to play roles in ion uptake (2, 4, 32) and acid-base regulation (11, 12, 16). According to genetic databases, there are over 12 isoforms of CA reported and/or predicted in fish species including fugu and zebrafish. Whether there are specific CA isoforms responsible for acid-base regulation and ion uptake in fish gill ionocytes is still being debated. Rahim et al. (30) first purified two distinct branchial and blood CA isoforms in rainbow trout and carp, and they identified the specific existence of a branchial CA in gill ionocytes (chloride cells) and pavement cells with isoform-specific antibodies. Esbaugh et al. (8) confirmed the specific expression of the CA isoform in trout gill cells by cloning and RT-PCR analysis, and they identified the isoform as trout cytoplasmic CA, which differs from another vertebrate CA2 on the basis of phylogenetic analysis. Immunocytochemistry and Northern blot data demonstrated the expression and function of CA2 in gill ionocytes of Osorezan dace (Triboleodon hakonensis) (16). Similarly, in zebrafish embryos, CA2 was also identified in skin ionocytes by in situ hybridization with an isoform-specific probe (6, 19). On the other hand, the existence of the CA4 isoform has been reported in gill pillar cells of the spiny dogfish (Squalus acanthias) (13) and in intestinal (11, 15) and renal cells of rainbow trout (11). Apparently, so far, no data are available to demonstrate the expression and function of CA4 or its equivalent in gill ionocytes, which is the major cell type responsible for acid-base regulation and ion uptake mechanisms in freshwater fish (9, 17, 20, 28).

The purpose of the present work was to use the zebrafish (Danio rerio) as the model to test whether some CA isoforms are specifically expressed in ionocytes and whether they func-
tion in Na\(^+\) uptake and acid-base balance in fish gills. The zebrafish was selected because of its rich genetic database and applicability for various molecular physiological approaches (20). In previous studies, a novel ionocyte, H\(^+\)-ATPase-rich (HR) cells, which was identified in the skin and gills of zebrafish (23), was demonstrated to be involved in acid-secretion and Na\(^+\) uptake mechanisms via H\(^+\)-ATPase and NHE (6, 18, 40). Specific aims of the present study were to 1) clone and sequence the full-length or partial cDNAs of the zca gene family in zebrafish (zca); 2) identify the specific zca isoforms expressed in skin and gill ionocytes by whole mount in situ hybridization; 3) determine mRNA expression patterns of the specific zca isoforms in various tissues of zebrafish by RT-PCR; 4) determine the cellular localization of zca mRNAs, H\(^+\)-ATPase, and Na\(^+\)-K\(^+\)-ATPase in zebrafish skin and gills; 5) elucidate the effects of translational knockdown of specific zca isoforms and Na\(^+\) influx, and mRNA expressions (using real-time PCR) of the zca isoforms, zatp6v1a (H\(^+\)-ATPase A-subunit) and zhhe3b; and 6) determine the effects of acclimation to ambient acid or low-Na\(^+\) on the mRNA expressions of the zca isoforms, zatp6v1a and zhhe3b.

MATERIALS AND METHODS

Experimental animals. Mature zebrafish (AB strain), obtained from stocks of the Institute of Cellular and Organismic Biology, Academia Sinica, were reared in circulating local tap water at 28.5°C. Fertilized eggs were incubated in local tap water (FW). Embryos were collected within 30 min after fertilization and were incubated in a Petri dish until the desired developmental stages. Fish were anesthetized with 100–200 mg/l of buffered MS222 (3-aminobenzoic acid ethyl ester; Sigma, St. Louis, MO) before sampling. The experimental protocols were approved by the Academia Sinica Institutional Animal Care and Utilization Committee (approval no.: RFIZO0HP2006086).

Acclimation experiments. Following a previous study (40), high-Na\(^+\) (10 meq/l) and low-Na\(^+\) (0.04 meq/l) artificial FW was prepared with double-deionized water with sufficient CaSO\(_4\)·2H\(_2\)O, MgSO\(_4\)·7H\(_2\)O, Na\(_2\)SO\(_4\), NaCl, K\(_2\)HPO\(_4\), and KH\(_2\)PO\(_4\) added. Other ion (Ca\(^2+\), Mg\(^2+\), K\(^+\), and Cl\(^−\)) concentrations and the pH of the media were the same. Local tap water (control, pH 6.7–6.9) and acidic FW (pH 4.00–4.05) were also prepared. The acidic medium was made by adding H\(_2\)SO\(_4\) to local tap water, and the concentrations of other ions were adjusted to maintain the proper ion concentrations, and acidic FW was continuously pumped into the experimental tank bottom with an electrical pump to maintain a stable pH. The pH values of all experimental solutions (using real-time PCR) of the zca isoforms, zatp6v1a (H\(^+\)-ATPase A-subunit) and zhhe3b; and 6) determine the effects of acclimation to ambient acid or low-Na\(^+\) on the mRNA expressions of the zca isoforms, zatp6v1a and zhhe3b.

Table 1. Primer sets for the RT-PCR analysis

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<th>Primer Sequence</th>
<th>Length, bp</th>
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<tr>
<td>zca2-like a</td>
<td>F 5′-CATCTGTGGAGCCGTTGCA-3′</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>R 5′-TTTGGTGGTCGTTTCCG-3′</td>
<td></td>
</tr>
<tr>
<td>zca2-like b</td>
<td>F 5′-TGGCCTTCCGATATGCTGCTTTT-3′</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>R 5′-CATGCGGCTTTATGACAAGG-3′</td>
<td></td>
</tr>
<tr>
<td>zca6a</td>
<td>F 5′-CGTGTGTGGAAAAGGCCATGTC-3′</td>
<td>153</td>
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<td></td>
<td>R 5′-AATATTGCGCCACGGCTGGA-3′</td>
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</tr>
<tr>
<td>zca15a</td>
<td>F 5′-TACAGAACACACTGTCATGTCG-3′</td>
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<td>β-actin</td>
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</tr>
<tr>
<td></td>
<td>R 5′-GATGCTGCAGACTGATGCTACTG-3′</td>
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</tbody>
</table>
and goat anti-mouse IgG conjugated with Texas red (Jackson Immuno-
were further incubated in goat anti-rabbit IgG conjugated with FITC
microelectrodes were backfilled with a 1-cm column of 100 mM
Sarasota, FL) with 1.1- and 1.5-mm inner and outer diameters,
capillary tubes (model TW 150-4; World Precision Instruments,
laterally in the chamber for the following measurement. Briefly,
numbers on the yolk sac were counted.
Live embryos were preincubated in zebrafish solution containing 0.5
monoclonal antibody against the
H\textsubscript{IA}), and a polyclonal antibody against the A subunit of killifish
PBST at room temperature for 10 min. Afterward, the embryos were immunore-
acted with an alkaline phosphatase-coupled anti-Dig antibody (1:
with 3% bovine serum albumin
3-chloro-3-indolyl phosphate for the alkaline phosphatase reaction.
Samples were observed with a stereomicroscope (SZX-ILLD100;
Olympus, Tokyo, Japan) or an upright microscope (Axioplan 2
samples were incubated with 3% bovine serum albumin

for 20 min), 2\times \text{ SSC} \text{ (at 65°C for 10 min)}, 2\times \text{ SSC} \text{ (at 65°C for 10 min), 0.2\times \text{ SSC} \text{ (at 65°C for 30 min, 2 times)}, and PBST at room

Whole mount immunocytochemistry. For triple staining of zca
mRNA, Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, and H\textsuperscript{+}-ATPase, some in situ-hybridized
samples were subjected to immunocytochemistry. After being washed
with PBS, samples were incubated with 3\% bovine serum albumin
and 5\% normal goat serum for 30 min to block nonspecific binding.
The samples were then incubated overnight at 4°C with an α5
monoclonal antibody against the α-subunit of the avian Na\textsuperscript{+}pump
(Developmental Studies Hybridoma Bank, University of Iowa, Ames,
IA), and a polyclonal antibody against the A subunit of killifish
H\textsuperscript{+}-ATPase (22). After being rinsed with PBS for 20 min, the samples
were further incubated in goat anti-rabbit IgG conjugated with FITC
and goat anti-mouse IgG conjugated with Texas red (Jackson Immuno-
research Laboratories, West Grove, PA) for 2 h at room tempera-
ture. For quantification of functional HR cells, concanavalin A
was used to label apical opening of HR cells following Horng et al. (18).
Live embryos were preincubated in zebrafish solution containing 0.5
mg/ml Alexa Fluor 488-conjugated concanavalin A (Invitrogen) for
10 min. After being washed, embryos were imaged, and the cell
numbers on the yolk sac were counted.

Surface \text{pH} of zebrafish embryos. By following previously de-
scribed methods (23, 34), an anesthetized zebrafish embryo was laid
laterally in the chamber for the following measurement. Briefly,
microelectrodes with a tip diameter of 3–4 μm were pulled from glass
capillary tubes (model TW 150-4; World Precision Instruments,
Sarasota, FL) with 1.1- and 1.5-mm inner and outer diameters,
respectively, then baked at 200°C overnight, and vapor-silanized with

Table 3. Probes for in situ hybridization

<table>
<thead>
<tr>
<th>Name</th>
<th>Length</th>
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<tr>
<td>zca2-like a</td>
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<tr>
<td>zca2-like b</td>
<td>nt 207–1260</td>
</tr>
<tr>
<td>zca4a</td>
<td>nt 640 bp</td>
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<tr>
<td>zca5</td>
<td>796 bp*</td>
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<tr>
<td>zca6</td>
<td>571 bp</td>
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<td>zca7</td>
<td>nt 144–1527</td>
</tr>
<tr>
<td>zca9</td>
<td>669 bp</td>
</tr>
<tr>
<td>zca10a</td>
<td>443 bp</td>
</tr>
<tr>
<td>zca14</td>
<td>666 bp</td>
</tr>
<tr>
<td>zca15a</td>
<td>nt 783–1277</td>
</tr>
</tbody>
</table>

*The numbers of nucleotides (nt) are unknown because only partial se-
quencies were cloned.
Table 4. Primer sets for the qualitative RT-PCR analysis

<table>
<thead>
<tr>
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</tr>
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<td>CA2-like a R</td>
<td>5'-CAGGAAAAGGCTTCTATTT-3'</td>
</tr>
<tr>
<td>CA2-like b F</td>
<td>5'-TGCTCTGTTAAGGGCTTCTATTT-3'</td>
</tr>
<tr>
<td>CA2-like b R</td>
<td>5'-CAGGAAAAGGCTTCTATTT-3'</td>
</tr>
<tr>
<td>CA4a F</td>
<td>5'-GGGTTTGGAAAGGGCATTCC-3'</td>
</tr>
<tr>
<td>CA4a R</td>
<td>5'-AATAAAGCTCCAGGCTTCA-3'</td>
</tr>
<tr>
<td>CA15a F</td>
<td>5'-TGGAGAAACAGATCATGGAGAC-3'</td>
</tr>
<tr>
<td>CA15a R</td>
<td>5'-TGTTGTTCTATTGCAGTCTC-3'</td>
</tr>
<tr>
<td>H^+ATPase F</td>
<td>5'-GAGGAAAAAGCAGCTGCCATTCCA-3'</td>
</tr>
<tr>
<td>H^+ATPase R</td>
<td>5'-CAACGCGAATATAAAGTTAGCATG-3'</td>
</tr>
<tr>
<td>NHE3b F</td>
<td>5'-TGACAGACAGGCTCTTCAGC-3'</td>
</tr>
<tr>
<td>NHE3b R</td>
<td>5'-TGTTGCTGTTTCAGTCTCC-3'</td>
</tr>
<tr>
<td>β-Actin F</td>
<td>5'-ATGGTTCAGATCCATCCATTCG-3'</td>
</tr>
<tr>
<td>β-Actin R</td>
<td>5'-GATGGTTCAGATCCATCCATTCG-3'</td>
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NHE, Na^+\text/H^+ exchanger.

ng/embryo) contained 0.1% phenol red, which was used as a visualizing indicator and was injected into zebrafish embryos at the one- to two-cell stage using an IM-300 microinjector system (Narishige Scientific Instrument Laboratory, Tokyo, Japan). For confirmation of the specificity and effectiveness of the MOs, the pCS2+zca-like a green fluorescent protein (GFP) and pCS2+zca15aGFP constructs were generated to synthesize capped mRNAs (cRNAs) by SP6 mMessage mMachine kit (Ambion, Austin, TX). Capped mRNAs at 250 pg/embryo were coinjected with or without the respective MO at the one- to two-cell stage.

Statistical analysis. Values are presented as means ± SD and were compared using Student’s t-test or ANOVA (Tukey’s pairwise comparison).

RESULTS

Phylogenetic analysis of the zca family. The latest version of the Ensembl zebrafish genome database (zv7, Ensembl release 48) was first surveyed to identify members of the carbonic anhydrase gene family. Excluding the receptor-type tyrosine-protein phosphatase (PTPRG) genes, 19 zebrafish carbonic anhydrase isoforms were found in the eukaryotic carbonic anhydrase gene family (Interpro Domain ID: IPR001148), and one of them, zCA6, cloned in the present study, has not been detected in other tissues, including the blood, gills, kidneys, moderately in the intestines, heart, and spleen, but were undetected in other tissues, including the blood, gills, kidneys, testis, and ovaries. Notably, zca15a was the major isoform expressed in the gills and showed only a very mild amount of transcripts in the spleen, muscles, and testis (Fig. 4).

Effects of knockdown of zca isoforms on embryo phenotypes. In preliminary experiments, 0.5, 1, 1.5, 3, 4, and 5 ng/embryo of zca2-like a and zca15a morpholinos were injected into zebrafish embryos. With injection of 1.5 ng/embryo, zca15a morphants showed about a 70% survival rate; 60% of the surviving morphants were small in size, and the rest had a malformed tail region. On the other hand, the phenotype and survival of zca2-like a morphants injected with 1.5 ng/embryo of morpholino appeared normal. Both zca2-like a and zca15a morpholinos did not affect the numbers of HR cells in zebrafish embryos (Fig. S3).

The specificity and effectiveness of the zca MOs were confirmed by injecting embryos with zca2-like a (or zca15a): GFP cRNA (Fig. S4). After injection of zca2-like a (or zca15a):GFP cRNA, the embryos showed strong GFP expression (Fig. S4, A and C). On the other hand, coinjection with the zca2-like a (or zca15a) MO was sufficient to abolish GFP

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</tr>
<tr>
<td>CA4a R</td>
<td>5'-AATAAAGCTCCAGGCTTCA-3'</td>
</tr>
<tr>
<td>CA15a F</td>
<td>5'-TGGAGAAACAGATCATGGAGAC-3'</td>
</tr>
<tr>
<td>CA15a R</td>
<td>5'-TGTTGTTCTATTGCAGTCTC-3'</td>
</tr>
<tr>
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expression in the embryos (Fig. S4, B and D), indicating the specificity and effectiveness of the MOs used.

**Effects of knockdown of zca isoforms on H⁺ activity on the embryonic surface.** A noninvasive H⁺-selective microelectrode was used to assay the effects of knockdown on H⁺ activity on the surface of zebrafish embryos. An external pH gradient (pH) at the surface of intact embryos was measured at a location near the lower part of the yolk sac, as reported previously (18) as showing the highest H⁺ activity (i.e., the lowest pH). As shown in Fig. 5, injection of zca2-like a morpholinos did not initially affect the surface pH gradient in morphants at 24 hpf, but it induced a significant decline in surface H⁺ activities from 48 hpf and later compared with that in the wild type.

Interestingly, zca15a morpholinos produced different effects on the surface pH gradient in morphants. As shown in Fig. 6, zca15a morphants exhibited an initial increase in surface H⁺ activities at 24 hpf, a recovery to the level of the wild type at 48 hpf, and a subsequent significant decrease compared with the wild type.
Na\(^+\) influx in zca2-like a and zca15a morphants. To examine the effects of the knockdown of zca isoforms on Na\(^+\) uptake function in embryos, a \(^{24}\)Na\(^+\) radioisotope tracer was used to measure the whole body Na\(^+\) influx in morphants at 96 hpf. As shown in Fig. 7A, zca2-like a morphants revealed a significantly higher Na\(^+\) influx than the phenol red-injected control and wild type. Similarly, a higher Na\(^+\) influx was found in zca15a morphants, compared with that in the wild type (Fig. 7B).

Expressions of relevant genes in zca2-like a and zca15a morphants. To further explore the possible mechanisms behind the functional changes in H\(^+\) activity and Na\(^+\) influx, qRT-PCR analysis was conducted to assay the expression profiles of the relevant genes including zca2-like a, zca15a, zatp6v1a, and znhe3b in morphants at 96 hpf. As indicated in Fig. 8, zca2-like a morpholinos caused significant increases in the expressions of zca15a (Fig. 8A) and znhe3b (Fig. 8C) but an evident decline in the transcription of zatp6v1a (Fig. 8B) in 96-hpf morphants. On the other hand, zca15a morpholinos did not affect zca2-like a (Fig. 8D) expression but induced a decrease in the expression of zatp6v1a (Fig. 8E) and an increase in that of znhe3b (Fig. 8F) in morphants.

Effects of environmental pH and Na\(^+\) concentration on relevant gene expressions in zebrafish gills. According to the above results, zCA2-like a and zCA15a appeared to be involved in acid-base regulation and Na\(^+\) uptake mechanisms in zebrafish HR cells. Further experiments were designed to test whether gill expressions (by qRT-PCR) of the two zca isoforms are regulated during acclimation to acid or low-Na\(^+\) environments, which was previously reported to affect H\(^+\) secretion and Na\(^+\) uptake functions of fish gills (20). As shown in Fig. 9A, neither an acid nor a low-Na\(^+\) environment produced a significant effect on the gill expression of zca2-like a.

On the other hand, both acid and low-Na\(^+\) environments caused evident enhancements, of approximately 1.7- and 1.8-fold higher than the respective control, in zca15a mRNA expression in zebrafish gills (Fig. 9B).

DISCUSSION

In the present study, two CA2-like isoforms (zCA2-like a and b), and two new prototype CA clades (zCA15a, b, c, and zCA16a, b, c) were identified and newly annotated. The two zCA2-like isoforms were classified as an outer group to the mammalian CA1/CA2/CA3/CA13 gene cluster (Fig. 1A) and are most similar to mammalian CA2. As to nomenclature, CA2 was previously mentioned in several teleost CA studies (16, 20, 24, 37, 40), and the names, CA2-like a and CA2-like b, are suggested, rather than creating a new number of CAs or directly adopting CA2 due to their outer group’s position to that of mammals. Considering other teleostean CA2-like genes published together, CA2-like a is mainly expressed in the gills and embryonic ionocytes in zebrafish (Figs. 2 and 3) and rainbow trout (8), whereas CA2-like b (also named CAHz) is a red blood cell-predominant isoform in zebrafish (Fig. 8F) and rainbow trout (7). In the case of CA4-related isoforms, two new prototype CA clades (CA4-like group 1 and CA4-like group 2) were first identified, and these two groups of isoforms were annotated as CA15 and CA16 in systematic nomenclature following the Zebrafish Information Network (ZFIN; http://www.zfin.org) annotation suggestions. The newly found 15th and 16th CA isoforms are most closely related to mammalian CA4 and teleost CA4 orthologues in sequence identities, phylogenetic linkages, and a conserved alpha_CA_IV_XV_like domain. Therefore, CA4, CA15, and CA16 may have evolved from a congeneric isoform in ancestor vertebrates. In the
current version of the zebrafish genome database, one-gene cluster was found in Scaffold Zv7_NA1067 with three CA isoforms of zCA15a, zCA15b, and zCA15c, which are usually considered to be a hallmark of tandem duplication of gene isoforms (1, 31). However, this gene cluster was found with only two corresponding isoforms (named CA15a/b/c and CA15d; Fig. 3B) in both green pufferfish (Tetraodon nigroviridis) and medaka (Oryzias latipes) in the current genome assembly, and the evolutionary relationship between these isoforms may need further investigation.

Tufts et al. (36) and Esbaugh et al. (8) pointed out the inappropriateness of directly applying mammalian CA nomenclature to teleosts. However, the phylogenetic analysis of the present study on additional teleostean CA isoforms provides additional clues to vertebrate CA's evolutionary history. The mammalian (or terrestrial) CA1/CA2/CA3/CA13 gene cluster was generated more recently after the divergence with the teleost common ancestor, and teleostean CA2-like a/CA2-like b gene duplication occurred in a whole genome duplication of the teleost common ancestor (21). To prevent confusion, the present analysis suggests annotating these isoforms as CA2-like a and CA2-like b rather than as cytoplasmic CA. In our previous studies (19, 20, 40), we annotated zCA2-like a as zCA2 and zCA15a as zCA4-like, because the nomenclatures were roughly made on the basis of BLAST (Basic Local Alignment Search Tool) searches.
Alignment Search Tool) similarities to known mammalian CA isoforms. These indicate the importance of the entire genome database and a functional genomic approach for identification and functional analysis of specific isoforms of ion transporters (see the discussion below).

CA has long been known to play important roles in acid-base balance and ion uptake in fish gills. So far, some studies using immunocytochemistry and in situ hybridization have demonstrated the existence of CA2-like (or cytoplasmic CA) (12) in gill mitochondrion-rich cells and/or pavement cells in rainbow trout (12, 30), flounder (Platichthys flesus) (33), mudskipper (Periophthalmodon schlosseri) (38), Osorezan dace (Tribolodon hakonensis) (16), and embryonic zebrafish (6). The present study confirmed the colocalization of zca2-like a mRNA in zebrafish skin and gill HR cells and further found an increase in zca2-like a-expressing cells following development, reflecting the enhanced acid-secretion ability of developing zebrafish (18). On the other hand, no convincing evidence for the expression of CA4 or equivalent CA isoforms in teleost gill ionocytes is available, although immunocytochemical studies indicated localization of CA4 in dogfish (Squalus acanthias) gill pillar cells (13) and cells in other osmoregulatory organs (kidneys and intestines) of rainbow trout (11, 15). The present study for the first time demonstrates the specific localization of zca15a mRNA in HR cells in the skin and gills of zebrafish. On the other hand, NHE3b (40) and NBC1 (16) were found to be colocalized in apical and basolateral gill HR cells. Taking all of these data together, the molecular evidence supports the proposed model for zebrafish HR cells, which is similar to human proximal tubular cells in the expressions of relevant transporters and enzymes (20, 40). zCA15a has a glycosylphosphatidylinositol lipid anchor, through which the enzyme may also be tethered to the outer leaflet of the plasma membrane, as are the trout and human CA4 in apical and basolateral membranes of kidney cells (11, 29). However, localization of the protein of zCA15a in HR cells with a specific antibody remains to be done in the future.

Knockdown of the translations of either zca2-like a or zca15a with specific morpholinos affected the H⁺ concentration around the surface of the yolk sac membrane and the whole body Na⁺ influx, providing molecular physiological evidence to support the roles of these two CA isoforms in the

Fig. 7. Effects of translational knockdown of zca2-like a (A) and zca15a (B) on the Na⁺ influx in embryos at 96 hpf. Phenol red, injection control. Values are means ± SD (n = 5). Different lowercase letters indicate significant differences (one-way ANOVA pairwise comparison). *Significantly different from WT (Student’s t-test, P < 0.05).

Fig. 8. Effects of translational knockdown of zca2-like a (A–C) and zca15a (D–F) on transcripts (quantitative RT-PCR) of relevant genes in 96-hpf embryos. A: zca15a. D: zca2-like a. B and E: zatp6v1a. C and F: znhe3b. Values are means ± SD (n = 4). *Significantly different from WT (P < 0.05, Student’s t-test).
mechanisms of acid-base regulation and Na⁺ uptake in zebrafish. The translational knockdown of zca15a showed a direct effect, an increase in the apical H⁺ concentration, initially at 24 hpf (~23.5 h after injection of the morpholino) (Fig. 6). However, compensatory responses were subsequently observed in both zca15a and zca2-like a morphants, which showed a decrease in the apical H⁺ concentration by 96 hpf (Figs. 5 and 6). The cytosolic CA2-like a hydrates CO₂ to form H⁺ and HCO₃⁻, and the proton recycles back out of the cell by apical H⁺-ATPase and NHE, as proposed in mammal renal Na⁺ secretion (18, 23), was also found in zca15a morphants at 96 hpf (Fig. 8E). On the other hand, both morphants showed upregulation of znhe3b (Fig. 8, C and F). Operation of znH3b appears not to contribute significantly to the H⁺ gradient outside of the apical side of HR cells, since a supplementary experiment showed that amiloride (an inhibitor of NHE) did not decline the H⁺ gradient, compared with bafilomycin A1 (a H⁺-ATPase inhibitor) which caused ~35% decrease (Fig. S5). It should be noted that the decline of H⁺ gradient in zca2-like a and zca15a morphants was approximately 20–36% (Figs. 5 and 6).

The decline in the extracellular H⁺ concentration at the apical side of HR cells reasonably favors the operation of NHE3b, which can be driven by the H⁺ gradient (25). The present data, presenting increased Na⁺ influx (Fig. 7A) and upregulation of znhe3b (Fig. 8C), support this notion. Congenital CA2 deficiency in mammals has reported to cause renal tubular acidosis (3). zca2-like a knockdown may induce a systemic acidosis, which may also contribute to the favorable gradient for the operation of znH3b in HR cells, but this needs further confirmation. In a recent work, Yan et al. (40) examined the mRNA expressions of gill zatp6v1a and znhe3b in zebrafish acclimated to a low-Na⁺ environment, and indicated that apical H⁺-ATPase was downregulated but probably maintained an intracellular H⁺ gradient to facilitate Na⁺ uptake via apical NHE3b, which may be the dominant player, and thus its function was enhanced. The present study supports this hypothesis by providing further molecular physiological data. In both zca morphants, downregulated zatp6v1a and upregulated znhe3b were accompanied by an increase in Na⁺ uptake and a decline in the apical proton concentration. All these demonstrate the partitioning and negative correlation of NHE3b and H⁺-ATPase for involvement in the Na⁺ uptake/acid-base regulation mechanisms in zebrafish HR cells.

Subsequent acclimation experiments further supported the roles of zCA2-like a and zCA15a in Na⁺ uptake/acid-base regulation mechanisms in zebrafish HR cells. After 1-wk acclimation to low-pH FW, fish had to compensate for internal acidosis by enhancing the proton secretion and HCO₃⁻ uptake, which is apparently achieved by stimulating H⁺-ATPase (40) and CA15a (the present study), respectively, in zebrafish HR cells. Stimulation of the expression and function of gill H⁺-ATPase in hypercapnia- or acid-acclimated fish has been reported in many previous studies (10, 14, 27, 28, 40). Enhancing CA4 expression to facilitate HCO₃⁻ uptake (via subsequent operations of cytosolic CA2 and basolateral NBC) as compensation for acidosis has been reported in fish kidneys (11), and the present study for the first time demonstrates a similar mechanism in fish gills. Interestingly, acclimation to low-Na⁺ FW also induced stimulation of zca15a expression (Fig. 9B). Apparently, the mechanism of apical CA4 facilitating the operation of NHE3 in mammal proximal tubular cells (29) also holds in zebrafish gill HR cells. Enhancement of zca15a expression in zebrafish HR cells decreases the apical proton concentration (as occurred in zca2-like a morphants at 96 hpf),
providing a more favorable gradient for the operation of zNHE3b to absorb more Na\textsuperscript{+} for compensation.

Interestingly, zca15a morphants showed no significant change in the expression of zca2-like a (Fig. 8D). CA2 mRNA expression also did not change in mice with genetic knockout of CA9 (26), which is a membrane-associated CA isoform like CA4 (29). On the other hand, zca2-like a expression was not changed in either the acid or low-Na\textsuperscript{+} environment (Fig. 9A). It is probable that the intact CA2-like a is sufficient to overcome the physiological defects caused by the translational knockdown of zca15a, and it may provide sufficient carbonic anhydrase activity to fulfill the physiological needs in zebrafish coping with different environments. Indeed, in mammalian kidneys, CA4 accounts for >95% of CA activity and shows the highest catalytic rate, whereas CA4 and other membrane-associated isoforms sustain the remaining 5% of activity (3, 29, 39).

In summary, the present study provides molecular physiological evidence for the existence of zCA2-like a and zCA15a in zebrafish HR cells and for the roles of the two CA isoforms in Na\textsuperscript{+} uptake/acid-base regulation mechanisms. Among the 20 isoforms identified in the present study, only 10 of them have been cloned and examined for mRNA localization. Whether any other(s) in the remaining 10 members is expressed in gill ionocytes and involved in ion regulation mechanisms remains to be studied in future.

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