Rhcg1 and Rhbg mediate ammonia excretion by ionocytes and keratinocytes in the skin of zebrafish larvae: H\(^+\)-ATPase-linked active ammonia excretion by ionocytes

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Shih T. Horng J. Lai Y. Lin L. Rhcg1 and Rhbg mediate ammonia excretion by ionocytes and keratinocytes in the skin of zebrafish larvae: H\(^+\)-ATPase-linked active ammonia excretion by ionocytes. Am J Physiol Regul Integr Comp Physiol 304: R1130–R1138, 2013. First published April 17, 2013; doi:10.1152/ajpregu.00550.2012.—In zebrafish, Rhcg1 was found in apical membranes of skin ionocytes [H\(^+\)-ATPase-rich (HR) cells], which are similar to α-type intercalated cells in mammalian collecting ducts. However, the cellular distribution and role of Rhbg in zebrafish larvae have not been well investigated. In addition, HR cells were hypothesized to excrete ammonia against concentration gradients. In this study, we attempted to compare the roles of Rhbg and Rhcg1 in ammonia excretion by larval skin and compare the capability of skin cells to excrete ammonia against concentration gradients. Using in situ hybridization and immunohistochemistry, Rhbg was localized to both apical and basolateral membranes of skin keratinocytes. A scanning ion-selective electrode technique (SIET) was applied to measure the NH\(_4^+\) flux at the apical surface of keratinocytes and HR cells. Knockdown of Rhbg with morpholino oligonucleotides suppressed ammonia excretion by keratinocytes and induced compensatory ammonia excretion by HR cells. To compare the capability of cells to excrete ammonia against gradients, NH\(_4^+\) flux of cells was determined in larvae exposed to serial concentrations of external NH\(_3\). Results showed that HR cells excreted NH\(_4^+\) against higher NH\(_3\) concentration than did keratinocytes. Knockdown of the expression of either Rhcg1 or H\(^+\)-ATPase in HR cells suppressed the capability of HR cells.

ionocyte; zebrafish; skin; keratinocyte; ammonia; rhesus; SIET

AMMONIA (INCLUDING BOTH NH\(_3\) AND NH\(_4^+\)) excretion by renal tubules of mammals plays a critical role in excreting metabolic acids and maintaining pH homeostasis (32). α-Type intercalated cells are thought to be responsible for acid/ammonium excretion in the collecting duct. With V-type H\(^+\)-ATPase and a Rhesus (Rh) glycoprotein (RhCG) in apical membranes of intercalated cells, H\(^+\) is pumped out of cells and combines with non-ionic NH\(_3\) to form NH\(_4^+\). This reaction maintains a favorable partial pressure gradient across apical membranes for NH\(_3\) diffusion through an NH\(_3\) channel, the RhCG (4, 6). In basolateral membranes, another Rh glycoprotein isoform, RhBG, was suggested to facilitate ammonia movement from the blood into intercalated cells (24, 27).

Unlike mammals, which convert metabolic ammonia into urea, most teleost fishes are ammoniotelic animals and excrete over 80% of the ammonia produced directly into the ambient water through their skin and gills (7, 30, 33). In recent years, four Rh protein isoforms (Rhag, Rhbg, Rhcg1, and Rhcg2) were identified in skin/gills of teleosts, including pufferfish (Takifugu rubripes) (19), zebrafish (Danio rerio) (3, 18), rainbow trout (Oncorhynchus mykiss) (21), killifish (Kryptolebias marmoratus) (9), and Japanese medaka (Oryzias latipes) (36). The significance of Rh proteins in fish has been comprehensively reviewed (10, 30, 35). In the gill epithelium of the pufferfish, Rhbg and Rhcg1 were respectively identified in pavement cells (PVCs) and ionocytes (also called mitochondrion-rich cells or MRCs), suggesting that both cell types are involved in ammonia excretion (19). It is generally believed that PVCs are the primary location for ammonia excretion since they cover over 90% of the gill surface (7). However, with the discovery of Rhcg1, ionocytes were also suggested to play a critical role in ammonia excretion. In zebrafish, Rhcg1 was identified in apical membranes of H\(^+\)-ATPase-rich (HR) cells (a subtype of ionocytes), which are acid-secreting ionocytes in gills and larval skin (5, 15). Shih and colleagues (25) used a scanning ion-selective electrode technique (SIET) and morpholino gene knockdown technique to show that H\(^+\)-ATPase and Rhcg1 in apical membranes play a coupling role in “acid-trapping ammonia excretion.” The H\(^+\)-ATPase pumps H\(^+\) onto the surface unstirred layer of the skin/gills to promote NH\(_3\) diffusion through Rhcg1 (25), similar to renal intercalated cells in mammals. This metabolon concept of Rh protein and other transporter was elaborated by several review articles (10, 30, 35).

HR cells of zebrafish were speculated to excrete ammonia against high external ammonia levels. Increases in Rhcg1 and H\(^+\)-ATPase transcripts were reported in zebrafish acclimated to high external ammonia, suggesting that mediation of ammonia excretion by HR cells was induced (3). However, there has been no convincing evidence to show that HR cells really do excrete ammonia against high external ammonia levels. The present study attempted to determine whether HR cells can actively excrete ammonia, and, if so, against how high of a gradient can HR cells excrete ammonia?

Although Rh protein isoforms have been identified in gills of several fish species, their cellular and subcellular localizations are not very clear. In zebrafish, Rhcg1 was consistently localized to apical membranes of HR cells (18); however, localization of Rhbg was not convincing. In previous studies, Rhbg was localized to PVCs of zebrafish gills by immunohistochemistry; however, its cellular localization in embryonic/larval skin is not clear (2). Since zebrafish embryos/larvae are an important model for functional studies, it is necessary to clarify localization of Rhbg in their skin.

In the present study, immunohistochemistry and in situ hybridization were used to demonstrate the location of Rhbg in...
zebrafish skin. We aimed to compare the roles of Rhbg and Rhcg1 in ammonia excretion by using morpholino oligonucleotides (MOs) to knock down specific genes and the non-invasive SIET to analyze NH4⁺ flux at specific skin cells. By comparing NH4⁺ flux of keratinocytes and HR cells, we demonstrated that HR cells can actively excrete ammonia, and both Rhcg1 and H⁻⁻ATPase are required for active ammonia excretion.

MATERIALS AND METHODS

Experimental animals and water preparation. Adult zebrafish (AB strain) were reared in circulating tap water at 28°C with a photoperiod of 14 h of light/10 h of dark. Fertilized eggs were incubated in normal water (NW) or high-ammonia (HA) water. NW contained (in mM) 0.5 NaCl, 0.2 CaSO₄, 0.2 MgSO₄, 0.16 KH₂PO₄, and 0.16 K₂HPO₄ (pH 7.0). (NH₄)₂SO₄ was added to NW to prepare 1 mM HA (pH 7.0) and 5 mM HA (pH 7.0). The media were changed daily to guarantee optimal water quality. The experimental protocols were approved by the National Taiwan Normal University Animal Care and Utilization Committee.

SIET. The SIET was used to measure NH₄⁺ activities and fluxes at the surface of zebrafish larvae. Glass capillary tubes (no. TW 150-4, World Precision Instruments, Sarasota, FL) were pulled on a Sutter P-97 Flaming Brown pipette puller (Sutter Instruments, San Rafael, CA) into micropipettes with tip diameters of 3–4 μm. These were then baked at 120°C overnight and coated with dimethyl chlorosilane (Sigma-Aldrich, St. Louis, MO) for 30 min. The micropipettes were backfilled with a 1-cm column of electrolytes (100 mM NH₄Cl) and front loaded with an 20- to 30-μm column of NH₄⁺ ionophore (ionophore I cocktail B, Sigma-Aldrich) to create an ion-selective microelectrode (probe). The details of the system were described in previous reports (15, 25). To calibrate the ion-selective probes, the Nernstian property of each microelectrode was measured by placing the microelectrode in a series of standard solutions (0.1, 1, and 10 mM NH₄Cl). By plotting the voltage output of the probe against log[NH₄⁺] values, a linear regression yielded a Nernstian slope of 58.2 ± 0.6 (n = 10).

Measurement of NH₄⁺ gradients and fluxes at the skin surface. The SIET was performed at room temperature (26–28°C) in a small plastic recording chamber filled with 1 ml of recording medium, which was prepared by adding 300 μM MOPS buffer and 0.3 mg/l MS222 (tricaine, Sigma-Aldrich). To measure larvae in HA water, (NH₄)₂SO₄ was added to the recording medium. The pH of the recording media was adjusted to 7.0 by adding an NaOH solution. The ion-selective probe was moved to the yolk-sac surface to record NH₄⁺ activities and then moved away (~1 cm) to record the background. In this study, ∆[NH₄⁺] was used to represent the NH₄⁺ concentration gradient between the yolk-sac surface and the background. To record the NH₄⁺ flux at the surface of HR cells and keratinocytes, the microelectrode was moved to a position ~2 μm above the surface of a cell. At every position, the voltage difference in microelectrode was recorded. At every position, the voltage differences were first converted into a concentration gradient ∆C (μM/cm²). ∆C was subsequently converted into an ionic flux using Fick’s law of diffusion in the following equation: J = D(∆C/∆X), where J (pA·cm⁻²·s⁻¹) is the net flow of the ion, D is the diffusion coefficient of the ion (2.09 × 10⁻⁵ cm/s for NH₄⁺), and ∆X (cm) is the distance between the two points. The detailed calculation of ionic flux was shown in previous reports (15, 25).

Morpholino design and microinjection. MOs were obtained from Gene Tools (Philomath, OR). Sequences of MOs against Rhbg and Rhcg1 were Rhbg (5′-CAGTGCCATGTCTCTACACGTGTTGAG-3′) and Rhcg1 (5′-CAGTGCCAAGTGTTCAAGTGTTGAG-3′). Standard control MO (5′-CCTTTCATTCTACTTACATTATA-3′) was also used as the control. The control MO provided by Gene Tools had no target and no significant biological activity. An MO solution was prepared with sterile water and contained 0.1% phenol red as a visualizing indicator. The MO was microinjected into embryos at the 1- to 4-cell stage with an IM-300 microinjector system (Narishige Scientific Instrument Laboratory, Tokyo, Japan). In a preliminary test, embryos injected with 4 ng of control MO showed no significant differences in survival rates, morphology, or NH₄⁺ gradients compared with wild-type (+) embryos.

Preparation of total RNA. Zebrafish larvae were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was purified following the manufacturer’s protocol. The total amount of RNA was determined by spectrophotometry (ND-1000, NanoDrop Technologies, Wilmington, DE), and the RNA quality was checked by running electrophoresis in RNA-denatured gels. All RNA pellets were stored at −20°C.

Reverse transcription. Total RNA extracted from zebrafish larvae was treated with DNase I (Qiagen, Hilden, Germany) to remove genomic DNA contamination, and then phenol-chloroform extraction and purification were used to stop the reaction. For complementary (c)DNA synthesis, 5 μg of total RNA was reverse transcribed in a final volume of 20 μl containing 0.5 mM dNTPs, 2.5 mM oligo(dT)₂₀, 250 ng of random primers, 5 mM dithiothreitol, 40 U of an RNase inhibitor, and 200 U of SuperScript III RT (Invitrogen) for 2 h at 55°C, followed by incubation at 70°C for 15 min. Total cDNA was stored at −20°C.

Real-time, quantitative PCR. mRNA expressions of target genes were measured by a real-time, qualitative (q) PCR with an ABI StepOne Plus sequence analysis system (Applied Biosystems, Foster City, CA) in a final volume of 10 μl, containing 5 μl of ×2 SYBR green master mix (Applied Biosystems), 5 ng of cDNA, and 50 mM of the primers pairs (for Rhbg, forward: 5′-GCTCAAAACAGGGGCTC-3′, reverse: 5′-TGGGCTTCTACGTTTGG-3′; for Rhcg1, forward: 5′-TGCAACTGTCAGGGTGG-3′, reverse: 5′-AGGATAAGCAGGGAGGAA-3′). The standard curve for each gene was constructed to be linear in presence of the ribosomal protein L13a (rpl13a) used as an internal control (forward: 5′-CCTCGCTGTCITTCGGCATTT-3′, reverse: 5′-CAGGCTACACCCCTCTTGTTTG-3′).

In situ hybridization. Rhbg probe fragments (nucleotides 1,011 to 1,433, accession no. NM_200701.2) were obtained by a PCR and inserted into a pGEM-T easy vector (Promega, Madison, WI). Digoxigenin (DIG)-labeled RNA probes were synthesized by in vitro transcription with T7 and SP6 RNA polymerase (Takara Bio, Tokyo, Japan), and the quality and concentration were examined with RNA gels. Zebrafish embryos were anesthetized on ice and fixed in a 4% paraformaldehyde (PFA) PBS (1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na₂HPO₄, and 0.002 mM KH₂PO₄, at pH 7.4) solution at 4°C overnight and then treated with methanol at 4°C overnight again. Afterward, samples were treated with a methanol series at concentrations of 100% to 25% in diethylpyrocarbonate (DEPC)-PBS (PBS with 0.1% Tween 20) for 10 min each. Samples were then hybridized with the prepared probe in hybridization buffer (60% formamide, ×5 SSC, 0.1% Tween 20, 500 μg/ml yeast RNA, and 50 μg/ml heparin) overnight at 65°C. The next day, samples were serially washed with 75% hybridization buffer in 25% ×2 SSC at 65°C up to 100% ×2 SSC, and finally with ×0.2 SSC for 5–10 min six times. Samples were blocked with 5% sheep serum in 2 mg/ml BSA (Sigma, St. Louis, MO) at room temperature for 4 h and then incubated with an anti-DIG antibody (1:5,000, in blocking solution) at 4°C overnight. After being washed with DEPC-PBST at room temperature for 15 min eight times, staining buffer (0.1 M Tris at pH 9.5, 50 mM MgCl₂, 0.1 M NaCl, and 0.1% Tween 20) was subsequently used for 5 min three times. Staining was conducted with a mixture of NBT and BCIP in 10 ml of staining buffer at room temperature from 10 to 30 min in the dark. The reaction was stopped by PFA and subsequently washed with methanol...
several times. Finally, samples were stored in PBS at 4°C in the dark until further examination and analysis. Images were obtained with an upright microscope (BX51W1; Olympus, Tokyo, Japan) equipped with a digital camera (Canon 50D, Canon, Tokyo, Japan).

**Immunohistochemistry.** Zebrafish embryos were fixed in 4% paraformaldehyde for 2 h at 4°C. After being washed in PBS, fixed embryos were treated with 100% ethanol for 10 min at −20°C and subsequently subjected to blocking with 3% BSA at room temperature for 30 min. For identification of Rhbg, embryos were then incubated with a polyclonal rabbit antibody (diluted 1:500 in PBS) developed against the Rhbg (amino acids 33 to 51, accession no. NP_956365.2; Genomics BioSci&Tech, New Taipei City, Taiwan) of zebrafish overnight. After being washed with PBS, embryos were blocked again with 3% BSA for further double immunostaining of Na+−K+−ATPase (NKA). Embryos were incubated with an α5 monoclonal antibody against α-subunit of avian NKA (Hybridoma Bank, University of Iowa, Ames, IA; at a 1:500 dilution) at room temperature for 2 h. Embryos were washed in PBS and then incubated with 1:200 PBS-diluted goat anti rabbit immunoglobulin G (IgG) (conjugated with Alexa Fluor 488, Molecular Probes, Carlsbad, CA) and goat anti-mouse IgG (conjugated with Alexa Fluor 568) for 2 h at room temperature. Embryos were finally stained with DAPI (Invitrogen, Carlsbad, CA) to identify nuclei. Images were acquired with a confocal laser-scanning microscope (TCS-SP5, Leica Lasertechnik, Heidelberg, Germany). For z-plane images, 30 serial sections (0.5 μm/section; total thickness 15 μm) of images were acquired and subjected to image reconstruction and analysis.

**Western blotting.** Proteins of 20 μg/well were loaded onto a 10% SDS-PAGE at 100 V for 1.5 h. After separation, proteins were transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany) at 30 V overnight. After being blocked for 3 h in 5% nonfat milk, blots were incubated with an anti-Rhbg antibody (overnight, 4°C, diluted 1:500) and with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (dilute 1:5,000; Millipore) at room temperature for another 2 h. Blots were visualized with an enhanced chemiluminescence system (Millipore). The image was captured using an ImageQuant LAS 4000 system (GE Healthcare, Buckinghamshire, UK). Intensities of the immunoreactive bands were quantified by densitometry (Chemigenius, Syngene, UK).

**Statistical analysis.** Data are expressed as means ± SE. Values from each condition were analyzed using a one-way ANOVA followed by Tukey’s pairwise comparisons. Student’s unpaired t-test (two-tailed) was used for simple comparisons of two means. In all cases, significance was accepted at a level of 0.05.

**RESULTS**

**Localization of Rhbg in larval skin.** The cellular localization of Rhbg was determined with in situ hybridization and immunohistochemistry. The Rhbg anti-sense probe labeled polygonal keratinocytes covering an entire larva (blue signals in Fig. 1, A and B). No detectable signal was found in ionocytes in the larval skin. An image of a sense probe is shown in Fig. 1C as a negative control.

In addition to mRNA localization, a specific Rhbg antibody was generated and used to label Rhbg protein localization. The specificity of antibody was assessed by Western blotting (Fig. 2A). Three major bands with molecular weights of 37, 51, and 58 kDa were revealed. The band of 51 kDa matches the predicted molecular weight of zebrafish Rhbg. The 58-kDa band may represent the glycosylated form of Rhbg as reported in previous studies (16, 22). The specificity of Rhbg antibody was also confirmed by Rhbg MO injection (see below). Immunohistochemical images showed that Rhbg was mainly located in both apical and basolateral membranes of keratinocytes (green signals in Fig. 1, D, F, and H) but not in ionocytes (red signals represent NKA-labeled NaR cells; an unlabeled apical surface indicates the location of other ionocytes). Apical and basolateral localization in keratinocytes was clearly revealed by the z-axis confocal images (Fig. 1H). Both of the apical and basolateral signals were remarkably attenuated by 2 ng of Rhbg MO (Fig. 1, E, G, and I).

**Ammonia excretion in Rhcg1 and Rhbg knockdown larvae.** MOs were injected into fertilized eggs to knock down the translation of Rhbg or Rhcg1. Survival rates of larvae injected with different doses of MOs are shown in Table 1. Injection with 1 or 2 ng Rhbg MO slightly decreased the survival rate, whereas 4 ng of Rhbg MO remarkably decreased it. However, injection with the Rhcg1 MO (1, 2, or 4 ng) did not decrease the survival rate. Therefore, 2 ng of Rhbg MO and 4 ng of Rhcg1 MO were used for experiments, and no significant defect was observed in those larvae injected with those MOs.

In our previous study with immunohistochemistry (25), the same Rhcg1 MO was shown to almost completely block Rhcg1 protein expression in HR cells of zebrafish larvae. In this study, the knockdown efficiency of the Rhbg MO was determined by Western blotting (Fig. 2A). With injection of 2 ng of Rhbg MO, ~88% Rhbg protein abundance (the 51- and 58-kDa protein) was suppressed in 4-days postfertilization (dpf) larvae (Fig. 2B).

Using SIET to measure the NH4+ gradient at the yolk-sac surface of larvae, we found that the NH4+ gradient decreased by ~35% in Rhcg1 morphants (MO-injected larvae) and by ~80% in Rhbg morphants (Fig. 3). NH4+ fluxes at the surface of HR cells and keratinocytes were also measured in Rhbg and Rhcg1 morphants (Fig. 4). In Rhbg morphants, NH4+ flux was significantly suppressed in keratinocytes (Fig. 4C) but elevated in HR cells (Fig. 4A). In Rhcg1 morphants, NH4+ flux was suppressed in HR cells (Fig. 4B) but did not change in keratinocytes (Fig. 4D). Relative mRNA levels of Rhbg and Rhcg1 in whole larvae were determined by a real-time PCR. Results showed that both Rhbg and Rhcg1 levels were significantly elevated in Rhbg morphants (Fig. 5A), and Rhcg1 but not Rhbg was elevated in Rhcg1 morphants (Fig. 5B).

**NH4+ flux of keratinocytes and HR cells in larvae subjected to high external ammonia.** To compare the capability of keratinocytes and HR cells to excrete ammonia, NH4+ fluxes were measured in larvae exposed to a series of external ammonia concentrations (0.1 to ~5 mM NH4+; n = 8 cells from 3–4 larvae). In addition, three groups of larvae, which had respectively been pre-acclimated to NW, 1-mM HA, and 5-mM HA for 4 days, were compared. Results showed that the NH4+ flux gradually declined from positive values (efflux) to negative values (influx) in keratinocytes and revealed the reverse concentration (RC) as the flux declined to 0 (Fig. 6A). The RC was used as a criterion to compare the capability of cell in NH4+ excretion. Apparently, HA acclimation shifted the curve upward and increased RC of keratinocytes.

A similar phenomenon was also found in HR cells of the NW group; the NH4+ flux declined to negative values as the external NH4+ increased and the RC was revealed (Fig. 6B). However, the curves of the two HA groups greatly differed. The NH4+ fluxes seemed to be relatively independent of external NH4+ levels, and the RC was not presented within 5 mM. Apparently, the RC of the same group was higher in HR cells than in keratinocytes.
Fig. 1. Whole-mount in situ hybridization and immunohistochemistry of Rhbg in 3-days postfertilization (dpf) larvae. A and B: the Rhbg antisense probe is labeled on polygonal keratinocytes (arrows, blue signals) covering the larval surface. C: the Rhbg sense probe was used as a negative control. D–I: confocal microscopic images of the immunohistochemistry in wild-type (WT) and Rhbg morphant (MO). Green signals showed that Rhbg is located in keratinocytes but not in ionocytes (D and F). Red signals revealed NaR cells. Other subtypes of ionocyte were not labeled by Rhbg or NKA and thus are shown as black holes surrounded by keratinocytes (arrows in F). The z-plane scanning image (H) shows that Rhbg was located in both apical and basolateral membranes (arrows) of keratinocytes. The green signals were remarkably decreased in Rhbg morphants (E, G, and I).
RCs of keratinocytes and HR cells. To shorten the duration of experiment for determining RC and obtain RC of individual cells, we sequentially measured NH4⁺ fluxes at the same cells in different NH4⁺ water by simply changing the medium from low to high concentrations (Fig. 7). RCs were derived from the regression line (broken lines) of the curves obtained from each cell (Fig. 7, A and B). To determine the RC of HR cells, the external NH4⁺ concentration was increased to 10 mM. Curves of keratinocytes and HR cells from the three groups are respectively shown in Fig. 7, A and B, and RCs from each cell are compared in Fig. 7C. The RC of keratinocytes was 0.7 ± 0.1 mM in the NW group, and it increased to 1.2 ± 0.3 mM in the 1 mM HA group and 4.2 ± 0.5 mM in the 5-mM HA group. The RC was remarkably higher in HR cells than in keratinocytes in all three groups.

RCs of HR cells in Rhcg1 and atp6v1a morphants. To test whether Rhcg1 and H⁺-ATPase are involved in the high

Table 1. Survival rate of Rhbg/Rhcg/MO-injected larvae

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<th>MO Dose</th>
<th>1 ng (%)</th>
<th>2 ng (%)</th>
<th>4 ng (%)</th>
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<tr>
<td>Ctrl</td>
<td>83.5</td>
<td>92.9</td>
<td>89.5</td>
</tr>
<tr>
<td>Rhbg MO</td>
<td>80.1</td>
<td>76.9</td>
<td>18.0</td>
</tr>
<tr>
<td>Rhcg1 MO</td>
<td>90.2</td>
<td>94.1</td>
<td>81.0</td>
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MO, morpholino oligonucleotide; Ctrl, control.
capability of HR cells to excrete ammonia, RCs were determined in Rhcg1 and atp6v1a (a subunit of H^+-ATPase) morphants. A real-time PCR was used to determine the expressions of Rhbg and Rhcg1 in 4-dpf larvae injected with 2 ng of Rhbg MOs or 4 ng of Rhcg1 MOs. Values are given relative to the amount of the WT. Data are means ± SE (n = 5). *Significant difference (Student’s t-test; P < 0.05).

**DISCUSSION**

The apical localization of Rhcg1 in ionocytes of pufferfish and zebrafish was clearly shown in previous studies (18, 19). However, the localization of Rhbg in fish gills is not yet clear. In pufferfish, Rhbg was immunolocalized to the basolateral side of PVCs in gills (19). In rainbow trout, Nawata and colleagues (21) also reported the localization of Rhbg in PVCs by analyzing separate gill cells, although apical or basolateral localization was not determined. In zebrafish, Braun et al. (3) used immunohistochemistry to show the localization of Rhbg in gill PVCs, whereas Rhbg mRNA was detected in unidentified cells in yolk-sac skin of zebrafish larvae (2). To further investigate the role of Rhbg in zebrafish, herein we used in situ hybridization and immunohistochemistry (with a zebrafish-specific antibody) to localize Rhbg and found that Rhbg mRNA/protein is expressed by keratinocytes (which are similar to PVCs in gills) but not in ionocytes of zebrafish larvae (Fig. 1). Confocal images revealed that Rhbg is mainly expressed in both apical and basolateral membranes of keratinocytes. Rhbg MO remarkably decreased the signals of Rhbg in both apical and basolateral membranes (Fig. 1). In pufferfish, Rhcg2 was found in the apical membranes of PVCs (19). In the case of zebrafish, however, expression levels of Rhcg2 in zebrafish gill and whole larvae were low (data not shown), suggesting that Rhcg2 is not a major form and is unlikely to play a critical role in zebrafish.

The transcript level of Rhbg in zebrafish larvae was remarkably higher than that of Rhcg1 (2), implying that Rhbg plays a dominant function in ammonia excretion. In this study, we found that knockdown of Rhbg caused a remarkable decrease (~80%) in ammonia excretion (Fig. 3). However, knockdown of Rhcg1 only decreased ammonia excretion by ~35%. Using SIET to examine individual cells, we found that ammonia excretion by keratinocytes was suppressed in Rhbg morphants (Fig. 4C), demonstrating the localization and function of Rhbg in keratinocytes. Interestingly, ammonia excretion by HR cells increased in Rhbg morphants, implying a compensatory up-regulation of ammonia excretion (Fig. 4A). This notion was supported by the elevation of Rhcg1 mRNA levels in whole larva homogenates of Rhbg morphants (Fig. 5). In contrast, knockdown of Rhcg1 only suppressed ammonia excretion by HR cells but did not increase ammonia excretion by keratinocytes (Fig. 4, B and D). Taken together, the role of Rhbg in ammonia excretion seems to be dominant over Rhcg1 in zebrafish larvae.

In a previous study on zebrafish larvae, it was reported that knockdown of either Rhbg or Rhcg1 caused similar impacts on ammonia excretion (2). This inconsistency could have been due to different approaches used to measure ammonia excretion. In that study, they measured the excreted ammonia accumulation from pooled larvae in a limited amount of water for 3 h to calculate the ammonia excretion rate. That method...
Another major finding in this study was a comparison of the ammonia-transporting capabilities between keratinocytes and HR cells. The RC of ammonia excretion was used as a criterion to compare the capability of cells. We showed for the first time that ionocytes (HR cells) are able to secrete ammonia against a higher external ammonia level compared with keratinocytes (Fig. 6). In larvae acclimated to NW (without the addition of ammonia), the RC of HR cells was \( \approx 3 \text{ mM} \), over 2 mM higher than that of keratinocytes. High-ammonia water (1 and 5 mM \( \text{NH}_4^+ \)) acclimation increased the RCs of both HR cells and keratinocytes, but RCs of HR cells were always higher than those of keratinocytes (Fig. 7). Accordingly, we suggest that HR cells are able to actively secrete ammonia. Since the ammonia concentration in the body fluid of larvae was not available in this study, we cannot conclude whether ammonia was passively or actively excreted by keratinocytes. However, the linear relationship between external \( \text{NH}_4^+ \) concentrations and \( \text{NH}_4^+ \) fluxes shown in keratinocytes (Figs. 6 and 7) implies that ammonia moves across keratinocytes by passive diffusion. If this is true, the RC would be close to the internal
ammonia level. The RC in keratinocyte of zebrafish larva was 
~0.7 mM, slightly higher than the common blood ammonia level observed in adult fishes (usually <0.5 mM; Ref. 7). This might have been due to the higher metabolic rate in the larval stage than in adult fish (1). Alternatively, it is possible that the internal ammonia of larvae is <0.7 mM and that keratinocytes can excrete ammonia against the gradient.

Since keratinocytes cover almost the entire larval surface, passive diffusion through keratinocytes is supposedly a highly efficient way to excrete ammonia into water. However, this pathway would be blocked if the external ammonia level is raised. After 1 or 5 mM NH$_4^+$ acclimation, we found that the RCs of keratinocytes turned out to be close (1 mM group) or lower (5 mM group) than the NH$_4^+$ concentration of the acclimation water, indicating that ammonia excretion from keratinocytes was difficult in high-ammonia water. In this situation, the role of HR cells is magnified by their capability to pump out ammonia against a high external ammonia level.

Our previous study showed that knockdown of either Rhcg1 or H^+-ATPase in HR cells caused a decline in ammonia excretion by zebrafish larvae (25). Herein, we further found that knockdown of either Rhcg1 or H^+-ATPase decreased the RC of HR cells to a level close to that of keratinocytes (Fig. 8), suggesting that both of these molecules are required for active ammonia transport by HR cells. The coupling of Rhcg1 and H^+-ATPase in the apical membrane probably functions as an “ammonia pump,” even though Rhcg1 alone is a passive NH$_3$ channel. With H^+-ATPase in the apical membrane, the H^+ gradient across the apical membrane probably provides the driving force to pump NH$_3$ out of cells. Acid-tapping ammonia excretion was proposed for a long time in a variety of animals from invertebrates to mammals (28, 29, 31, 33). However, to our knowledge, no convincing evidence has been proposed to demonstrate this mechanism to be effective for active ammonia transport.

In addition to H^+-ATPase, the Na^+/H^+ exchanger (NHE) was also suggested to be involved in ammonia excretion in mammalian kidneys and fish gills/skin (17, 26, 36). In seawater pufferfish, the expression of NHE3 was induced during high-ammonia exposure, indicating that the NHE is involved in ammonia excretion in seawater (20). However, in freshwater rainbow trout, expression of the NHE (NHE2 and NHE3) was unchanged after acclimation to high-ammonia water (34). Our unpublished data also showed that NHE3 transcripts did not change in zebrafish acclimated to high-ammonia water. However, some species do not have H^+-ATPase on the apical side of ionocytes, such as medaka (14) and killifish (12). Whether NHE in these species can directly transport NH$_4^+$ or indirectly transport H^+ for NH$_3$ trapping needs to be further investigated.

In some seawater species or euryhaline species in seawater, ionocytes were suggested to actively excrete ammonia through transporting NH$_3$ with K^+ transporters such as Na^+-K^+-ATPase (NKA) and the Na^+-K^+-2Cl$^-$ cotransporter (NKCC). For example, the addition of an NKA inhibitor (ouabain) reduced the ammonia excretion by the mudskipper (Periophthalmodon schlosseri) (23). Recent studies on pufferfish and climbing perch (Anabas testudineus) showed that gene/protein expressions of NKCC and NKA were induced after high-ammonia exposure (11, 20). It is likely that NKA in the basolateral membranes of HR cells (13) is also involved in the active ammonia transport of zebrafish. Whether a specific isoform of NKA in HR cells is involved in ammonia transport needs to be tested.

This study demonstrated that Rhbg facilitates ammonia excretion by skin keratinocytes of zebrafish larvae. HR cells can excrete ammonia against higher external ammonia levels compared with keratinocytes. Both Rhcg1 and H^+-ATPase in HR cells are required for active ammonia excretion.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


